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CONTENTS

AGE DEPENDANT HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE DOES MAMMARY GLAND

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Abstract

This study was done on 15 female rabbits (does) . The female rabbits (does) were collected from the Taif governorates. The mammary gland of the young age mainly consisted of lobules of adipose tissues. The primordia of the alveoli consisted of undifferentiated stratified epithelial cells with basophilic nuclei . The development of the mammary gland started to grow at the pregnant stage and completed at the late stage of pregnancy of the doe. During pregnancy the development of the parenchyma took place fastly and the mammary gland became mature few days before parturition. Myoepithelial cells appeared as elongated cells with long irregular nuclei, centrally located and acidophilic cytoplasm. It situated in the inter alveolar septum. Most of the alveoli in the adult lactating doe lined by simple squamous epithelium and their lumen filled with milk secretions . The lobules of the mammary gland at senile age shrinked and decreased in size and separated to located in the center of the lobules. The alveoli of the shrinked mammary gland lobules showed collapse and the intra lobular ducts showed narrowing the lumen. Some alveoli filled with casiated milk secretion (corpora amylecia).

Key words. *Mammary gland-age dependant-alveoli-does*

Introduction

The productions of domestic rabbits are used for many purpose, meat, fur, laboratory diagnosis and research. Rabbit's meat of all ages shows high values for human consumption, it has a higher percentage of protein than other meats and the meat itself is highly digestible, for this reason it is often recommended for sick people (Sandford and Woodgate , 1974 and Zotte, 2002). The doe (female rabbit) has four or five pairs of nipples. The mammary gland develops in the last week of pregnancy. Suckling is stimulated by a pheromone produced by a gland near the nipple. Rabbit milk is richer than cow's milk, it has an unusually low lactose content and a very high protein and fat content.13% protein, 9% fat, 1% lactose and 2.3% minerals (Richardson, 2000).

During the lifetime of the animal the mammary gland probably undergoes more and greater changes in size, structure, composition and activity than any other tissue or organ. These changes start during fetal life and continue even after the gland has reached maturity since it waxes and wanes during successive reproductive cycles (Knight and Peaker,1982).

The mammary gland is a modified sweat gland. It is a compound tubule-alveolar gland, which is divided into lobules by interlobular connective tissue. The mammary gland consists of parenchyma (alveoli), stroma (connective tissue), ducts, vessels and nerves (Dellmann and Carithers 1996; Bacha and Bacha 2000 and Salomon et al., 2008).

No true development occurs before pregnancy. Although some ducts are formed and the alveoli primordia. (Cowie et al., 1980 and Salama ,2004). In mice and rabbits the earliest changes are brought about by morphogenetic movement of cells rather than by proliferation (Balinsky, 1950); this pattern may be common to all species.

It has been estimated that 94% of all mammary growth takes place during gestation in the hamster (Sinha et al., 1970), 78% in the mouse (Brookreson and Turner, 1959) and sheep (Anderson, 1975b), 66% in the rabbit (Lu and Anderson, 1973) and 60% in the rat (Griffith and Turner, 1961). During gestation the duct system increases in size and complexity and

epithelial cells proliferate, displacing adipose tissue and forming the first true lobulo-alveolar tissue. Contrary to the belief of early workers proliferation continues throughout the whole of gestation, and sometimes into lactation. For a comparative review of mammogenesis in many species (Cowie et al., 1980).

Materials and methods

The does were collected from taif governorates and divided into 5 groups represented the ages and physiological state. At I month (young), 5 months (immature) , 8 month (adult pregnant) , 12 months (adult lactating) and 24 months (senile). The mammary glands was removed from rabbits under ether anesthesia preserved in 10% formalin, bouin's solution, dehydrated, cleared in xylol and cut at 5micron . These techniques were done according to (Bancroft et al.,1994).

Results

The mammary gland of the young age (1 month) consisted of lobules of adipose tissues (Fig.1). Numerous arterioles and venules located in the CT between the lobules . The adipose tissue was negative alcianophilic reaction while the CT fibers were faint alcianophilic reaction . The CT fibers were moderate positive PAS reaction .

The mammary gland of the immature doe showed diminished of the adipose tissue to be few adipocyte cells at the periphery of the lobules. Each lobule consisted of numerous fibroblast cells and CT fibers that surround the primordial of the alveoli (Fig. 2). The primordia of the alveoli consisted of undifferentiated stratified epithelial cells with hyperchromatic nuclei at the basal polygonal cells and dense basophilic nuclei at the superficial cells (Fig. 3). Some primordial characterized by star shape lumen while the others still appeared as solid masses of cells without lumen . The undifferentiated cells of the primordia and the mesenchymal cells were positive PAS reaction while the CT fibers were faint to negative reaction .

The development of the mammary gland started to grow at the pregnant stage and completed at the late stage of pregnancy of the doe. During pregnancy the development of the parenchyma took place fastly and the mammary gland became mature few days before parturition. The alveoli lined by high cuboidal epithelium with centrally located, basophilic nuclei and faint basophilic cytoplasm (Fig.4). The intralobular duct developed and lined with pseudostratified epithelium. The duct lumens contain milk secretion rich in plasma cells (Fig.5). Some adipocyte cells still located between the mammary gland lobules .The alveoli varied in size , it appeared as ovoid in shape or large with irregular contour. It contained eosinophilic mass in the lumen . The mammary gland of the pregnant stage was negative to alcian blue reaction while it took positive PAS reaction. It was strong positive PAS in the epithelium of the alveoli, duct epithelium and milk secretion while faint PAS reaction in the CT .

The size of the mammary gland lobules increased in the lactating does while the CT decreased. The intralobular duct of the adult lactating doe was lined by simple cuboidal epithelium and filled with milk secretions . The interlobular duct lined by stratified columnar epithelium. It located in the interlobar CT septa .Most of the alveoli lined by simple squamous epithelium and their lumen filled with milk secretions (Fig.6). Myoepithelial cells appeared as elongated cells with long irregular nuclei, centrally located and acidophilic cytoplasm. It situated in the inter alveolar septum (Fig.7). The mode of milk secretion was

apocrine mode. The epithelium cells extruded to the lumen. The CT decreased to be very fine fibers extended between the alveoli while the CT surround the intralobular duct was also diminished . Positive PAS reaction in the epithelial lining of the alveoli while faint PAS reaction in the CT surround the duct . At the late stage of lactating prior to the next pregnancy. The milk secretion filled with numerous lipid globules. It filled the alveoli and the ducts .

Few days before parturition, the mammary gland showed increase in the interlobular CT. Some mammary gland lobules showed shrinkage in size. Some lobules appeared as highly lactating while the adjacent lobules appeared as inactive lobules (dry period). Most of alveoli were stopped milk secretion to prepare their selves to the next milk stage. It characterized by irregular lumen, increased interalveolar CT and the epithelium of the alveoli returned back to be cuboidal in shape (Fig.8).

The mammary gland of the senile doe was greatly decreased in size in comparison to the previous lactating stage. Numerous adipose tissues occupied the architecture of the mammary gland (Fig.9). The lobules of the mammary shrinked and decreased in size and separated to located in the center of the lobules. The CT greatly increased in the lobules, the interlobular septa and the interlobular duct lamina propria. The alveoli of the shrinked mammary gland lobules showed collapse and the intra lobular ducts showed narrowing the lumen (Fig.10). It showed positive PAS reaction in the collapsed alveoli and intralobular ducts . while the intralobular and interlobular CT showed faint PAS reaction (Fig.11) . Some alveoli filled with casiated milk secretion (corpora amylecia), and the adipose tissue increased between the lobules (Fig.12).

Discussion

The mammary gland is one of the hormone dependant organs. Its growth, structure and function are closely-related to the age of animals and to the level of hormones in blood serum. Since each age group possesses special levels of hormones, the effect of age, climate and hormones were taken as parameters to show their effect on the mammary glands. (Salama,2004).

The development of mammary glands is the most important physiological process of lactating, which is mainly regulated by the hypothalamus-pituitary neuroendocrine system (Han et al. 2006). The mammary develops under hormonal control of estrogen (E2), progesterone (P), growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH) Boue (2003). E2 is an effective mitogen for mitosis of mammary gland cells. It also stimulates the growth and maturation of the mammary duct (Chen et al., 2002 and Russo et al., 2005).

The mammary gland of does was formed of 4 quarters and each quarter was drained by a teat. Each quarter was surrounded by a capsule-like sheet of CT. Thick septa containing blood vessels and nerves extended to divide each quarter into lobules. Most of the lobules were occupied by fat cells. Only a few number of lobules were found to be occupied by a mammary tissue. It was represented by branching ducts extending from the teat canal. The ducts were lined by a stratified epithelium and supported by a fibro-cellular stroma. Quite similar results have been described previously in the mammary gland of buffalo-calves (Moussa, 1977) and in infants up to 2 years of age (Anbazhagan et al, 1991).

The existence of alveoli in the glands of immature animals was found to be variable. Nosier et al., (1974) and Moussa (1977) in buffalo-calves demonstrated glandular units which are lined with cuboidal epithelium.

In infants, Anbazhagan et al., (1991) described few buds lined with stratified epithelium. Contrary to these findings, the present study showed no alveoli but rarely buds. They appeared as solid masses of cells growing from the sides of pre-existing ducts. The primordia of the mammary gland appeared at the immature age as solid masses of stratified cells around star shape lumen. These findings were similar to the results of (Salama,2004) in buffaloes. The development of the mammary glands was completed at the pregnancy stage. As the solid masses primordial alveoli increased and became alveoli lined by simple cuboidal to low columnar epithelium. These results were supported by the findings of (Imagawa et al., 1994) who stated that once pregnancy started that full development of the mammary gland is achieved. While (Lu and Anderson, 1973) stated that the development of the rabbit mammary gland was 66% and the full development completed after parturition.

The parenchyma was gradually increased till it occupied most of the lobules. The inter and intralobular stroma were simultaneously decreased to constitute a smaller part of the gland. It had been estimated that the glandular : CT ratio was 5.9 : 1 in cows (Solov'eva, 1964) and the glandular tissue formed 20% of the gland in goats (Parmar et al, 1986) and 23.58 % in she-camels (Ibrahim et al, 1990).

During pregnancy the development of the parenchyma took place fastly and the mammary gland became mature few days before partiuration. Similar findings had been reported in Bentivoglio (1986) who reported that the alveoli were developed during pregnancy and started to secrete milk between 85-30 days prepartum in goats. In cows and during the first 3 months of gestation, the mammary glands were consisted of ducts only and well developed alveoli were not formed until the 8^{th} month (Hammond, 1927), (Giese,1986) in sows also decided that the alveoli did not develop until the last trimester of pregnancy.

It had been found that some alveoli in the mammary glands of the does synthesis and secrete milk during late pregnancy. The secretion appeared as an acidophilic PAS-positive substance which frequently contained detached nuclei and some migrating cells. The cells around this secretory product were usually columnar-shaped and possessed acidophilic vacuolated cytoplasm. These results were supported by the findings of (Salama,2004) in buffaloes and Bentivoglio (1986) in goats . Milk secretion in the lumen of alveoli may be referred to as colostrum (Moussa, 1977). The plasma cells that were frequently seen in milk during pregnancy might be the source of gamma globulin of colostrum (Marx and Cole, 1965 and Salem et al, 1983).

The mammary gland alveoli lined by simple columnar epithelium (synthesis phase), simple cuboidal (secretory phase) and simple squamous in (inactive phase) as reported by (Salama,2004). The alveoli epithelium rest on myoepithelial cells which were elongated cells with irregular oval nuclei .These resulted were in agreement of the results mentioned by (Barone 1990; Dellmann and Carithers 1996; Salama, 2004 and Salomon et al., 2008). In contrast to these findings (Kurosumi et al.,1968) stated that the secretory epithelium of the rat mammary gland consists of glandular cuboidal epithelium , myoepithelial cella and temporary wandering cells which migrated from the surrounding connective tissue and blood vessels.

The existence of myoepithelial cells seemed to be a constant finding in the mammary alveoli. It was flat cells with elongated and intended nuclei, basophilic in reaction and light

acidophilic cytoplasm. Similar findings were achieved by Moussa (1977) who recorded an interrupted layer of myoepithelial cells around the alveoli of pregnant buffaloes. The cells were found to occur internal to the basement membrane of the alveoli (Richardson, 1949 and Salem et al, 1983) and were believed to be derived from the outer layer of immature ducts (Linzell, 1955).

Many functions had been attributed to the myoepithelial cells ; required to squeeze the alveoli (Richardson, 1949) and to replace cells that were degenerated and detatched during the process of involution in rodents (Holst et al., 1987). The myoepithelial cells didn't developed until lactation started. As it didn't recognized in young and immature ages. This believe was augmented by (Carolyn and Radnor 1972). After parturition, the mammary glands of the does reached their maximum growth and started to secrete milk. It had been found that the enlargement of buffalo glands was solely due to enhanced growth of the parenchymal tissue. The glandular stroma, on the other hand, was reduced greatly into thin partitions between the lobules and fine strands between the adjacent alveoli. It had been estimated that the parenchyma accounted for 85.85% of the mammary glands in cows (Krastev and Koven, 1964), 79.32 % in she-camels (Ibrahim et al, 1990) and 80% in goats (Parmar et al, 1986).

The intralobular duct was lined by pseudostratified epithelium then decreased to be high cuboidal epithelium and it has secretory activity similar to the cells of the alveoli . This result was in agreement with the result of Richardson (1947) and Salama (2004).

The mammary gland showed involution few days before second time of pregnancy. It characterized by involution revealed regression of most of the alveoli and increased amount of CT. The persisting alveoli were very small in size and their epithelium reverted to the non functioning state. Naito et al., (1955) and Naito (1958) stated that regression is characterized by decreased number of alveoli / lobule and decreased number of cells / alveolus. Reduction of the number and size of the alveoli and increased amount of CT in the regressing glands was found to be a general pattern in most domestic animals (El-Ghousien, 1972, Nosier, 1973, Moussa, 1977, Michel, 1981 and Salem et al., 1983).Also these results were augmented by the findings of (Feng et al., 1995; Jaggi et al., 1996 and Quarrie et al., 1994). While (Li et al., 1997 and Lund et al., 1996).

At the senile age, the mammary glands of the does showed disappearance of most of the alveoli and tubules. The involuted structures were replaced by newly-formed CT. Only few non-functioning units were seen embedded in a reticular net containing numerous fibroblasts, macrophages and plasma cells. The parenchyma was greatly reduced into a few numbers of small lobules and the CT formed the main bulk of the mammary tissue. Each lobule was occupied by a few numbers of tubular structures. It had been found that all alveoli and tubules and most of the intralobular ducts were disappeared. The persisting ducts possessed wide lumina lined with simple cuboidal epithelium. The cell's cytoplasm was homogenous and acidophilic and the centrally-located nuclei are darkly-stained. The intertubular stroma was still delicate but showed a marked cellular infiltration. The cells might aggregate to obscure the underlying fibrocellular framework. Frequently one of the persisting tubules contained a homogenous acidophilic substance. These might be corpora amylecea ; a structure which was commonly demonstrated in the glands of senile animals. These results were augmented by the findings of (Cowie and Tindal 1971, Dellmann and Brown 1976, Moussa,1977 and Salama, 2004).

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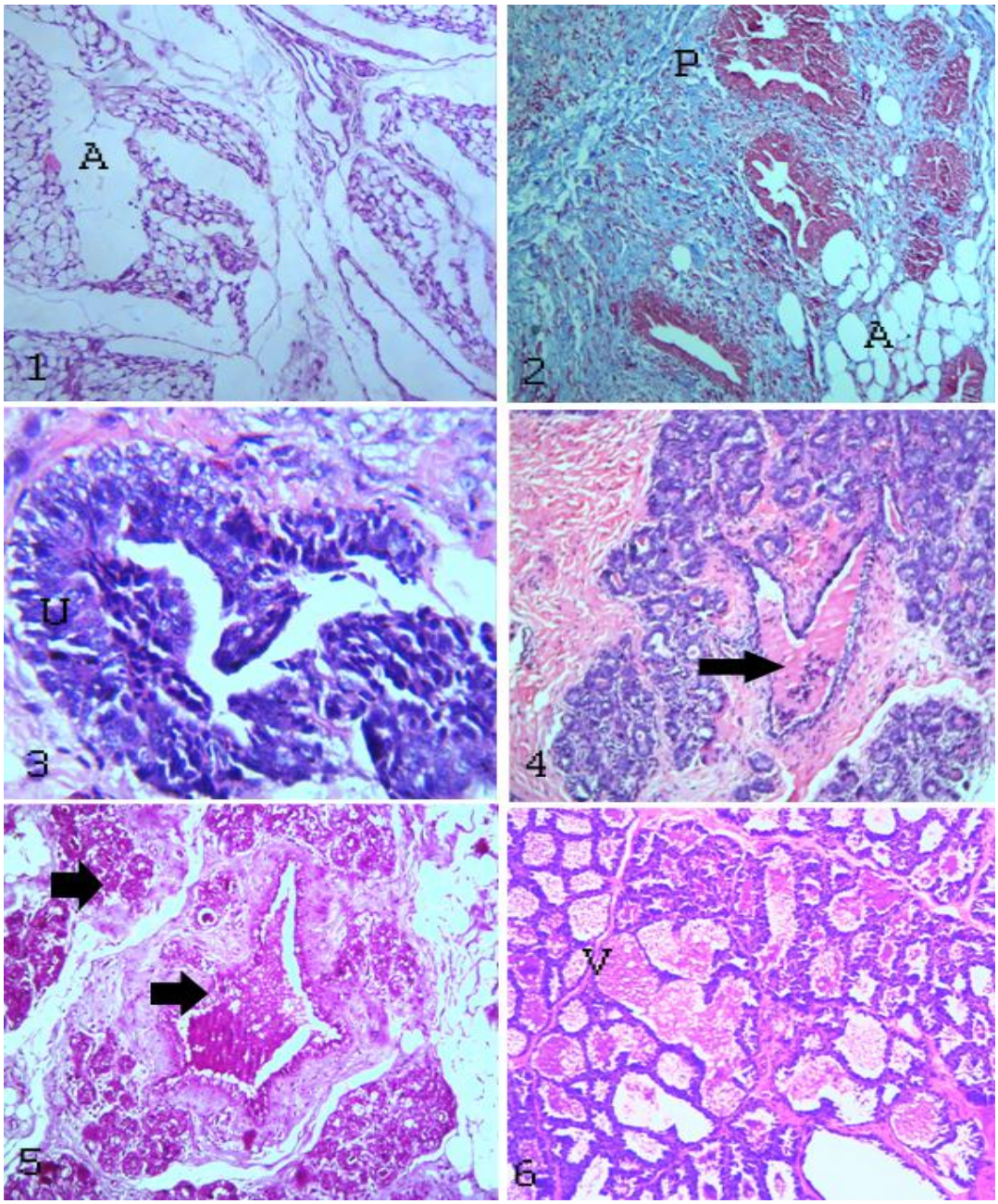

Plate.1. photomicrograph of mammary gland in young age (1) showing,the gland consisted of lobules of adipose tissues (A).H&E.x10. in (2), the primordial of the mammary alveoli consisted of star shape lumen surrounded by undifferentiated cells (P), the adipose tissue still persist (A). Crossmon's trichrome .X10. in (3), the primordial consisted of mass of undifferentiated stratified cells with centrally basophilic nuclei (U).H&E.X40. In (4) the intralobular duct contained colostrums (arrow). H&E.X10. In (5), Positive PAS in the developing alveoli and colostrums (arrow).PAS.X10.In (6) the alveoli of the lactating mammary gland lined by simple squamous epithelium and filled with milk secretion (V).H&E.X40.

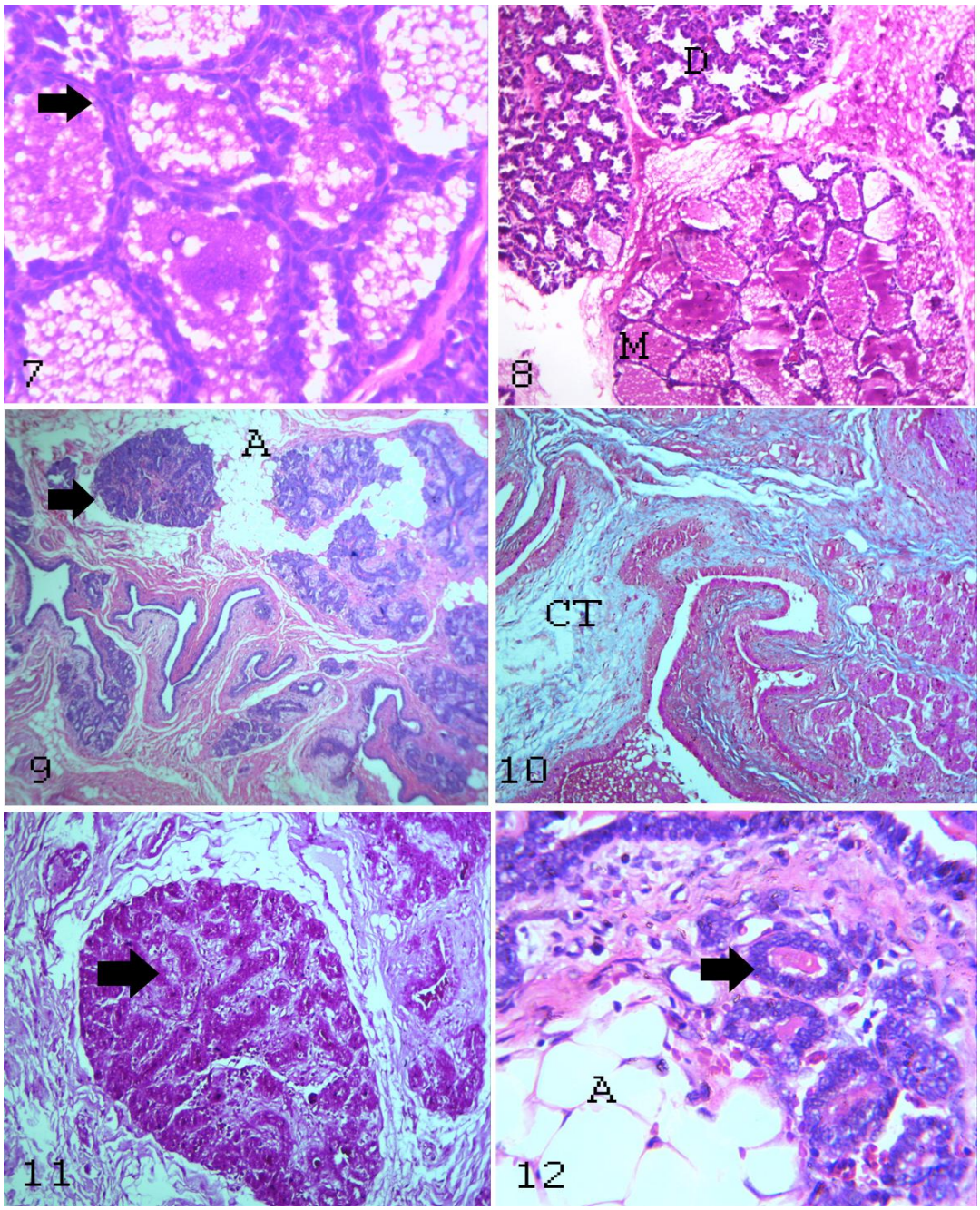

Plate.2. photomicrograph of mammary gland in adult lactating age (7) showing, the myoepithelial cell (arrow) H&E.X40. in (8), the dry mammary tissue (D) and lactating mammary tissue (M).H&E.x10.In (9), the mammary tissue in senile age showing shrinked lobules (arrow), and appearance of adipose tissue(A). H&E.x10. in (10), the CT increased between the lobules (CT). crossmon;s trichrome .X10 In (11), the shrinked lobules were PAS positive reaction (arrow). PAS.X10. In (12), appearance of corpora amyelcia (arrow) and adipose tissues replaced the mammary tissues.H&E.X40.

HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE OVARY OF THE RABBITS AT HIGH ALTITUDE

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Abstract
This study was done on 5 groups of female rabbits (does) representing the different physiological pattern of the female life. The female rabbits (does) were collected from the Taif governorates. The ovaries were collected for histological and histochemical examination. The ovary consisted of external cortex and internal medulla. The ovary covered by pseudostratified columnar epithelium in young age which changed to columnar epithelium then to cuboidal epithelium in the adult age. The medulla consisted of CT rich in blood vessels, lymphatic vessels and nerve fibers. The cortex filled with follicles at different stages of development, primordial follicle, primary follicles, secondary follicles, tertiary follicles and mature follicle. Corpus haemmorhgicum, corpus luteum and corpus albicans are ovarian structure located in the cortex according to the physiological, cyclic and age of the does. CL consisted of light large and dark small lutein cells. CH consisted of reminant of the mature graffian follicle with blood ooze from the surrounding blood vessels. CA consisted of structure less destructed CA or CL with invading numerous CT.

Introduction

The productions of domestic rabbits are used for many purpose, meat, fur, laboratory diagnosis and research. Rabbit's meat of all ages shows high values for human consumption, it has a higher percentage of protein than other meats and the meat itself is highly digestible, for this reason it is often recommended for sick people (Sandford and Woodgate , 1974 and Zotte, 2002).

The health effects of high altitude-induced hypoxia which affect a large number of people live at high altitudes and many others like to visit such areas for trekking, climbing or athletic training. The composition of the air stays the same but the total barometric pressure falls as altitude increases. As a result, the partial pressure of oxygen decreases and a state of hypoxia occurs (Michiels, 2004). Certain biochemical, physiological and microanatomical responses occur during acclimatization and adaptation to chronic hypoxia of high altitude (Ward et al., 1989). Therefore, the adaptive processes that occur in response to hypoxia indicate complex modifications in the homeostatic steady state of endocrine and metabolic functions (Michiels, 2004). The citizen which live in high altitude have the ability to modify their body biochemistry and hormone status (Palinkas et al., 2007).

The ovaries are two in each side. It located on the lateral wall of the pelvis. It supported by the broad ligament. The ovary composed of cortex externally and medulla internally. The surfaces of the ovaries covered by simple cuboidal or columnar epithelium above CT fibers thick tunica albuginea. The cortex contain interstitial CT. The cortex parenchyma consists of primordial, primary, secondary, tertiary follicles, mature follicles, corpus luteum and atretic follicles. The medulla consists of loose connective tissue rich in blood and lymph vessels. The ovary functions as an organ of reproduction and also as a gland of internal secretion.. It secrete the mature graffian follicles (Exocrine function) and non-steroidal hormones that act locally (Endocrine function) (Hodges et al.,1985).

Over the portions of the ovary covered by low columnar or cuboidal epithelium, cells frequently appeared to be detaching from the surface. Tunica albuginea, a poorly vascularized, dense, irregular collagenous connective tissue capsule. Each ovary was subdivided into the highly cellular cortex and medulla, which consisted mostly of a richly vascularized loose connective tissue. The cortex, which was surrounded on the outside by the surface epithelium, contained germ cell clusters, some primordial, primary, secondary, tertiary and atretic follicles, and dilated blood vessels (Bjersing et al,1981 and Ozdemir et al., 2005).

Bruin et al.,2002, reported that the primordial follicles located under tunica albuginea and extended deeply for a short distance into the cortical tissue. The primordial follicle consists of a primary oocyte surrounded by a single layer of flattened follicular cells which are cuboidal in shape. Primary follicles consists of a primary oocyte surrounded by a simple cuboidal or columnar follicular cells.. Secondary follicles consists of a primary oocyte surrounded by a stratified epithelium of granulosa cells. Tertiary follicles were composed of a primary oocyte surrounded by a stratified epithelium of follicular cumulus cells. The stratum granulosum was surrounded by the theca, which in tertiary follicles differentiates into two layers: an inner vascular theca interna and an outer supportive theca externa. The theca interna cells were spindle-shaped and located in a delicate reticular fiber. The theca externa consisted of a thin layer of loose connective tissue with fibroblasts arranged concentrically around the theca interna.

The zona pellucida is structureless layer which surround the oocyte. It is glycoprotein in nature and has fundamental role in during fertilization and early development (Dunbar et al., 1981; Sacco et al., 1981; Hedrick and Wardrip, 1987; Yurewicz et al., 1987 and Wassarman, 1988), such as species-specific attachment and binding of spermatozoa (O'Rand, 1988), induction of the acrosome reaction (Bleil and Wassarman, 1983 and Cherr et al., 1986) and the block to polyspermy (Braden et al., 1954). Zona pellucida is a potent heteroimmunogen and share common antigenic determinants . Zona antisera possess contraceptive activity in vitro and in vivo (Sacco, 1981; Dunbar, 1983; Maresh and Dunbar, 1987and Henderson et al., 1988). Human and porcine zona share common antigens (Sacco, 1977 and Koyama et al., 1985) and antiserum to porcine zona demonstrates contraceptive efficacy in humans (Trounsonet al., 1980 and Henderson et al, 1987a).

In the ovary of unmated or un-stimulated rabbits, therefore, functional corpora lutea (CL) should not be present and, in circulating plasma, progesterone concentration should remain at basal level. (Boiti et al., 1996).

Materials and methods

The does were collected from Taif governorates and divided into 5 groups represented the ages and physiological state. At I month (young), 5 months (immature) , 8 month (adult pregnant) , 12 months (adult lactating) and 24 months (senile). The ovaries were removed from rabbits under ether anesthesia preserved in 10% formalin, bouin's solution, dehydrated, cleared in xylol and cut at 5micron . These techniques were done according to (Bancroft et al.,1994).

Results

The ovary of the young doe consisted of cortex and medulla. The cortex located in the outer part while the medulla located in the center of the ovary. The ovary lined by pseudo stratified columnar epithelium (Fig.1). The cortex consisted of numerous primordia cells surrounded by mesenchymal cells, it mainly represented the bulk of the cortex (Fig. 2). Fine collagen fibers extended between the cortical follicles (Fig. 3). Primary follicles were located at the edge of the cortex. The medulla consisted of CT rich in blood vessels, nerves and lymphatic in the center of the ovary (Fig. 4). The medulla, epithelium lining of the ovary and the granulosa cells were strong PAS positive reaction (Fig. 5), while the CT fibers between the follicle was faint PAS positive (Fig. 6).

The ovary of the immature doe covered by pseudo stratified columnar epithelium. The tunica albuginea located beneath the covering epithelium, it consisted of thick layer of CT (Fig.7). The secondary follicles were appeared and they consisted of ovum in the center surround by eosinophilic yolk material and surrounded by shiny eosinophilic structure (zona pellucida). One row of low columnar cells surrounds the zona pellucid that represented the (corona radiata), it characterized by acidophilic cytoplasm and basely situated basophilic nuclei . Small, round cells with centrally located basophilic nuclei surround the corona radiata that represented (granulosa cells) .Fine fibers appeared and surround the follicle in circumference manner might represent the primordia of the theca externa cells (Fig. 8). The tertiary follicles were appeared and characterized by well organized corona radiata cells and increased number of granulosa cells, increased number of theca cells and the theca cells were organized to differentiated into theca interna and theca externa. The antral cavity appeared and filled with antral fluid (Fig. 9). The antral cavities were appeared numerous and large in size, while the granulosa cells increased and fill the spaces between the antral cavities (Fig. 10). The zona pellucida was strong PAS positive reaction while the theca and the CT between the follicles were faint PAS reaction (Fig. 11). Some follicles showed atresia and surrounded by numerous CT with disruption and desquamation in the granulosa cells (Fig. 12). The epithelium lining of the ovary became high cuboidal epithelium. The mature follicles appear at the late stage of the immature age. It characterized by well organized, granulosa cells, theca cells and appearance of cumulus oophurus, which hanged up the oocyte inside the antral cavity. The mature follicle characterized by positive PAS reaction in the oocyte, granulosa and theca cells (Fig. 13).

The ovary of the pregnant doe showed increased in size. The covering epithelium lining decreased in size to be simple cuboidal epithelium rest on thick tunica albuginea. The medulla characterized by well developed blood vessels (Fig.14). The corpus haemorraghicum was formed after ovulation. It consisted of rest cells of the mature graffian follicle except oocyte which emerge from the stigma in the wall of the graffian follicle. Some granulosa cells showed disruption (Fig. 15). The blood oozes from the blood vasculature and fill the antral cavity of the corpus haemmorhgicum (Fig. 16). After fertilization the corpus luteum (CL) was formed. It formed from the cells of the granulosa and theca cells. It persisted during the period of pregnancy. It separated from the neighboring follicles by CT fibers (Fig. 17). The CL consisted of small and large lutein cells. The large lutein cells appeared pyramidal or oval in shape, large in size with faint basophilic cytoplasm and large basophilic nuclei while the small lutein cells appeared small ovoid cells with dark basophilic cytoplasm (Fig. 18). The CL characterized well organized to light large lutein cells in the middle of the CL while the dark small lutein cells located in the periphery of the CL (Fig. 19). Strong PAS reaction in

the tunica albuginea ovarian epithelium, CT fibers, zona pellucid and theca cells (Fig. 20), while faint PAS reaction in the granulosa cells. No metachromatic reaction was detected in the ovary (Fig. 21).

The lining epithelium of the ovary of lactating doe was cuboidal in shape rest on thick tunica albuginea (Fig.22). The blood vessels of the medulla showed thick wall and the CT fibers between the cortical follicle was increased .The tunica albuginea and the covering epithelium of the ovary was PAS positive while faint reaction in the CT of the cortex (Fig.23). Some follicles filled with 9-10 oocytes inside one follicle. It characterized by thick theca cells (Fig. 24). The cortex filled with numerous atretic follicles between the other developing follicles (Fig.25). The lutein cells, large and small showed vacuolation and sparsely desquamation in the cytoplasm with eccentric pyknotic nuclei (Fig.26). The reminants of the CL escape to the surface of the ovary and invaded by CT cells and fibers (Fig.27).

The ovary of the senile doe showed increase in the thickness of the tunica albuginea with increased CT fibers between the cortical follicles. Some cells of the covering epithelium showed desquamation (Fig.28). Numerous follicles showed disruption and atretic characters (Fig.29). The cytoplasm of the primordial cells showed sparsely degranulation and the nuclei showed pyknosis (Fig.30). Some follicles showed atresia with lymphocytic infiltration (Fig.31 and32). The other follicles showed normal pattern of development. The medulla blood vessels showed congestion (Fig.33). Numerous lymphocytic infiltrations occupy most of the medulla architecture. The corpus albicans located in the periphery of the ovary cortex under the tunica albuginea. It consisted of numerous CT that replaced the follicular cells. It was positive PAS reaction (Fig.34). All the atretic follicles, covering epithelium and tunica albuginea were positive PAS reaction (Fig.35).

Discussion

The ovary of the rabbit was consisted of cortex externally and medulla internally. It covered by pseudostratified columnar epithelium in the young age and became cuboidal epithelium in the mature ages. The cortex surrounded by tunica albuginea which consisted of CT. The ovary had the same oriental structure in all rabbit species and other animals except mares. This finding was supported by the result of (Bjersing et al., 1981; Junqueira, and Carneiro,2003 and Ozdemir et al., 2005). In contrast to this finding (Ozdemir and Dinc, 2002 and 2003) reported that the surface epithelium was simple cuboidal in young animals and simple squamous in older animals.

The changes in the epithelial shape in the covering epithelium from pseudostratified to cuboidal epithelium in the young and adult age due to cyclic changes and mitotic divisions during estrous cycle. This result was supported by the findings of (Ozdemir and Dinc, 2002 and 2003) in guinea pig and (Weakley, 1969) in hamster.

The medulla consisted of highly vascularized CT rich in blood vessels, lymph vessels and nerve fibers. This finding was in agreement with (Numazawa and Kawashima, 1982) in mouse , (Ozdemir and Dinc, 2002), in guinea pig and (Hafez, 1970 and Dellmann and Brown, 1981) in domestic animals.

The cortexes contain different follicles at different stages of development. Primary, secondary, tertiary and mature graffian follicles. Corpus luteum , corpus albicans and corpus haemmorrhgicum were prominent in the cortex. Numerous polyovular follicles were demonstrated in the cortex of the lactating does. Similar findings were evident in many

animals as the ovaries of some rodents and hamster (Bodemer et al., 1959; Bodemer and Warnick, 1961 and Weakley, 1969), guinea pig (Collins and Kent, 1964). In contrast to these results no polyovular follicles not detected in guinea pig (Ozdemir and Dinc 2002), and (Odor and Blandau 1968).

The oocytes surrounded by follicular cells, these cells appeared cuboidal or columnar in shape (corona radiata layer and granulosa cell layers). Zona pellucida was PAS positive materials. Similar findings have also been observed in rat (Sotelo and Porter,1959), in mouse (Odor and Blandau, 1968) and guinea pig (Ozdemir and Dinc, 2002).

The primordial follicles represented about a third of the follicles. While the majority of the follicles represented by the developing follicles up to the mature one. The sizes of oocytes, oocyte nuclei, and their ratios did not change with follicle stage up to the primary stage. Most of the follicles in the does rabbits were in the intermediary stages as that of the bovine ovaries, more than 80% of follicles were found to be at the intermediary or primary stage (Van Wezel and Rodgers, 1996).

The corpus luteum was formed after induced ovulation in does. It consisted of small and large lutein cells. The large cells were located externally, large in size with granular cytoplasm, while the small ones were located internally, small in size with small nucleus. These cells originated from theca and granulosa cells as that reported by (Buffet and Bouchard, 2001). The corpus luteum has a principal role in production of steroidal hormones, including estradiol, progesterone and androgen (Buffet and Bouchard, 2001).

The corpus luteum give progesterone hormone which is responsible for calming of the uterus during pregnancy and maintenance of embryos during pregnancy and differentiation between fertile and infertile cycles (Niswender et al.,2000). The steroidogenic cells in the luteum body are denominated large lutein cells (LLC) and small lutein cells (SLC), which can be distinguished by their size and other functional and structural characteristics (Alila and Hansel, 1984 and Fields and Fields, 1996).

Many atretic follicles were reported in the contour of the rabbit cortex. These follicles were strong PAS positive reactions. The atretic follicles represented different failed follicles to reach maturity (O'shea, 1970). Signs of atresia remained obscure, similar findings of a low rate of atresia have been reported (Himelstein-Braw et al.,1976). Apparently, resting follicles accumulate little damage, which may be associated with their low metabolic rate (Okatay et al.,1997). The increase in atresia may occur only after the initiation of rapid follicle growth.

The granulosa cells surround the oocyte and the antrum cavities. It was polyhedral to polygonal in shape with centrally located basophilic nuclei and acidophilic cytoplasm. Similar results were stated by (Nottola et al., 2006). While (Dhar et al., 1996; Gersak and Tomazevic, 1999 and Whitman et al., 1989), identified different granulosa cell subpopulations, corresponding to different stages of luteal differentiation

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List of Figures

Plate.1: photomicrograph of the young rabbit's ovary showing; in fig.1, the ovary consisted of outer cortex (C) and inner medulla (M), the covering epithelium is pseudostratified columnar epithelium (P) . H&E.X10. In.2, numerous primordial follicles surrounded by mesenchymal cells (arrow). H&E X40. In 3, fine collagen fibers extended between the cortical follicles (arrow) . Crossmon's trichrome X10. In 4, primary follicles were located at the edge of the cortex (o). The medulla consisted of CT rich in blood vessels, nerves and lymphatic (arrow). Crossmon's trichrome. X10. In 5, the medulla, epithelium lining of the ovary and the granulosa cells were strong PAS positive reaction (arrow).PAS.X10.In 6, the CT fibers between the follicle was faint PAS positive (arrow).PAS.X40.

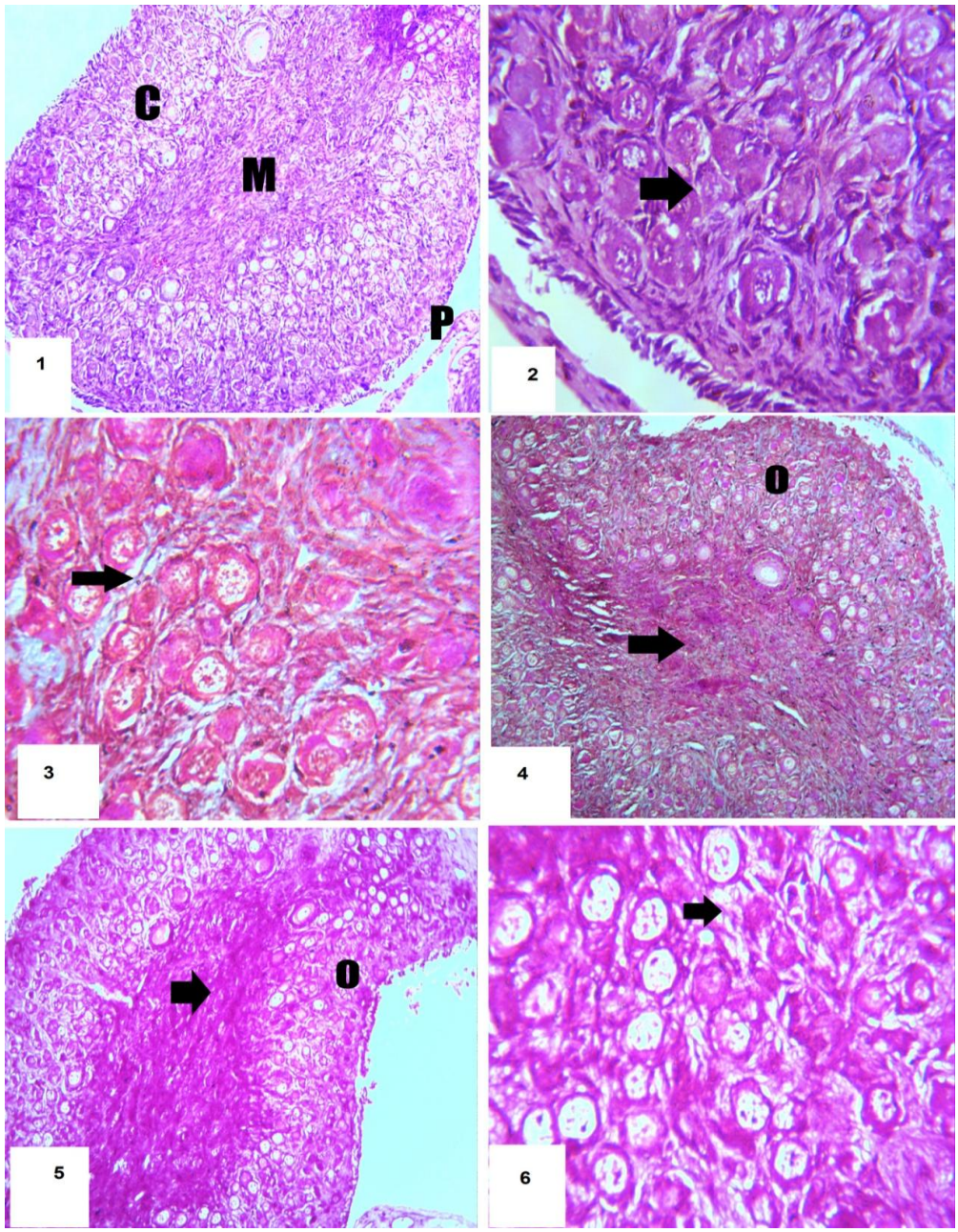
Plate.2: photomicrograph of the immature rabbit's ovary showing; in fig.7, the tunica albuginea (arrow) which consisted thick layer of CT. H&E.X10. In.8, in secondary follicle , zona pellucida (z), corona radiata (c) , granulosa cells (g) and theca externa primordia (arrow). H&E X40. In 9, the tertiary follicles characterized by well organized corona radiata (c) and increased number of granulosa cells (g). The antral cavity appeared and filled with antral fluid (f) . H&E X10. In 10, the antral cavities were appeared numerous and large in size (f), while the granulosa (g) cells increased and fill the spaces between the antral cavities Crossmon's trichrome. X10. In 11, the zona pellucida was strong PAS positive reaction (zp) while the theca and the CT between the follicles were faint PAS reaction (arrow).PAS.X40.In 12, no metachromatic granules were detected. Toulidine blue X40. In 13, some follicles showed atresia and surrounded by numerous CT with disruption and desquamation in the granulosa cells (arrow). Crossmon's trichrome.X40.In 14, mature follicle characterized by positive PAS reaction in the oocyte, granulosa and theca cells (arrow).PAS.X40.

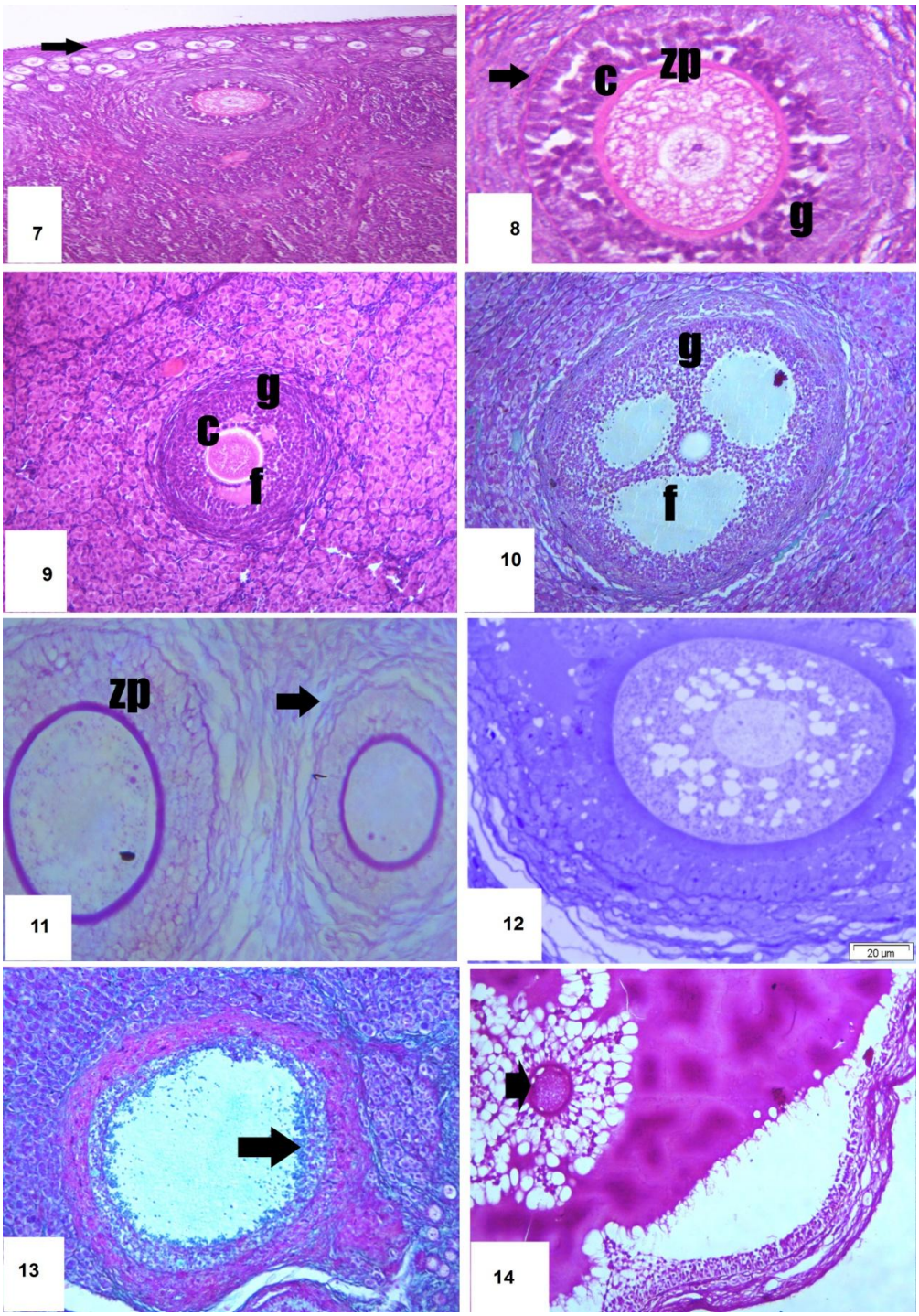
Plate.3&4: photomicrograph of the pregnant rabbit's ovary showing; in fig.15, well developed blood vessels in the ovary medulla (arrow). Crossmon's trichrome.X10. In.16, the rest of the mature follicle forming the CH (arrow). H&E X40. In 17, fully developed CH (ch) . H&E X40. In 18, the CL separated from the ovarian structure by CT (arrow) . H&E X10. In 19, The CL consisted of small and large lutein cells (CL) . In 20 , the large lutein (L) cells appeared pyramidal or oval in shape, large in size with faint basophilic cytoplasm and large basophilic nuclei while the small lutein (S) cells appeared small ovoid cells with dark basophilic cytoplasm. H&E X40. In 21, mature follicle characterized by positive PAS reaction in the oocyte, granulosa and theca cells (arrow).PAS.X40. In,22, no metachromatic granules were detected in the ovary.TB.X40.

Plate.5: photomicrograph of the lactating rabbit's ovary showing; in fig.23, thick tunica albuginea(arrow) and thick blood vessels in medulla (bv). Crossmon's trichrome.X10. In.24, the epithelium was positive PAS (arrow) while the CT was faint reaction (f). PAS X40. In 25, some follicles contains numerous oocytes (o) with thick theca cells (arrow) . H&E X40. In 26, numerous atretic follicles between the other follicles (arrows). H&E X10. In 27, The CL showed vacuolation (v) and sparsely desquamation (d) in the cytoplasm with eccentric pyknotic nuclei .Masson trichrome.X40.In 28, CT fibers invade the CL (arrow). H&E.X40.

Plate.6: photomicrograph of the senile rabbit's ovary showing; in fig.29, thick tunica albuginea(arrow) and desquamated some cells of the covering epithelium (d). H&E.X10. In.30, atretic follicles between the other follicles (arrows) . H&E X40. In 31, the cytoplasm of the primordial cells showed

sparsely degranulation (sd) and the nuclei showed pyknosis. H&E X40. In 32, Some follicles showed lymphocytic infiltration (L). H&E X40. In 33, Some follicles showed atresia (arrows) .H&E.X40.In 34, medullary blood vessels showed congestion (arrows). H&E.X40. 35, positive PAS reaction in the atretic follicles (arrows). PAS.X40. 36, positive PAS reaction in the covering epithelium atretic follicles (arrows). PAS.X10.

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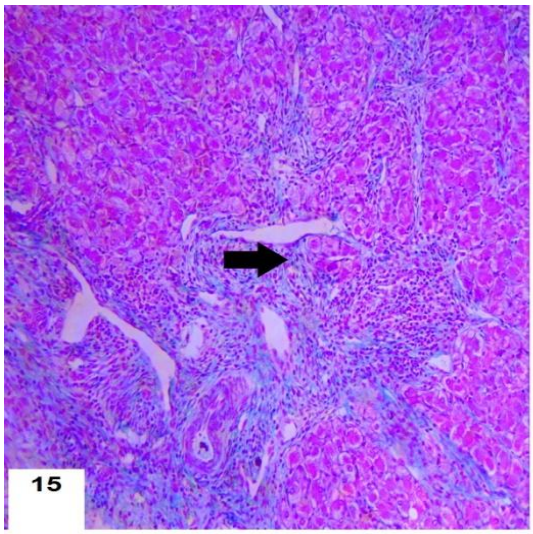
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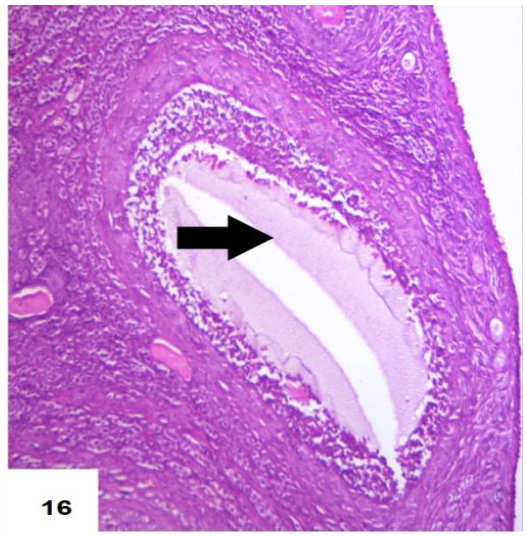
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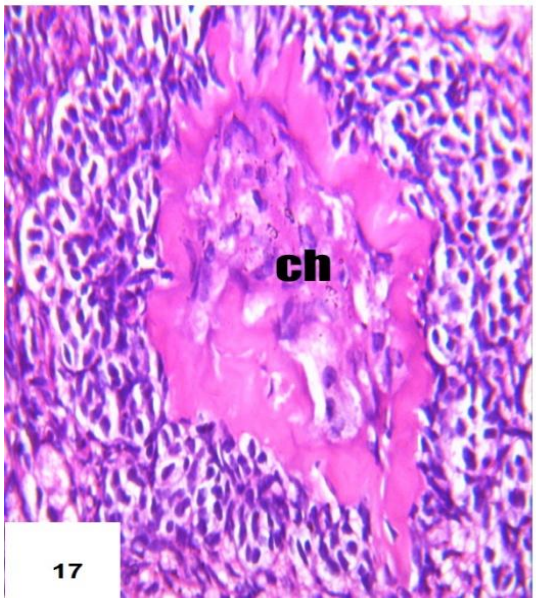
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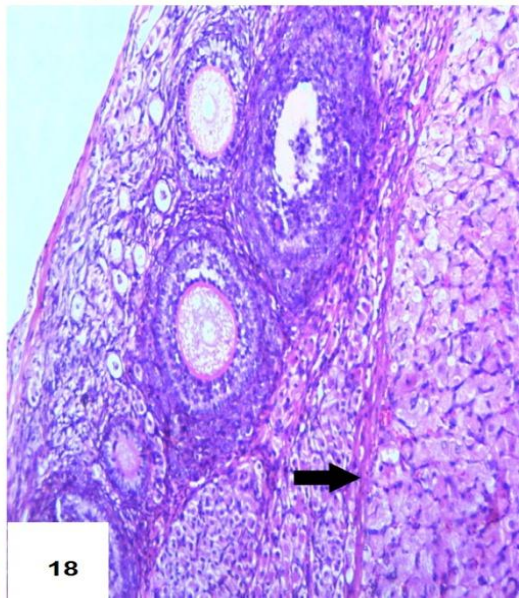
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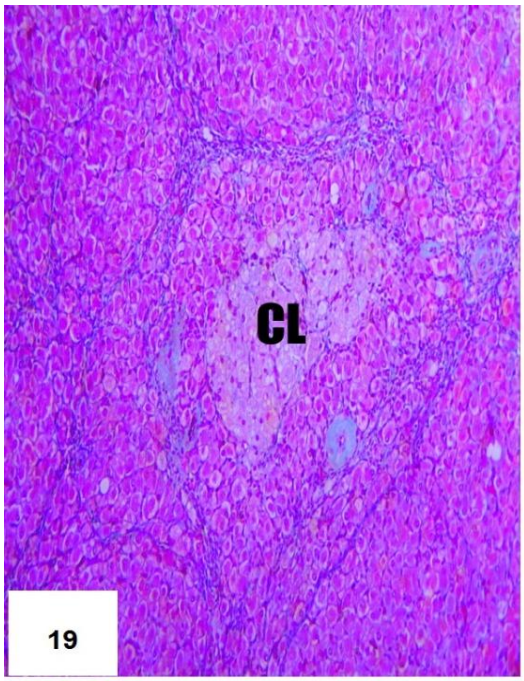
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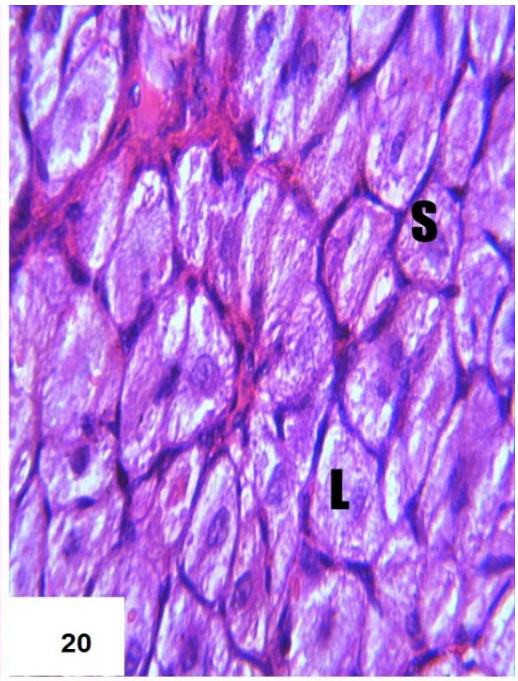
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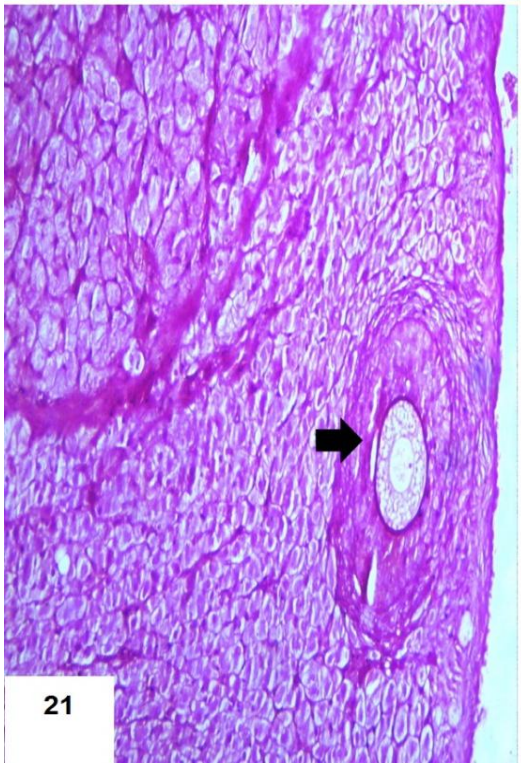
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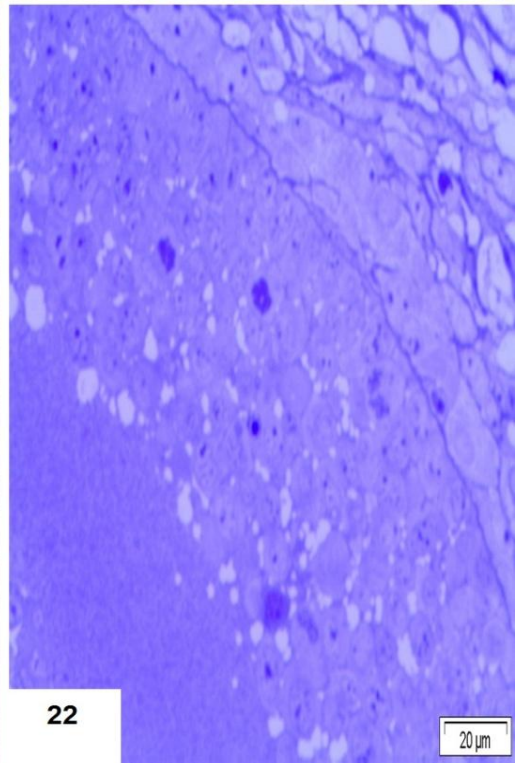
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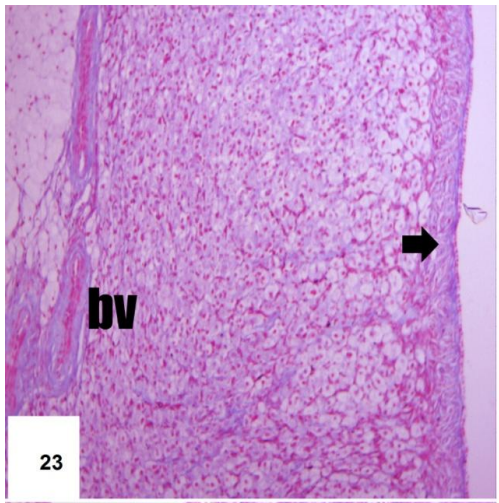
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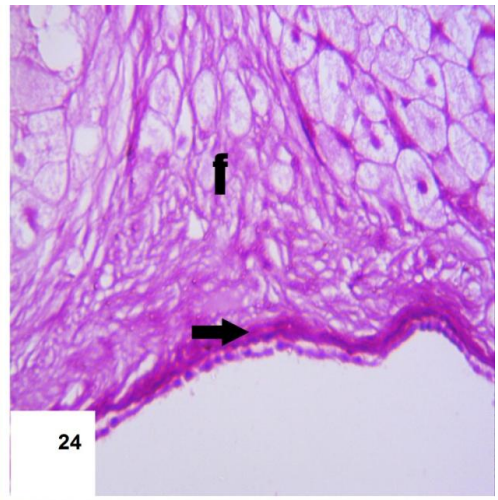
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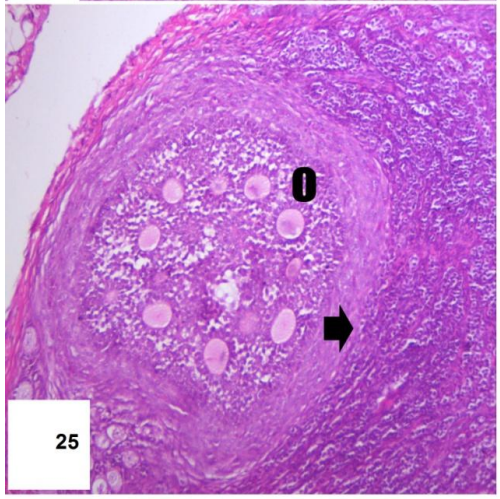
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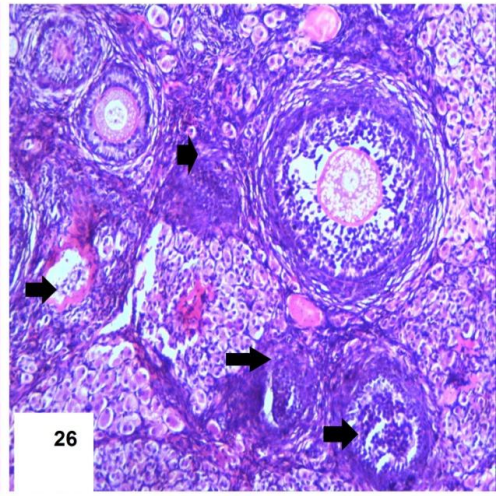
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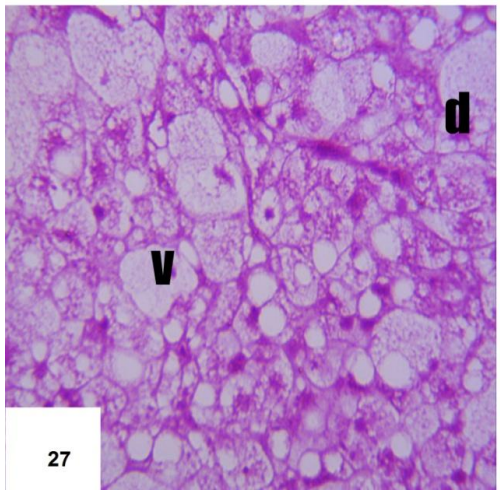
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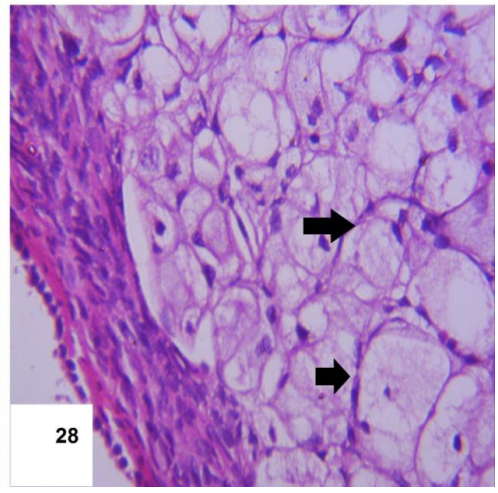
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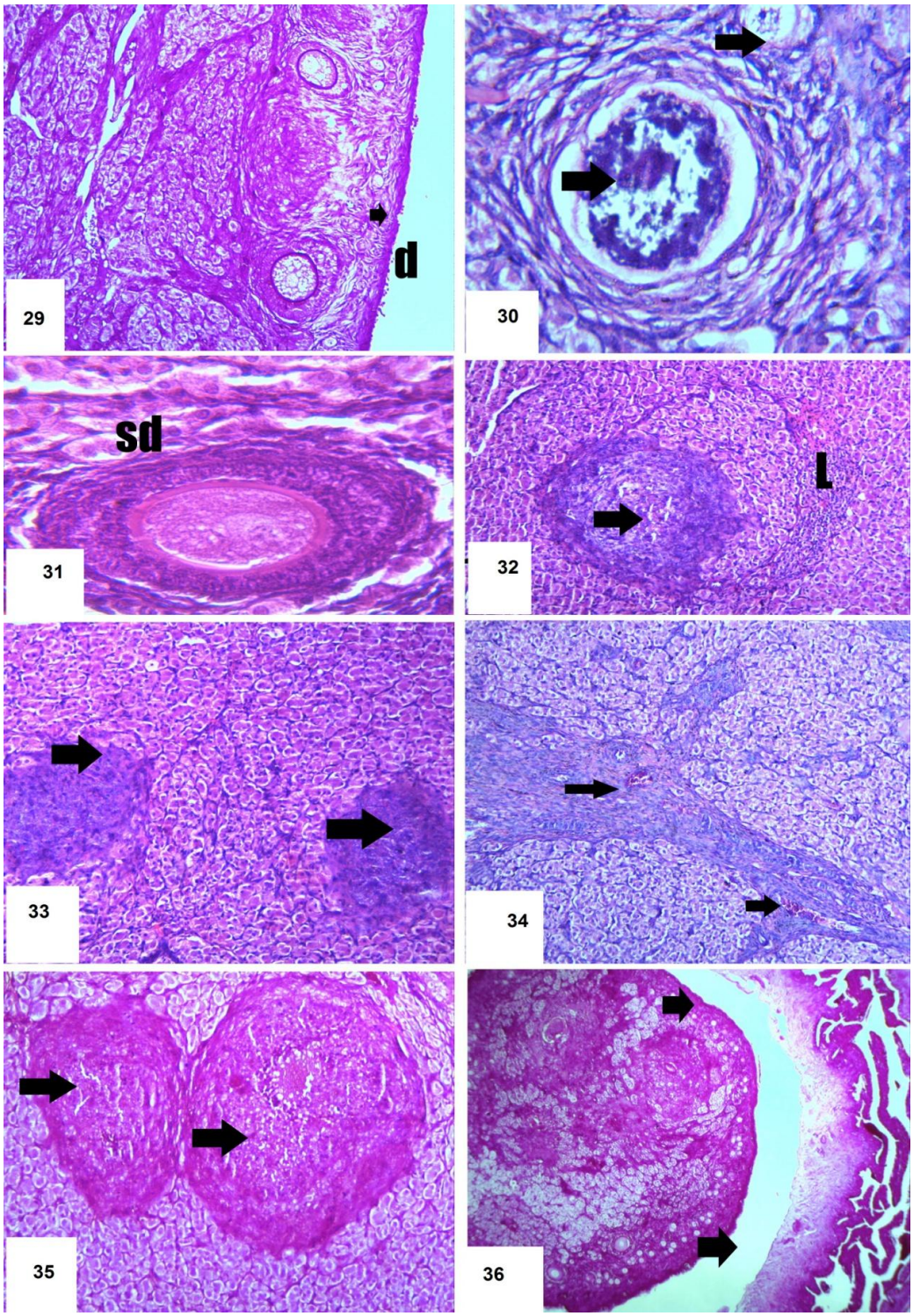
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IMPROVEMENT OF NEW ZEALAND RABBIT BUCKS PERFORMANCE BY L. TYROSINE SUPPLEMENT

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Abstract

Delayed puberty and reduced fertility during summer months considered one of the most problems encountered in rabbit production. Therefore, this study aimed to improve the performance of male New Zealand rabbits using L. tyrosine. For this purpose, 30 male New Zealand rabbits of two months age are used. They were randomly assigned into two groups: a control and L. tyrosine treated one (100 mg/kg b.wt). After one month, three bucks from each group was slaughtered and half of the treated group was administered a further similar oral dose of L. tyrosine while the second half was left without further treatment. Weekly blood samples collected from all groups7 for hormone assay of testosterone, T_3 and T_4. The results revealed that total body weight and serum testosterone, T_3 and T_4 significantly increased with advancing age in all groups. L. tyrosine induced a further, dose dependent, significant increase in body weight, GSI and serum testosterone, T_3 and T_4 as compared to control. Testes descent in the scrotum at the end of the 3rd month age of the control group and two weeks earlier in L. tyrosine treated one. Semen collection was firstly performed at the middle of the 4th month age of L. tyrosine treated group and one month later in the control one. L. tyrosine induced a significant increase in semen volume, sperm count, mass and individual motility and a live sperm percentage but abnormalities were significantly decreased ($P < 0.01$). Histological sections in testes, at the end of the 3rd month age, showed free spermatozoa in the lumen of seminiferous tubules in the treated group but it reaches to the spermatocyte stage only in the control one. The study recommends the addition of L. tyrosine in a dose of 100 mg/kg b. wt. to induce puberty and to improve fertlizability of male New Zealand rabbits during summer months

Introduction

Young rabbit is born and the testes hidden inside the abdominal cavity. They descend in the scrotum shortly before puberty at an age of about 3-4 months (Lebas *et al.*, 1986). Puberty of male rabbit defined as the stage when the endocrine function of the testes first becomes evident or it is the first time when the male becomes capable for producing spermatozoa in the ejaculate (Donovan and Bosch, 1965). It has been indicated by Sand (1979) that, puberty occurs a month or even more before rabbits attain sexual maturity and there is an age difference between different breeds of rabbits. Newzealand white bucks reached puberty at five months of age (Peter *et al.*, 1979). The growth of rabbit testes follows a sigmoid curve increasing rapidly during puberty (Gaddum, 1964). The weight of the individual Newzealand white rabbit testes was estimated to be in an average of 3.08 $\pm$ 0.1 g at about 4-5 months of age (Amann, 1970).

In New Zealand bucks, spermatogonial mitosis first appeared at 7-8 weeks of age and Leydig cell differentiation should precede the onset of spermatogenesis (Gondos *et al*., 1972). Spermatocytes were seen at 9-12 weeks of age, spermatid maturation stage was from 13-14 weeks of age and spermatozoa appeared in tubular lumen at the end of the 14th week (Gondos *et al.*, 1973).

Daily rhythm in the metabolism of dietary amino acids led to the discovery of a relationship between nutrient intake and neurotransmission (Wurtman *et al.*, 1981). L-tyrosine is a semi essential amino acid involved in formation of catecholamine from adrenal

gland and thyroxin from thyroid gland (El-Amrawi, 2008). L. tyrosine is an aromatic amino acid, blood-borne metabolites involved in the formation of catecholamines, thyroid hormones and protein synthesis (Harper *et al.*, 2000). Dopamine, one of the hypothalamic catecholamines, is effective in controlling prolactin secretion through activation of prolactin inhibiting factor (Kamberi *et al.*, 1971). It is also involved in activating the release of growth hormone (Muller, 1973).

L-Tyrosine is considered safe for all animal species, provided that the conditions of use are respected, i.e. supplementation of conventional diets with 0.5 % L-tyrosine for food-producing animals and 1.5 % for non-food-producing species (EFSA ,2013).Exogenous *L. tyrosine* was found to decrease the age of puberty in rats (Hammerl and Rüsse, 1987) and increase pulse frequency in growth-restricted lambs (Hall *et al.*, 1992). It also hasten puberty and increase body weight of female New Zealand rabbits (Abo-Elroos, 1992 and Omara *et al.*, 2005). *L. tyrosine* was also found to improve testosterone level, reaction time and semen picture of both bulls and buffalo-bulls (El-Amrowi *et al*., 1995).

The percentage of does kidded twins and triplets were higher among does treated with L-tyrosine than control group. Mortality rate was significantly lower in the does treated with L-tyrosine compared with control group (Abu El-Ella et al., 2011). Treated cows with L-tyrosine showed lower somatic cell count in milk, Postpartum estrous interval was shorter, Number of services per conception was less and Conception rate increased compared with those of control Friesian dairy cows (Gabr, 2012)

One of the problems encountered in New Zealand rabbits rearing is the decreased reproductive performance during hot months of the year (May-August). Therefore, the present study aimed to investigate the effect of oral administration of *L. tyrosine* on the induction of puberty and hormonal levels of T_3, T_4 and testosterone as well as semen picture in New Zealand bucks during hot months of the year.

Material and methods

Experimental animals:

This experiment was carried out at the period from March until July 2012. A total number of 30 male New Zealand rabbits of 1000 $\pm$ 50 g body weight and 50 $\pm$ 5 days age were used. They were acclimatized for one week. Rabbits were allocated into two groups a control (11 bucks) and treated groups (19 bucks). The treated group was administered orally *L. tyrosine* 100mg/kg b.w. (Serva, Heidelberg, Germany) according to El-Amrawi, (1997) at the age of two months. One-month post *L. tyrosine* administration, three bucks from control and treated groups were slaughtered for weighing testes and preparing histological sections from testes. Half of the treated groups (N = 8) was administered a further similar oral dose of *L. tyrosine* while the other half was left without further treatment. After another one month three bucks from each group were slaughtered. The remaining bucks (5 in each group) were used for semen collection and evaluation. During the experimental period, rabbits were fed on a diet composed of 16.76% crude protein, 2.36% ether extract, 12.62% crude fibers and digestible energy 2600 k. calories /kg in addition to the nutritional requirements of vitamins and minerals according to NRC (1977).

Blood samples:

Weekly blood samples were collected from ear vein of rabbit bucks for 10 weeks beginning from day of first oral administration of *L. tyrosine*. Serum samples were used for hormonal assays of testosterone, T_3 and T_4.

Histological samples:

Three bucks from control and treated groups were decapitated after one and two months from first dose. Testes were exteriorized, weighed to calculate the gonadosomatic index (GSI) $\frac{\text{Testes weight in gm x 100}}{\text{Total body weight in gm}}$.

Testes of the 4th month age were fixed in 10% neutral formalin for histological sections. 5-7 micron thickness sections from testes were prepared and stained with haematoxylin and eosin (Drury and Wallington, 1980) for studying the possible changes in spermatogenesis in testes.

Hormonal assays:

Testosterone (Active® testosterone RIA DSL-4000), thyroxine (T_4) (Active® thyroxine DSL 3200) and triiodothyronine (T_3) (Active® T_3 RIA DSL 3100) were measured by radioimmunoassay kits purchased from Diagnostic System Laboratories Inc. (DSL) according to Reiter and Grumbach (1982), Engler and Burger (1984) and Yalow and Berseon (1971) respectively.

Semen collection and evaluation:

From the day of first oral dose of *L. tyrosine*, bucks were observed and inspected (digital palpation) daily for the descent of the testes into the scrotum. Trials for semen collection from all buck groups using artificial vagina (A.V.) and live doe (to calculate the reaction time) were done. Semen was collected twice weekly at morning. After regularity of semen collection, each ejaculate was directly transferred to water bath at 35°C while various evaluation examinations were made after removal of the gel. The volume of ejaculate was recorded by graduated collecting tubes. Mass motility (0-5), individual motility percentage and sperm abnormalities were determined according to Salisbury *et al.* (1978). Sperm cells concentration was determined using Neubauer haemocytometer. The percentage of a live sperms was determined in Eosin-Nigrosin stained films according to Swanson and Bearden (1951). Semen evaluation was done for four months after the first dose of *L. tyrosine*.

Statistical analysis:

The obtained data were statistically analyzed according to Harvey (1990).

Results

Table (1) showed that one dose of *L. tyrosine* induced a significant increase in total body weight and testes weight as compared to control. Second dose of *L. tyrosine* induced a further increase in these parameters and GSI.

Serum testosterone level (ng/ml) was gradually increased with advancing age in control group. As compared to control group, *L. tyrosine* induced a significant, dose dependent, increase in serum testosterone level along the experimentation period (Table 2).
Serum T_3 and T_4 concentrations were significantly increased in all groups with advancing age until the end of the 4th month age and then after decreased. *L. tyrosine* treatment resulted in a significant increase in both T_3 and T_4 levels as compared with the control (Tables 3 and 4).

Concerning the reproductive traits of New Zealand bucks, it was noticed that, descent of testes was completed at the end of the 3rd month age in control group and two weeks earlier in *L. tyrosine* treated groups. Semen samples was firstly collected from *L. tyrosine* treated bucks in the middle of the 2nd month post treatment (4th month-age) but it occurs one month later in the control groups. *L. tyrosine* induced a dose dependent increase in semen

volume, sperm cell concentration, a live sperm percentage and mass and individual motilities with significant reduction in reaction time and total abnormalities percentage as compared to control (Tab., 5).

The histological sections showed that, in the seminiferous tubules, the spermatogenesis proceed to the spermatocyte stage only in the control group (Fig.1) at the end of the 3rd month age but free spermatozoa were observed in the lumen of seminiferous tubules of the treated groups at the same age (Fig. 2).

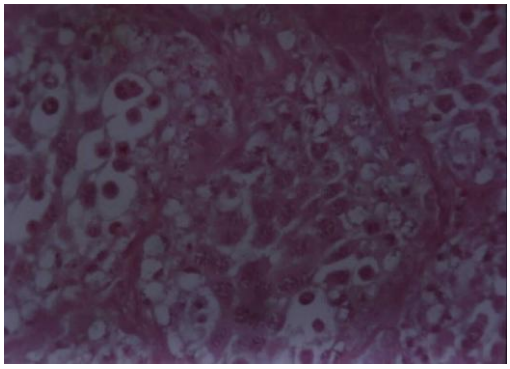

Fig. 1: Cross section in testes of control New Zealand rabbit at the 3rd month age showing seminiferous tubules with developmental stages reach to spermatocytes stages only (H & E x400)

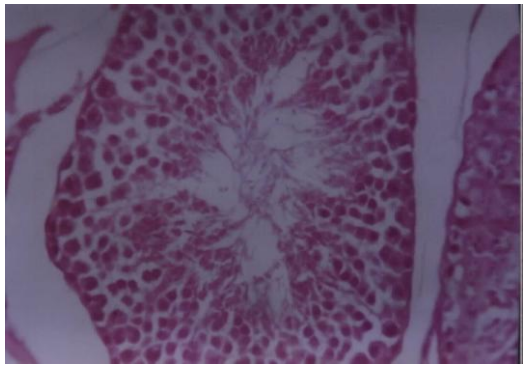

Fig. 2: Cross section in testes of the treated New Zealand rabbit at the 3rd month age showing free spermatozoa in the lumen of seminiferous tubules (H & E x400)

Discussion

One of the problems encountered in rabbit breeding is the decreased fertility and productivity in summer months of the year. Searching for solutions to this problem is necessary. *L. tyrosine* is one of the most prominent candidates under study to solve this problem. The amino acid tyrosine, a blood-borne metabolite, may be involved in stimulating GnRH release because availability of tyrosine influences synthesis of norepinephrine (Acworth *et al.*, 1988) a neurotransmitter that stimulate GnRH release (Terasawa *et al.*, 1988).

In the present study, *L. tyrosine* induced, a dose dependent, significant increase in total body weight (Table, 1) and serum levels of T_3 and T_4 (Table 3 and 4 respectively). These results were in accordance with Abo-Elroos (1992) and Omara *et al.* (2005) who recorded that the serum levels of T_3 and T_4 increased with advancing age and *L. tyrosine* induced a superimposed increase in such values. Tyrosine acts as a growth promoting factor as it activates the hypothalamic catecholamine, dopamine which in turn activates the release of growth hormone and TSH (Muller, 1973), in addition to its importance in formation of thyroid hormones and protein synthesis (Harper *et al.*, 2000). The thyroid gland's activity correlates with body weight as it is responsible for the basal metabolic rate. Increasing body weight requires more energy demands and more energy expenditure from mitochondria with increasing demands for more thyroid hormones (McDonald and Pineda, 1989 and Omara *et al.*, 2005). In addition, thyroid hormones stimulate appetite, enhance growth and stimulate most metabolic activities and metabolic rate. In addition, thyroid hormones stimulate

secretions of other glands and also increased the needs of tissues for hormones (Guyton and John, 1996). Increasing the circulating tyrosine by supplementation in the present study will be insured by increasing the level of TSH from anterior pituitary that stimulates the thyroid gland to secrete T_3 and T_4 (Harper *et al.*, 2000). Furthermore, koritschoner *et al.* (2001) recorded that thyroid hormones are major regulators of energy metabolism and T_3 upregulates tub-mRNA neuronal cells and defects in thyroid status are frequently associated with changes in body weight. Moreover, tyrosine was recorded to have a powerful antioxidant activity trapping free radicals (Van Overveld *et al.*, 2000), this may explain the increase in body weight after tyrosine supplementation in the present study. Abo-Elroos (1992) recorded that the pars distalis of male *L. tyrosine* treated rabbit's pituitary gland exhibited an increase in size and number of somatotrophes, lactatrophs and LH gonadotrophs cells that secrete growth hormone, prolactin and gonadotropins respectively. Consequently, *L. tyrosine* stimulates release of growth hormone, TSH and GnRH that are involved in increasing general metabolism and body weight gain and increase testicular activity. Release of FSH and LH (ICSH) would increase testosterone secretion and release. The decrease in T_3&T_4 levels after the 4^{th} month age was due to the known negative correlation between temperature and thyroid gland activity (May month).

Bearing in mind that ensuring the testicular descent into the scrotum and on completing testicular development, the testes begin to be functioning (Hafez, 1987). From the clinical observations in the present study, it has been noticed that, the tyrosine-treated rabbits were able to do successful mounting with the spermatozoa in their ejaculate earlier than that of the control. This finding added a further support to the suggestion that tyrosine might induce early puberty in male rabbits as a growth promoting factor (Muller, 1973) and a stimulant to the GnRH (Kamberi *et al.*, 1971) which potentate the testicular function.

It is of interest to notice that, the testes size of rabbit throughout the experimental period appeared remarkably larger in the *L. tyrosine* treated group than that in the control. This finding came to establish the close relationship between the testes weight and body weight, and also between the testes weight and the age of the animal (Amann, 1970). However, the magnitude of significance due to the effect of tyrosine superimposed that due to age. This may be due to the importance of tyrosine as a stimulant releasing factor of growth hormone, TSH, T_4 and protein synthesis (Harper *et al.*, 1980). This was clearly evident in this study in which L. tyrosine induced an increase in GSI (table 1) and the histological findings (Fig. 2) that showed free spermatozoa in the lumen of seminiferous tubules while it reached to spermatocyte stage only in the control group(Fig. 1) .

Regarding the seminogram (Tab. 5) and serum testosterone level (Tab. 2) in the present study it is obvious that *L. tyrosine* induced a significant increase in serum testosterone level and ejaculate volume, sperm count/ml, mass and individual motilities and alive sperm percentage while total abnormalities was significantly decreased as compared to control.
Hall *et al.* (1992) found a linear correlation between plasma and hypothalamic concentrations of tyrosine. *L. tyrosine* is the precursor of catecholamines which stimulate the hypothalamus to release GnRH via adrenergic receptor mechanism (Arthur, 1989). Release of GnRH stimulates the pituitary gland to secrete FSH and LH, the latter stimulates the Leydig cells to secrete testosterone (Anderson, 1992).

El-Amrawi (1997) attributed the increase in libido of bulls following oral administration of *L. tyrosine* to the stimulatory effect of GnRH on pituitary gonadotorpin secretion which initiated the production of androgen-binding protein, transferrin and induced

the maximum level of serum testosterone responsible for the improvement of libido. Shishkina (1988) recorded that the disturbed relationships between noradrenaline system of the brain and hypothalamo-pituitary-testicular complex is presumably one of the causes of associate changes in the reproductive system during selection for the domestic type of behaviour.

The increase in sperm cell concentration and ejaculate volume after *L. tyrosine* treatment in this study may be due to the effect of FSH, LH and testosterone hormones on spermatogenesis and effect of adrenaline and noradrenaline on smooth muscles in the wall of seminiferous tubules and epididymis (El-Amrawi, 1997).

In accordance with our results (Tab. 2&5) the reaction time and ejaculate volume were improved after injection of testosterone (Idris, 1977) which increases the sexual desire (Arthur, 1989). Farahat *et al.* (1979) cited that the increase in ejaculate volume after injection of GnRH might be due to the effect of testosterone on accessory glands' secretion. Moreover, Nasr *et al.* (1994) reported that, the reaction time, ejaculate volume and libido of bulls were improved after treatment with GnRH. El-Amrawi (1995) found a positive correlation between testosterone and total sperm count and a negative correlation between testosterone level and reaction time.

In the present work, *L. tyrosine* improved both individual and mass motilities of sperms. This improvement may be due to the stimulatory effect of *L. tyrosine* on GnRH release and consequently increase in FSH, LH and testosterone (Hafez, 1987; Roa, 1990, Nasr *et al.*, 1994 and El-Amrawi, 1995). Concerning the percentage of live sperm cells, it was noticed that, L. *tyrosine* increased the live sperm percentage. In consistence with our results, GnRH injecitoin improved the percentage of live sperm cells in azoospermia men (Gottaz *et al.*, 1992) and bulls (Nasr *et al.*, 1994). Furthermore, Abo-Elroos (1992) concluded that *L. tyrosine* acts as hormonal inducing factor and a synergistic transmitter between the hypothalamus, pituitary gland and testes.

The results of this study revealed that, there is a decrease in the sperm abnormality in *L. tyrosine* treated group. This result coincides with that of Idris (1977) who found a decrease in primary cell abnormalities after injection of testosterone. On the contrary, Nasr *et al.* (1994) found no variation in sperm abnormalities under injection of GnRH.

It could be concluded that oral administration of *L.tyrosine* led to an increase in body weight and improvement in libido and semen picture in male New Zealand rabbits, it could be effectively used to improve fertility during summer months.

Table 1. Total body weight, testes weight and GSI of New Zealand bucks treated with *L. tyrosine*.

		Total body weight (g)		Testes weight (g)		GSI %	
Two months		1256.7±40.9	f	--	-	--	=
Three months	Control	1756.7±57.3	e	4.93±0.27	e	0.280±0.02	c
	One dose *L. tyrosine*	1990.0±44.8	d	6.14±0.43	d	0.309±0.03	c
	Two doses *L. tyrosine*	--		--		--	
Four months	Control	2256.7±63.6	c	6.61±0.22	c	0.293±0.03	c
	One dose *L. tyrosine*	2696.7±87.4	b	9.90±0.95	b	0.367±0.03	b
	Two doses *L. tyrosine*	2960.0±99.5	a	14.04±1.31	a	0.474±0.04	a

Values are mean ± S.E.

Means with different letters in each column are significantly different at $P < 0.01$.

Table (2): Serum testosterone level (ng/ml) in control and L. tyrosine-treated New Zealand bucks

	0 days (2 month)	1 w	2 w	3 w	4 w (3 months)	5 w	6 w	7 w	8 w (4 months)	9 w	10 w
Control	0.250 q ± 0.05	0.357 qp ± 0.12	0.470opq ± 0. 17	0.540 nop ± 0.11	0.663 mno ± 0.09	0.737 lmn ± 0.08	0.817 klm ± 0.09	0.933 jkl ± 0.09	1.143 hij ± 0.11	1.243 hi ± 0.11	2.120 de ± 0.12
One dose *L. tyrosine*	0.266 q ± 0.06	0.667mno ± 0.07	0.833 klm ± 0.09	1.007ijk ± 0.12	1.117 hij ± 0.11	1.307 h ± 0.12	1.550 g ± 0.17	1.837 f ± 0.17	2.190 de ± 0.21	2.567 c ± 0.25	3.177 b ± 0.23
Two doses *L. tyrosine*	0.267 q ±0.05	0.657 mno ± 0.08	0.813 klm ± 0.09	0.957 jkl ± 0.09	1.163 hij ± 0.11	1.763 gf ± 0.16	1.970 ef ± 0.18	2.227 d ± 0.13	2.580 c ± 0.24	3.170 b ± 0.31	3.900 a ± 0.36

Values are mean ± S.E. W = weeks after treatment
Means with different letters in the table are significantly different at $P < 0.01$.

Table (3): Serum T_3 level (ng/dl) in control and *L. tyrosine*-treated New Zealand bucks.

	0 days (2 months)	1 w	2 w	3 w	4 w (3 months)	5 w	6 w	7 w	8 w 4 months	9 w	10 w
Control	107.3 l ±10.8	121.0 lk ± 10.9	146.3 jk ± 13.5	167.7 ij ± 15.2	183.3 hi ± 17.3	197.0 hi ± 20.2	215.3 gh ± 13.5	232.7 g ± 11.6	240.3 g ±12.8	242.0 g ±18.5	241.0 g ± 22.6
One dose *L. tyrosine*	111.0 l ±9.8	150.0 jk ± 10.4	190.0 hi ± 13.8	240.0 g ±11.6	289.7 f ± 20.6	309.7 f ± 13.9	343.0 cd ± 27.1	371.3 bc ± 15.9	353.3 cd ± 20.3	329.7 de ± 17.2	303.3 ef ±19.5
Two doses *L. tyrosine*	108.7 l ± 11.0	147.0 jk ±10.5	183.3 hi ±15.2	237.0 g ± 20.5	285.0 f ± 17.5	331.0 de ± 22.3	369.7 bc ± 16.4	410.3 a ±19.8	390.7 ab ± 27.1	370.0 bc ± 14.6	354.0 cd ±22.3

Values are mean ± S.E. W = weeks after treatment
Means with different letters in the table are significantly different at $P < 0.01$.

Table (4): Serum T_4 level (µg/dl) in control and *L. tyrosine*-treated New Zealand bucks.

	0 days (2 months)	1 w	2 w	3 w	4 w (3 months)	5 w	6 w	7 w	8 w (4 months)	9 w	10 w
Control	1.22 o ± 0.07	1.32 o ± 0.11	1.52 no ± 0.09	1.66 mn ± 0.13	1.76 mn ± 0.12	1.92 klm ± 0.07	2.01 klm ± 0.16	2.15 jkl ± 0.12	1.98 klm ± 0.14	1.81 lmn ± 0.09	1.73 mn ± 0.15
One dose *L. tyrosine*	1.22 o ± 0.12	1.89 klm ± 0.13	2.46 ghij ± 0.16	2.85 ef ± 0.24	2.94 e ± 0.23	2.84 ef ± 0.17	2.71 efg ± 0.15	2.67 fgh ± 0.11	2.52 fhi ± 0.22	2.35 hij ± 0.23	2.21 ijk ± 0.21
Two doses *L. tyrosine*	1.21 o ± 0.09	1.81 lmn ± 0.15	2.51 ghij ± 0.11	2.91 e ± 0.23	3.47 d ± 0.19	3.89 bc ± 0.30	4.27 a ± 0.31	4.16 ab ± 0.16	3.97 abc ± 0.18	3.76 cd ± 0.33	3.48 d ± 0.24

Values are mean ± S.E. W = weeks after treatment
Means with different letters in the table are significantly different at $P < 0.01$.

Table (5): Seminogram and reaction time in control and *L. tyrosine*-treated New Zealand bucks.

		2nd month post treatment (April)		3rd month post treatment (May)			4th month post treatment (June)		
	Control	One dose	Two doses	Control	One dose	Two doses	Control	One dose	Two doses
Volume (ml)	No semen	0.9± 0.034 d	1.1± 0.077 c	0.7± 0.044 e	1.2± 0.071 c	1.6± 0.093 a	0.7± 0.060 e	1.1± 0.034 c	1.4 ± 0.110 b
Sperm count(million/ml)	No semen	569.7± 11.17 c	680.0± 23.4 a	380.0± 19.8 e	490.0± 24.5 d	620.0± 24.7 b	310.0± 11.4 f	470.0± 17.9 d	590.0± 20.3 bc
Mass motility (0-5)	No semen	3.80± 0.07 c	4.20± 0.09 ab	3.20± 0.09 d	3.80± 0.11 e	4.33± 0.08 a	3.40± 0.10 d	4.00± 0.19 bc	4.03± 0.15 bc
Individual motility (%)	No semen	82.0± 3.6 a	92.7± 6.6 a	72.0± 2.9 bc	85.7± 5.1 a	89.0± 4.3 a	70.0± 3.6 c	82.3± 5.6 ab	88.0± 7.2 a
Alive sperm (%)	No semen	87.0± 3.3 ab	90.7± 4.3 a	80.7± 2.8 bc	90.0± 5.1 a	92.7± 3.8 a	77.0± 2.9 c	88.0± 4.1 a	92.0± 5.0 a
Total abnormality	No semen	5.3± 0.27 bc	4.7± 0.22 cd	9.1± 0.34 a	5.1± 0.21 c	4.3± 0.19 b	9.7± 0.62 a	5.9± 0.31 b	4.80± 0.16 cd
Reaction time (sec.)	No semen	55.0± 2.2 bc	42.0± 3.7 cd	99.0± 4.2 a	50.3± 1.9 cd	40.0± 2.1 d	95.0± 7.2 a	65.0± 5.8 b	49.7± 3.2 cd

Values are mean +_S.E.

Means with different letters in each raw are significantly different at $P < 0$.

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EVALUATION OF THYROID AND OVARIAN HORMONES IN RABBITS AT HIGH ALTITUDE

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Abstract

This study was done on 25 female rabbits (does) . The female rabbits (does) were collected from the Taif governorates. The serum was collected for evaluation of thyroid and ovarian hormones at high altitude.The serum level of (T3 and FSH) of the rabbit doe showed gradual decrease from young up to senile age while the serum level of (T4) showed gradual decrease from young up to mature pregnant age then it showed increase in the adult lactating age and significant decrease in senile age. he serum level of estradiol showed gradual decrease from young up to senile age while The serum level of progestone showed increase from young age to immature age then the hormone showed evoke at mature pregnant age then it showed decrease at adult lactating age and another evoke at senile age.

Introduction

The productions of domestic rabbits are used for many purpose, meat, fur, laboratory diagnosis and research. Rabbit's meat of all ages shows high values for human consumption, it has a higher percentage of protein than other meats and the meat itself is highly digestible, for this reason it is often recommended for sick people (Sandford and Woodgate , 1974 and Zotte, 2002).

The thyroid gland is the largest endocrine gland in the body. It located at the third tracheal ring below the thyroid cartilage, consists of two lobes connected by narrow isthmus. (Parchami and Dehkordi, 2012).

Thyroid function is manufacturing of thyroid hormones which are; T3 (triiodothyronine) & T4 (thyroxine) via follicular cells which represented the bulk cells of the thyroid follicles. Thyroid hormones are important in stimulating enzymes essential for glucose oxidation, thereby controlling cellular temperature and body metabolism. (Moura et al.,1987).

The ovaries are two in each side. It located on the lateral wall of the pelvis. It supported by the broad ligament. The ovary composed of cortex externally and medulla internally. The ovary functions as an organ of reproduction and also as a gland of internal secretion.. It secrete the mature graffian follicles (Exocrine function) and non-steroidal hormones that act locally (Endocrine function) (Hodges et al.,1985).

High altitude induces various physiological changes in sea level natives, both on acute and more prolonged exposure, including control of hormonal secretion. A substantial amount of work has been done on hormones regulating water and electrolytes handling or stress hormones at high altitude. Other hormones dealing with metabolic regulations have been scarcely examined in hypoxic conditions. Most studies showed increase in the (T3 and T4) although TSH secretion did not showed any changes at high altitude (Barnholt et al., 2006), while other papers showed decrease in thyroid hormones (Sawhney and Malhotra,1990).

Certain biochemical, physiological and microanatomical responses occur during acclimatization and adaptation to chronic hypoxia of high altitude, so the aim of the present

work is to evaluate the thyroid and ovarian hormonal changes (T3, T4, TSH, estrogen and progesterone).

Materials and Methods

This study was done on 5 groups of female rabbits (5 does in each group) representing the different physiological pattern of the female life. The female rabbits (does) were collected from the Taif Governorates (at 2512 meter high altitude at temperature (24-19 °C) , atmospheric pressure(365-367 atm) and RH (31-30 %). The collected rabbits belong to order Lagomorpha and family Leporidae. They collected and raised at Al-shafa rabbits farm.

Experimental design

Five Groups of Female rabbits were classified according to age and physiological status young age-immature- mature pregnant – adult lactating - senile).

Blood samples were collected from jugular vein of the rabbits under ether anesthesia (Boussarie, 1999). Then, the blood was centrifuged at 4000 rpm for 15 min and serum was collected for hormonal assay.

Results

The thyroid hormone (T3) was 121.6333± 0.338428 in young age, 120.4667±0.351188 in immature age, 119.4±0.4 in mature pregnant age, 119.6±0.4 in adult lactating age and 117.9667±0.378594 in senile age. The serum level of (T3) showed gradual decrease from young up to senile age but not significant (Table.1 and Fig.1).

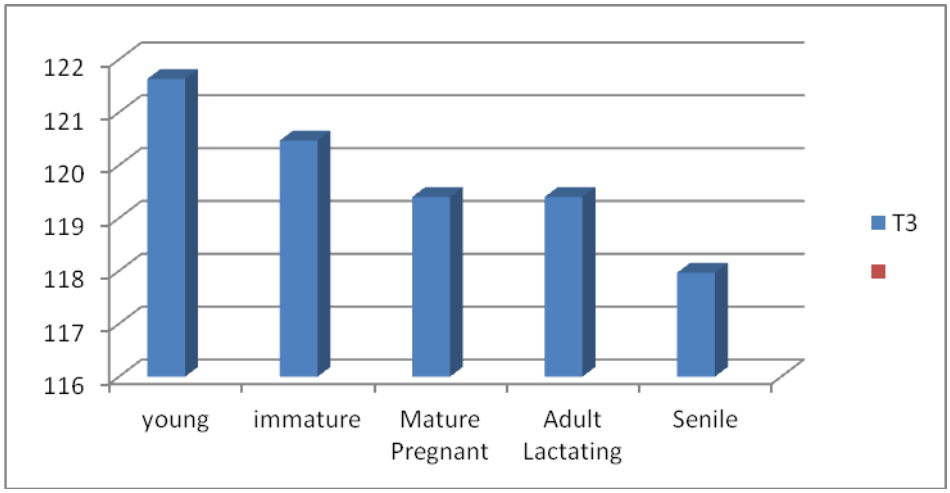

Fig.1: chart showing the serum level of (T3) at high altitude during different age.

The thyroid hormone (T4) was 4.456667± 1.069268 in young age, 4.3±0.608276 in immature age, 4.086667±0.080829 in mature pregnant age, 4.13±0.417971 in adult lactating age and 3.823333±0.254231in senile age. The serum level of (T4) showed gradual decrease

from young up to mature pregnant age then it showed increase in the adult lactating age and decrease in senile age. These changes were significant (Table.1 and Fig.2).

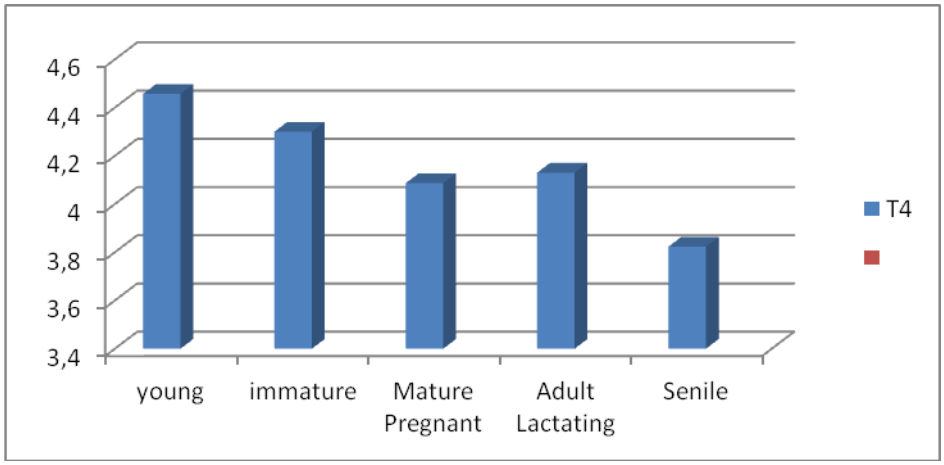

Fig.2: chart showing the serum level of (T4) at high altitude during different age.

The thyroid hormone (TSH) was 2.436667±0.60302 in young age, 1.89±0.363868 in immature age , 1.77±0.331512 in mature pregnant age, 1.46±0.130767 in adult lactating age and 1.306667±0.106927 in senile age. The serum level of (TSH) showed gradual decrease in TSH from young up to senile age. These changes were significant (Table.1 and Fig.3).

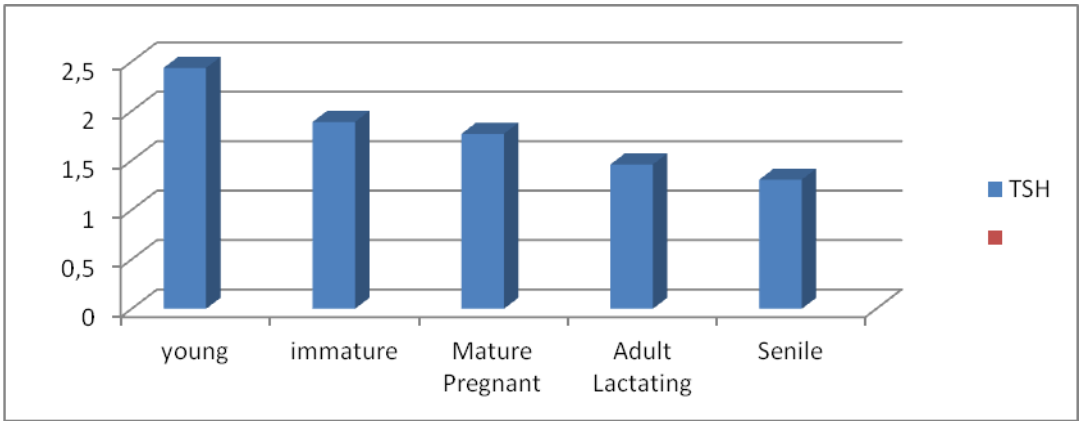

Fig.3: chart showing the serum level of (TSH) at high altitude during different age.

The estradiol hormone was 628.6667±23.07235 in young age, 555.6667±32.1455 in immature age, 535.3333±21.73323 in mature pregnant age, 509.3333±9.609024 in adult lactating age and 493.6667±13.6504 in senile age. The serum level of estradiol showed gradual decrease from young up to senile age. These changes were significant (Table.1 and Fig.4).

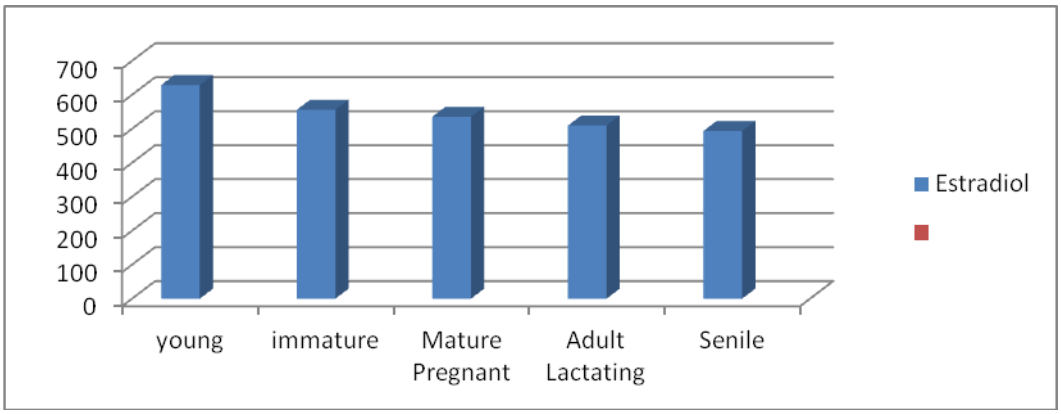

Fig.4: chart showing the serum level of estradiol at high altitude during different age

The progesterone hormone was 22.85667±0.176163 in young age, 22.93333±0.723418 in immature age, 23.62333±0.831284 in mature pregnant age, 23.14±1.160517 in adult lactating age and 23.83333±0.321455 in senile age. The serum level of progestone showed increase from young age to immature age then the hormone showed evoke at mature pregnant age then it showed decrease at adult lactating age . The hormone level showed another evoke at senile age. Significant changes in immature and senile age (Table.1 and Fig.5).

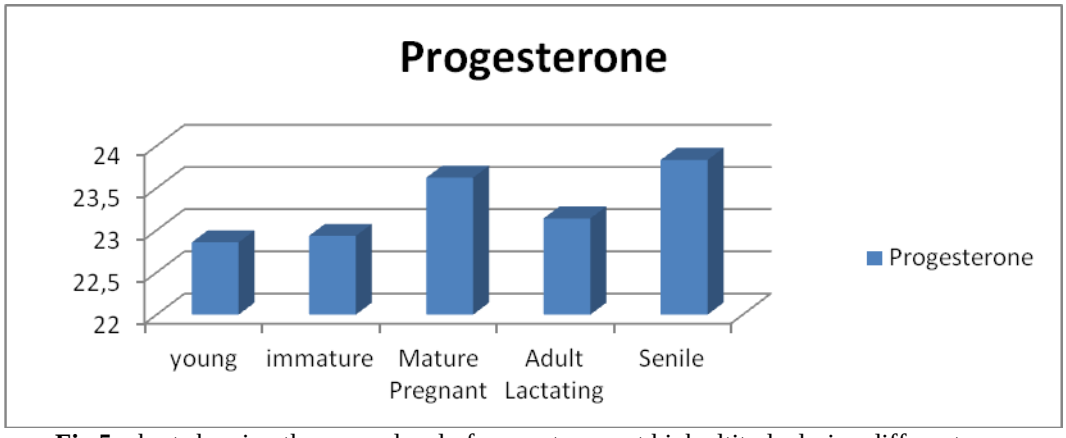

Fig.5: chart showing the serum level of progesterone at high altitude during different age.

Table.(1): Serum evaluation of hormones at high altitude

	young	immature	Mature Pregnant	Adult Lactating	Senile
T3 dl/ng	121.6333± 0.338428	120.4667±0.351188	119.4±0.4	119.4±0.6	117.9667±0.378594
P<0.05		0.268953166002754 3	0.741207308908814 9	0.758227715458080 1	0.063646663795972 77
T4 dl/ng	4.456667± 1.069268	4.3±0.608276	4.086667±0.080829	4.13±0.417971	3.823333±0.254231
P<0.05		0.020340001898674 54	0.040594010066266 2	0.016815395560309088	0.022413885807281 07
TSH ml/ng	2.436667±0.60302	1.89±0.363868	1.77±0.331512	1.46±0.130767	1.306667±0.106927
P<0.05		0.007152120255869413	0.008191341423056 91	0.008129271858356 255	0.013203020191325 573
Estradiol ml/pg	628.6667±23.07235	555.6667±32.1455	535.3333±21.73323	509.3333±9.609024	493.6667±13.6504
P<0.05		0.035944330094193 365	0.007538842658045 83	0.001244373851924 051 7	0.001007697875258 8
Progesterone-ml/ng	22.85667±0.176163	22.93333±0.723418	23.62333±0.831284	23.14±1.160517	23.83333±0.321455
P<0.05		0.030499152762722 397	1.0	0.488310018627448 5	0.008428195031397 107

Discussion

The reduced availability of oxygen owing to low barometric pressure is the basic problem associated with high altitude (HA). The acute exposure to reduced partial pressure of oxygen at (HA) decreases arterial oxygen saturation, stimulates the sympathoadrenal system, and provokes shifts in substrate metabolism (Mazzeo and Reeves,2003).

Our results indicated gradual decrease in the level of plasma T_3 and T_4, and it reached the maximum decrease at senile age. These observations in rabbits are in agreement with those of Martin et al., (1971) in rats who observed a 54% reduction in plasma protein bound iodine (PBI) and of Connors and Martin (1982) who recorded a marked decline in plasma T_3 and T_4 in rats exposed to chronic hypoxia for 5 weeks. These results indicate that repeated short-term hypoxic exposure is able to diminish the effect of chronic hypoxia on thyroid function. However, our findings of decreased thyroid activity in rabbits exposed to hypoxia are contradictory to that of Nelson and Anthony (1966) who observed unaltered thyroid activity in rats exposed to simulated altitude of 5486 m. Lowering serum level of T4 and T3 lead to hypofunction and failure of the thyroid activity (Ingbar and Woeber 1981).

Our results also reported decrease in the serum level of TSH. This hormone is secreted from the anterior pituitary as stimulating hormones for thyroid function through negative feedback mechanism (Hershman and Pekary 1985). More recently, evidence for thyroid hormone inhibition of thyrotrophic releasing hormone (TRH) release has become

available (Rondeel et al., 1988). On the other hand (Benso et al., 2007) showed an increase in free thyroxine and decrease in free tri-iodothyronine (T3) but the level of TSH not changed.

The serum level of female hormone showed fluctuation in rabbits does serum at high altitude due to cyclic changes. So the estimation of the hormones affected by the cyclic phase of the rabbit estrous cycle. These results were supported by the findings of (Escudero et al.,1996) reported that during early follicular phase serum estradiol levels were significantly higher at high altitude than at sea level ($P < 0.05$). During the late follicular phase the production of estradiol was higher at sea level than at high altitude ($P < 0.05$). The peak of serum estradiol was at day -1 in Lima and in day 0 at high altitude. At ovulation, the serum estradiol levels in women at sea level were 55.1% of the peak, but remained at high levels (80% of the peak) in women at high altitude ($P < 0.05$). The second increase of serum estradiol occurred earlier at sea level than at high altitude. From days +12 to +15, there was a significant decline in serum estradiol levels in women at sea level ($P < 0.05$) but not in those from high altitude ($P > 0.05$). Serum progesterone levels at days +5, and +8 to +12 were significantly higher at sea level than at high altitude.

Our results showed increase in the level of progesterone during the life time of the rabbits at high altitude. This increase is due to progesterone is known to increase hypoxic ventilatory responses with an improvement in oxygen saturation and a reduction in hematocrit for persons residing at 3100 m (Kryger et al.,1978) and to benefit patients with hypoventilation syndromes and sleep apnea (Milerad et al.,1985).

In animals, progesterone reduces brain edema, (Roof et al.,1992) possibly by tightening the blood brain barrier through the inhibition of Na1/K1-adenosine triphosphatase (ATPase). Progesterone stimulates an estrogen-dependent receptor at hypothalamic sites and influences the respiratory center via a neural pathway (Bayliss et al.,1992). Progestrone made improvement in peripheral oxygen saturation (Wright et al., 2004)and (Benso et al., 2007). On the other hand high altitude induced increase in progesterone levels but no change in pituitary, gonadal, and adrenal hormones in subjects who had a prolonged stay at high altitude but were not performing any physical activity (Basu et al.,1997). In contrary to our findings (Escudero et al., 1996) showed decrease in the level of progesterone at high altitude.

The level of estrogen showed fluctuation in serum level. It always decreased in high altitude in rabbits. These results were augmented by the findings of (Escudero et al. 1996). While (Benso et al., 2007) showed no significant changes in the serum level of estradiol at high altitude.

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DISRUPTED SPERMATOGENESIS, A CASE STUDY OF *AZOOSPERMIA* FOLLOWING FIELD EXPOSURE TO METHYL BENZIMIDAZOL-2-YLEARBAMATE(MBY)

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Abstract

A fungicide, Occidor, carbendazim 50%wp,Methyl Benzimidazol-2-Ylearbamate (MBY), is widely used as a specific fungicide in Sharkia, Egypt in field crops, fruit, citrous and ornamental trees and vegetables. Thirty-one mature males, with ages ranged from 27 to 49 years were occupationally exposed to such fungicide during application, to control fungal diseases. They live in at same geographical area. These men didn't feel testis swelling and/or pain during or after the exposure, and they had suffered from infertility on an azoospermic background according to their medical reports. A case among the men is in a direct contact with Occidor for 11 years business, especially, he used his olfaction and gustation to confirm that the fungicide in not adulterated. He had suffered from infertility since and during his 7 years marrige, both of his libido and socio-sexual behavior are normal. Microscopic evaluation of biopsy sections revealed; absent spermatogenic cells, Short Sertoli cells and few spermatogonia were remaining and lining the seminiferous tubules. The basal lamina was relatively thickened, with relative peritubular numerous mast cells. Most of the epithelium was disrupted with complete arrest of spermatogenesis, except the presence of a tiny epithelial areas lining some tubules which still exhibiting appearance of spermatogenesis. Medical reports confirmed that the Sertoli cell syndrome was prevalent characteristic of the biopsy sections, in addition to an insignificant increase of FSH level. In conclusion, these findings could address for the 1st time, the human testicular response to in-field MBY exposure reported. It could be considered the environmental socio-andrological study in a trial to correlate the extended azoospermia to the previous and successive exposures to Carbendazim preparation, Occidor 50%, as well as, disrupting and arresting the spermatogenesis which preceded azoospermia and infertility among carbendazim-exposed humans. These findings simulate and in parallelism with the prevoius MBC-induced disrupted spermatogenesis in rats.

Keyword*: Azoospermia, Occidor (50% carbendazim, MBY), Carbendazim (MBC), Sertoli's cell syndrome (SCO), Gonadotoxins*

Introduction

There are increasing concerns on the adverse effects of environmental chemicals and/or hazards on the functional structure of male reproductive glands. Studies have expressed increasing focusing on the potential of substances in the environment to disrupt endocrine systems in humans and wildlife (Kogevinas, 2001; Weber et al., 2002). Some of the chemicals that are known to affect testicular functions and reproduction in humans via endocrine mechanisms include pesticides, polychlorinated biphenyls, dioxins, alkylphenols (nonylphenol and octylphenol), phthalates, drugs both under prescription and abuse, naturally occurring plant oestrogens (phytoestrogens) and mycotoxins (Monoski et al., 2002). Moreover, reports suggested a possible decline in human semen quality during the last 50 to 60 years (Carlsen et al., 1992; Auger et al., 1995).

MBC is a toxicant to the male reproductive system of rodents and dog, It produces hypospermatogenesis and multinucleated giant cells (U.S. EPA, 1979; Carter and laskey,1982; Linder et al 1988). It is either methyl 2-bezimidazole carbamate (MBC) or methyl 1H-bezimidazole-2-ylearbamate (MBY). Carbendazim is used as a reliable control for protecting the fruits and orchard from fungi. The physical form of Carbendazim is a white powder with negligible odor. Several MBC formulations are commercially used

internationally e.g.; Delsane M fuyngicide, wettable powder contain 10% MBC and 64% maneb, Delsane MX fungicide, a wettable power containing 6.2% MBC and 73.8% mancozeb and Granosan seeds fungicide, a powder containing 15% MBC and 60% maneb for dry treatment of seeds, colour seeds red (Dupont, Technical data sheet for MBC, 1983).

In Egypt, the used Carbendazim preparation in sharkia governorate is Occidor 50% Carbendazim (methyl benzimidazol-2-ylearbamate, MBY). It is a specific fungicide against and control fungal diseases (i.e. Basidomycetes, Ascomycetes and Fungi impertecti) in field crops, fruit, citrous and ornamental trees and vegetables.

Most of chemical substances suppressing the spermatogenesis are unsuitable for human use, either due to general toxicity or potential mutagenecity (Styles, 1973a). Studies on the effects of benzimidazole fungicides on the testis have shown that these agents cause cleavage of the apical cytoplasmic processes of Sertoli cells and sloughing of immature germ cells (Parvinen and Kormano, 1974). Also, most of compounds known to induce male infertility disrupt spermatogenesis (Jackson, 1970). Furthermore, The effects of MBC were severe in adult male rats received a dose 400 mg for 10 successive days , causing retardation of and disrupting the spermatogenesis in 4 of 12 rats (Carter et al 1987). Moreover, a single least effective 25mg/kg MBC dose had the potential to induce pre-mature release of spermatides in some tubules (Abuel-Atta, 1992).

Hess, Moore, Forrer linder and Abuel-Atta (1991), recorded that the atrophic tubules with only SertoIi cells remaining in the epithelium, were surrounded with thickened basement lamina, at day 70 post-treatment with 400mg benomyl /kg dose. Meanwhile, The short term response following 2 hours post-25 mg MBC exposue in rats were described in cross sectioned tubules as; sloughed multinucleated giant round and elongated spermatides in tubular lumina, leaving vacuoles across the seminiferous epithelium (Abuel-Atta,1992). Moreover, Azoospermia was found only when greater than 80% of the semininferous tubules were atrophies, which occurred and most frequent in 4 of 12 testis from orally exposed rats to the same dose (Hess, Moore, Forrer, Linder and Abuel-Atta 1991). Clinical and pathologic study of 2 male patients 33 and 37-year-old presented with infertility, showing bilateral Sertoli cell only syndrome. The Sertoli cell only syndrome is an entity in which seminiferous tubules show a total absence of germ cells with present Sertoli cells alone (Jalón Monzón A et al., 2003).

Biopsy seminiferous tubules with Sertoli cells only and spermatogenic arrest patterns were demonstrated in obstructive azoospermia with a significant increase in both peritubular and interstitial mast cells (Roaiah et al., 2007). The current investigation is focused on the cyto-archeticuture of the seminiferous tubules with special emphasis on the spermatogenic process of the biopsy from the azoospermic human case. We are trying to address a direct and/or indirect correlation between azoospermia with the random and repeated professional and behavioral exposures of related people to , and in contacts to the used fungicide Occidor in fields and in business, as well as on the literature background of experimental MBC-studies.

Material and methods

The studied cases are farmers living and working in a small village in Sharkia governorate. Based upon several medical reports of thirty-one mature male humans with ages rangedfrom 27 to 49 years, they are still suffering from infertility and azoospermia. Their businesses are still citrus orchards living, farming and/or trading with Occidor, Carbendazim

50% (MBY). They didn't feel neither testicular swelling and pain nor poisoning symptoms during/or after the exposure. From the surprising point of view, the male human who buys and sells Occidor was suffering infertility and azoospermia, he had dealt such business 11 years ago. He had married 7 years ago. He is still infertile. He had previously repeated fungicides professional exposure during his dealing with Occidor 50%. The H-&E- stained biopsy paraffin sections are thoroughly evaluated using a bright field light microscope.

Results

The seminiferous tubules of a fertile human are characterized by; irregular luminal epithelial surface, the presence of normal functioning Sertoli's cell with indented lightly stained oval-pearl-shaped nucleus and the presence of cells of spermatogenic lineage; spermatogonia, primary and secondary spermatocytes cells in recesses among these supporting cells, and different spermatides (pre-mature, elongating and round spermatides) in the adluminal sertoli cells compartment. The luminal surfaces of the tubules is irregular due to the presence some epithelia as wedged areas across the tubular epithelium, showing different cell associations (stages). Such seminiferous stages are 6 in mature human testis (Figs, 1, 2, 3, 5, 6).

In comparison to the normal histology of mature fertile man, most seminiferous tubular of the studied case were free from spermatozoa. Individual sperms were scattered (20-30/ C. S. of a tubule) nearby and sometimes in contact with the luminal epithelial surface. These tubules were lined with a low stratified seminiferous epithelium, its thickness was similar to each other. Sometimes some tubules appeared with different epithelial thickness. The lining epithelium appeared as sertoli cells and few spermatogenic cells in some tubules, but most of tubular epithelium was free from spermatids, showing maturation arrest. It was mostly free from primary and secondary spermatocytes.

Most of the cross-sectional areas of the tubules were lined with mainly short acidophilic only Sertoli cells with clear vacuoles and dense nuclei, sometimes spermatogonia beneath them. They were resting on a relatively thickened basal lamina. Round and/or elongating spermatids were missing from the most of the tubules or partially from some ones. Few multinucleated giant cells were observed across the epithelium or exfoliated in the lumina. Some tubules possessed a few and/or very few spermatogonia with dense nuclei (Fig; 7&8).

The majority of the cross-sectioned tubules didn't show spermatogenic stages across their lining epithelium, however, tiny areas of some ones showed apparently active or less active epithelium with presence of some elongated spermatids and/or polarized round ones, exhibiting a little complete and incomplete spermatogenic activity. More the 95% of the seminiferous tubules showed arrested a disrupted spermatogenesis. Some tubules were atrophied. The pre-spermiated spermatozoa were absent, however, premature elongated spermatids or spermatozoa appeared attached to luminal surface of the semineferous epithelium. Peri-tubular mast cells were present, but its number was relatively increased. The human who buys MBC and sells it to farmers is suffering from infertility and azoospermia, with an insignificant FSH increase less than the reference level.

Discussion

Although there is a clear progress in medical care, azospermia is still andrologic problem in front the infertile males and community. In turn, the probabilities for male fertility to repreduce become very low and very hard to be and to continue. Such cases negatively

affect those suffering individuals exerting a questionnaire, how will a family or a society renews itself?

Clinical and pathologic study of 2 male patients 33 and 37-year-old presented with infertility, showing bilateral Sertoli cell only syndrome, in which, seminiferous tubules show Sertoli cells with total absence of germ cells (Jalón Monzón et al., 2003), such finding comes in close to seminiferous tubules of the case study, which were lined with sometimes only Sertoli cells with a relative decrease of sereies of spermatogenic cells. Sometimes, most of primary and secondary spermatocytes were missing, which might reflects how severe azoospermia occurred on bases of the In-field exposure, as well as, negative intoxication through air-born exposure of men to occidor 50% carbendazim.

Sertoli cells of the biopsy were abnormally organized; dense nuclei and vacuolated cytoplasm, resting on a relatively thickened basal lamina in some tubules. Similar results were mentioned by Carter et al (1987), Hess et al (1991) and Abuel-Atta (1992).

Beside the thickned basal laminae, there were numerous mast cells. The tubules with Sertoli cell only and spermatogenic arrest patterns were demonstrated in obstructive azospermia with a significant increase in mast cells (Roaiah et al., 2007). Mast cells are significantly higher in defective spermatogenesis than in normal one. Their product tryptase activate fibroblasts and promote collagen synthesis. They could be involved in the etiology of defective spermatogenesis and testicular dysfunction. The number of total mast cells was significantly higher in defective spermatogenesis than in normal spermatogenesis (Apa et a., 2002)**.**

Hess et al (1991) described that more than 80% of the seminiferous tubules were atrophied with disrupted spermatogenesis, azoospermia was found only when greater than 80% of the semininferous tubules were atrophies, which occurred most frequent in 4 of 12 testis from orally exposed rats to the same dose. Similar findings and more than 95% of tubules appeared inactive regarding occidor-induced abnormal changes in seminiferous tubules. Moreover, Naki and Hess (1997) stated that there is a possibility of recovery of seminiferous epithelium that showed sloughing, except when efferent ductules are occluded, however, abnormal spermatids could be produced in the recovered epithelium due to the extended action of Carbendazim. Meanwhile in our investigation, only a few tubules exhibited a little but incomplete spermatogenic activity, these areas were limited to the few tiny areas of the epithelium, which still with active, less active or recovered spermatogenic epithelium, that might interpret the presence of individual spermatozoa in contact to the luminal surface of the tubule epithelium**.**

Sertoli's cells contain abundant MTs in its apical cytoplasm (Russell et al, 1981), Which appear to be involved in the control of spermiation (Russell, 1984), and when disturbed, cause disorganization of the seminiferous epithelium (Abuel-Atta,1992 and Hess et al, 1991). Benomyl and benomyl's metabolite (MBC) are widely used as a fungicide on food crops and ornamental plants. Because benomyl inhibits formation of microtubules in fungi, it has also suggested to induce hypospermatogenesis in rats (Ireland et al, 1979). The presence of several multinucleated giant spermatids and other exfoliated cells among the findings of the case study might be an indication of dissociation of the microtubules system inside Sertoli cell cytoskeleton by a microtubules poison like benomyl metabolite (MBC) or its formulations. Furthermore, Zhang et al., (2004) added, disruption of spermatogenesis may be due to a disruption in the physiology and/or morphology of the blood-testis barrier. Such data might let

Sertoli's cells unable to create a a specific micro-environment essential to accommodate and support spermatogenesis**.**

Azoospermia and a serum FSH level (>2-3 times reference range) within the reference range suggest possible spermatogenic failure or obstruction. Also, FSH elevation of greater than 2.5-3 times of reference range is diagnostic for disrupted spermatogenesis and its failure; however, plasma testosterone levels are typically normal. Findings on testicular biopsy may include severe hypospermatogenesis, maturation arrest-spermatid stage, maturation arrest-spermatocyte stage, or SCO syndrome. The latter syndrome must present as azoospermia; however, a minority of men with such syndrome has foci of spermatogenesis in a testis predominately suffered from SCO.

The most common presentation involving Sertoli-cell-only (SCO) syndrome is a azoospermic young man seeking evaluation for infertility, his semen analysis demonstrating azoospermia, may be due to spermatogenic failure or obstruction. Examples of causes of spermatogenic failure include genetic factors, hormonal factors, idiopathic factors, toxin exposure, history of radiation therapy, and history of severe trauma. These conditions may be associated with SCO syndrome. Less commonly, these men may have severely decreased sperm densities of less than 1 million sperm per ml. In the latter situation, the testes have foci of SCO syndrome and hypospermatogenesis. Sperm production may be patchy and heterogenous among the seminiferous tubules of azoospermic men. The causes of male infertility are heterogeneous but more than 50% of cases have a genetic basis. The most common cytogenetic defects associated with non-obstructive azoospermia are numerical and structural chromosome abnormalities, including Klinefelter syndrome (47,XXY) and Y chromosome microdeletions. Klinefelter syndrome is characterized by progressive testicular failure causing androgen deficiency and azoospermia in most patients (Kim and Mobley, 2009).

Our case study is considered the 1st environmental socio-andrologic study for an human being has unawareness of the dangerous risk with the hazardous use of Occidor; smelling , tasting and negative Carbendazim-inhaling during contacts with it or during buying , selling and spraying plants with such antimycotic chemicals. Moreover, peoples who live in houses in carbendazim- sprayed farms cultivated with citrous trees or others may be very easily targets to MBY, inducing infertility of individuals in direct and/or indirect contacts to preparations of such fungicides in commercial stores and/or MBY-treated farms. The human who buys MBY and sells it to farmers is suffering from severe azoospermia.

The seminiferous epithelium of the biopsy section was only sertoli cells in most of seminiferous tubules, on a background of the medical reports for seminal analysis, FSH level and microscopic examination.

From the surprising and interesting point of view,. It could address for the first time, a human testicular response to the In-Field Carbendazim exposure, for a period of 11 successive years. That azoospermic patient had married from a fertile young lady since 7 yrs of the total (11yrs) exposure period, and he still azoospermic. What was extra-surprising and confirming our expectations, is that patiant is always smelling and/or tasting the occidor 50% carbendazim in the his market before buying it, as on-himself-biologic proof that the commercial MBY formulation is it and is not sheeted with other powders.

The extensive Occidor use and contacts are sychoronized with the unawareness of people with MBC early and delayed effects, which may increase the hazardous risk and the expected antifertility on male humans and animals. Such findings might be due to the

previous exposures to Occidor 50% carbendazim with indefinite cumulative doses and unknown inter- and post-exposure time, which merits further investigations. We are trying to address a direct and/or indirect correlation between the microscopic appearance of the seminiferous tubules of the azoospermic case with the delayed impact due to hazardous use of the professional and behavioural exposure to antimycotic chemical Occidor 50%, on the literature background of MBC-azoospermia . So, the random and prolonged , may be successive exposures to Occidor 50% could be considered one of the professional exposure disease, having the potential to induce azoospermia among people in direct or indirect contact to such fungicide

In conclusion: The present study could address the 1st environmental-socio-infertility investigation among people previously exposed to the fungicide Occidor. Such study is considered the basic scientific background for the spermatogenic failure of most of the seminiferous stages, as well as, disrupting and arresting the spermatogenesis which precedes the azoospermia and infertility among the exposed humans. Such exposures to toxicants may negatively affect the reproductive health males. The use or the contact with Occidor 50% carbendazim is safe clinically except the induced azoospermia, which raises proposals for the possible following recommendations.

Recommendations:

1. If a couple is considering In-Vitro Fertilization and intracytoplasmic sperm injection (IVF & ICSI), a micromanipulation technique in which a single sperm is injected into an oocyte, they should be offered genetic testing with Klinefelter syndrome (47,XXY) and Y chromosome microdeletions assay and karyotyping before IVF & ICSI, to avoid defrauding the azoospermic patiants**.**
2. Future studies to collect sperm concentrations from the other azoospermic men after they become away and safe from contacts or inhalation of MBC preparations to see if there well be a hopeful change of an improved spermatogenesis depending upon the amount of time exposed or post exposure**.**
3. Because the use of MBY is safe and without clinical symptoms after poisening, a strong recommendations have be considered with the awareness of people with hazardous use of such harmful fungicide carbendazim
4. Chemosteralization and control of rats in fields, factories and homes, since the MBY is azoospermia and infertility inducing chemical, so the use of MBC formulations as baits or a chaw for rats is very much promising to control their population dynamics, which is in need for a further research proposal and investigations.
5. Male contraceptive method; Because of MBY exposure is safe clinically, a single or multiple doses could have the potential to induce azoospermia and infertility among male human. That merits a further future studies experimentally**.**

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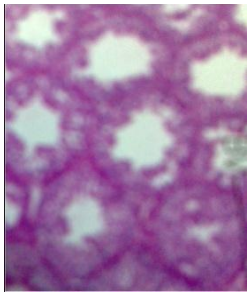
Figures

1, normal human seminiferous tubules, from Ross and Reith, 1985
2&3, normal human seminiferous tubules, Mescher2010
4, control rat seminiferous tubule, from Abuel-Atta, 1992
5&6, human seminiferous epithelium, Hess, 1998
7, seminiferous tubule of azoospermic man, Silber1 et al., 1997
8, azoospermic seminiferous tubule, the MBY-exposed case study
9, 12, 13,14,15, MBC-exposed rat seminiferous tubule, Abuel-Atta, 1992
10, 11, rat seminiferous epithelium different stages, Hess, 1998
16, atrophied tubules, some recovered ones, 70 days post-MBC exposed rat, Hess et al., 1991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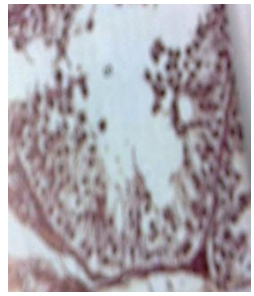
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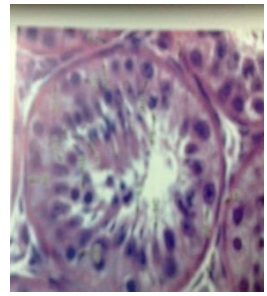
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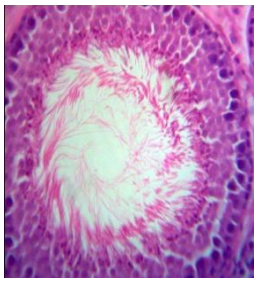
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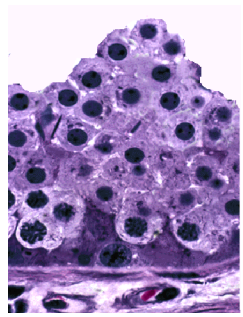
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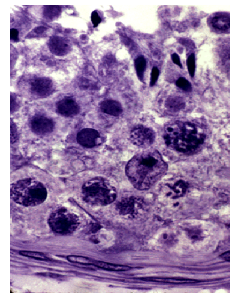
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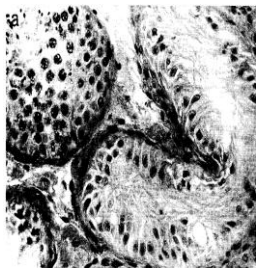
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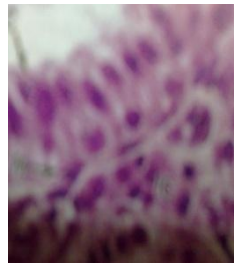
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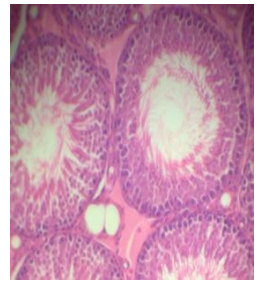
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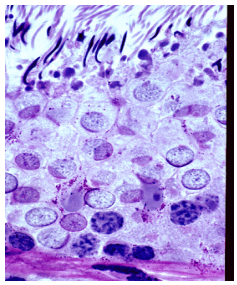

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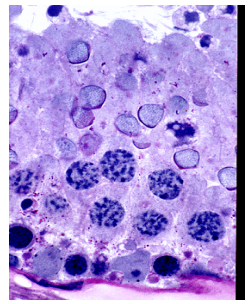

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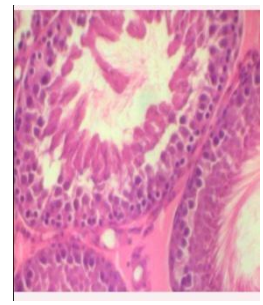

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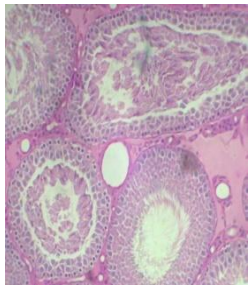

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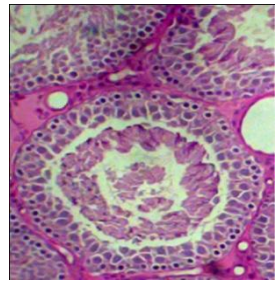

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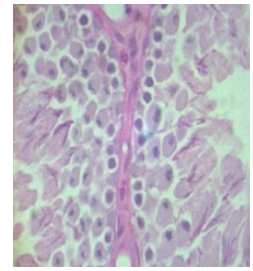

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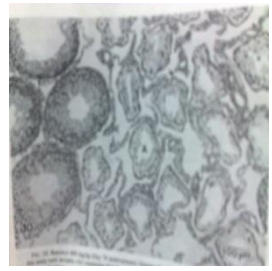

16

EFFECT OF PROBIOTIC FERMENTED SOY MILK AND GAMMA RADIATION IN AMELIORATING OXIDATIVE STRESS AND APOPTOSIS ON NITROSOUREA-INDUCED MAMMARY CARCINOGENESIS

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Abstract

Background and aim of the work: Antioxidants can reduce damage produced by low doses of radiation on living cells. This study was designed to investigate the effects of fermented soy milk (FSM) and low dose of gamma radiation on carcinogenic effect of N-methyl-N-nitrosourea (MNU). Materials and methods: Female rats were divided into 8 groups: group (1): control, group (2): injected with MNU, group (3): whole body exposed to low dose of gamma radiation (0.5 Gy), group (4): given FSM orally, group (5): given FSM and MNU, group (6): received FSM and exposed to gamma radiation, group (7): given FSM, MNU and exposed to gamma radiation. Results: Fermented soy milk exerted significant, ameliorative effect on glutathione peroxidase, superoxide dismutase and catalase activities, lipid peroxidation and TNF-α level and lipid profiles in rats injected with MNU. Combined treatment of FSM and low dose of gamma radiation markedly elevated GSH level, ameliorated MNU effect on cell cycle phases Go/1, S, G2/M and induce apoptosis via activation of caspase-3. Conclusion: FSM consumption with exposure to low doses of gamma radiation reduced carcinogenesis and oxidative stress effects induced by MNU in the mammary tissues.

Key words: *fermented soy, N-methyl-N-nitrosourea, mammary gland, cell cycle, TNF- α, gamma radiation, antioxidant state.*

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in female worldwide. The significance of nutrition in protecting living organisms from the toxic effects of environmental carcinogens has gained increasing attention due to less toxicity and high efficacy against various diseases. The intake of soy and soy-based products is associated with a lower risk of several types of cancers, including breast cancer. There are many functional ingredients contained in soy foods such as soy protein, isoflavones, saponins, phytic acid, phytosterol, and phenolic acid.The chemopreventive effects of soybean and soy containing food products may be related to genistiein, daidzein and glycitein (1).

Human have always been exposed to various natural sources of ionizing radiation emitted by the isotopes present in the earth's crust, air, water and biosphere, and also originating from the outer space. In some parts of the globe the level of this natural background radiation is significantly higher than the world average with no adverse health effects. Today, people can be additionally exposed to "man-made" radiation delivered at high doses (e.g., during radiotherapy and radiation accidents as well as after detonations of nuclear weapons) or low doses (e.g., during production and distribution of radioactive materials and use of radiation sources for industrial and medical purposes). The low-level environmental and occupational exposures are much more common and distributed over much larger populations than the high-level exposures(2).

Soybean fermentation by a system of Lactobacillus and yeast consists of a mixture of soybean extracts and the secondary metabolites of these microorganisms. In addition fermentation increased the bioactive isoflavoneaglycone than its unfermented counterpart. It

has been used in clinical trials to prevent cancer and cardiovascular disease progression due to its antioxidant activity (3), antimutagenic effect also for the reduction of chemotherapy side effects (4).

The lactic acid bacteria have cancer chemopreventive properties and act through diverse mechanisms, including alteration of the intestinal microflora, enhancement of the host's immune response, and antioxidative and antiproliferative activities (5). Some reports also claim that soymilk fermented with probiotic bacteria has some advantages: a reduced content of oligosaccharides, enhanced antioxidant activities, and improved flavor and sensory characteristics (6, 7). There is evidence suggesting that combining several probiotic bacteria will achieve stronger effects than single-strain probiotics (8).

Fermentation of Soy products using different types of microorganisms changes chemical components of soy and increase the soluble nitrogen compounds such as riboflavin, niacin, pantothenic acid, biotin, folic acid and nicotinic acid (9,10).

This work aim is to investigate the protective role of FMS and low dose of gamma radiation in reducing tumor incidence and progress induced by N-methyl-N-nitrosourea (MNU).

Material and method

Animals

Rats used in this study were Virgin female Sprague-Dawley at 42 days of age, with body weight of 130-150g. Rats were purchased from the Egyptian Holding Company for Biological Products and Vaccines (Cairo, Egypt). Animals were housed under standard conditions of light and temperature and allowed free access to standard pellet diet and tap water. Animals were randomly divided into eight groups (n=8).

Fermented soy milk (FSM)

Soy milk was purchased from Soy factor, food technology institute Agricultural research center, Giza, Egypt. The fermented soy was prepared using microorganisms: *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus lactis, Bifidobacteria* (**11**).FSM was diluted with distilled water to 2%and administrated orally at dose equivalent to 0.2 ml/kg body wt. daily.

Gamma radiation

Irradiation of rats was carried out using a Canadian Gamma cell-40 (137 Cs) at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. Rats whole body were exposed to gamma rays and received a dose rate of 0.461Gy/minute, calculated according to the Dosimeter department in the NCRRT.

Experimental design and sample collection

The N-methyl-N-nitrosurea (MNU) (Sigma–Aldrich, Diesenhofen, Germany) was injected intraperitoneally (I/P) twice (50 mg/kg/body weight each), between postnatal days 10 and 30.

Female rats were divided into 8 groups at the beginning of the experiment: group (1): served as negative control and orally received saline, group (2): Rats were injected with MNU, group (3): Animals were exposed to whole body gamma radiation (0.5 Gy), group (4): Rats were given FSM orally via gastric tube (20ml/kg), group(5): rats were given FSM and injected with MNU, group (6): Rats were received FSM (20ml/kg) and exposed to (0.5 Gy) gamma radiation, group(7):Rats were injected with MNU and given FSM before and after

injection, group (8): Rats were given FSM, injected with MNU and exposed to gamma radiation (0.5Gy). At the end of the experiment (13 weeks) animals were anesthetized and sacrificed, Heparinized blood samples were collected from the heart. And mammary glands tissues were dissected.

Evaluation of tumor necrosis factor- alpha (TNF-α):

TNF-alpha concentration in rat mammary gland was measured using the "Assay Max Rat TNF-alpha ELISA kit of murine monoclonal antibody". (ASSAYPRO, 41 Triad South Drive St. Charles, MO 63394, USA).

Evaluation of apoptosis and cell cycle analysis by flow cytometry:

Flow cytometric analysis was performed for cell cycle analysis and evaluation of apoptosis. Mammary glands were cut into small pieces and fixed in 70% ethanol in phosphate buffer saline for 1 h on ice, incubated with 50 µg/ml RNase A at 37ºC overnight, stained with 50 µg/ml propidium iodide and subjected to flow cytometric analysis using FACS Calibur. Cells were then analyzed for green (FITC, indicating DNA fragmentation detection) and (PI, allowing DNA quantification) red fluorescence by flow cytometry using a Becton Dickinson® FAC Star Plus flow cytometer. Apoptotic cells were identified in a DNA histogram as a sub-G1 hypodiploid population was obtained with a computer program for Dean and Jett mathematical analysis (12).

Antioxidant parameters:

Lipid peroxides content was determined according to the method of Yoshioka *et al.* (1979) (13) using 1,1,3,3-tetraethoxypropane as a standard. GSH content was determined according to the method of Beutler *et al.* (1963) (14). Glutathione peroxidase determined according to the method of Paglia and Valentine (1967) (15). Catalase activity was estimated according to the method of Sinha et al., (1972) (16).

Evaluation of lipid profile:

Triacylglycerol and cholesterol concentration were determined according to the method of Young *et al.*,(2001) (43). While HDL concentration were determined according to Gordon *et al*., (1977) (44) and (LDL-C) was calculated according to Friedwald *et al*., (1972) (45) from the equation.

Pathological study:

Rats mammary gland tissues were fixed in 10% neutral formalin buffer, and then embedded in paraffin wax. Specimens were dehydrated through graded alcohol, cleared in xylene and embedded in paraffin. Sections of 5µm-thickness were cut and stained with Heamatoxylin and eosin (H&E) according to Bancroft and Gamble (2008) (17).

Statistical analysis:

Experimental data were analyzed using one way analysis of variance (ANOVA) using SPSS (statistical package for social sciences, 1999; ver.10.0), and the significance among the samples was compared at $P \leq 0.05$. Results were represented as mean ±SD (n =8).

Results

In the present study, MNU intoxication induced significant biochemical alterations in the blood, causing a significant increase in the GSH content and GPx and CAT activities compared to that of control. Oral administration of fermented soy milk (FSM) after MNU injection, caused significant reduction in antioxidant enzymes GPx and CAT compared to

MNU treated group. Whole body irradiation with low dose of gamma radiation (0.5 Gy) markedly ameliorated GPx, and CAT while increased SOD activities with significant increase in GSH level compared to MNU. Combined treatment of both FSM and gamma radiation to MNU treated group significantly increased GPx, SOD and GSH compared to MNU and significantly reduced CAT, Table (1).

Table I: The effect of fermented soy milk and and/ or -irradiation on glutathione peroxidase, superoxide dismutase, catalase activities and glutathione (GSH) level in the blood.

Groups	GPx (mU/mL)	SOD (U/ml)	Catalase (U/L)	GSH (mg/dl)
Control	6.4 ±0.78	24.3 ± 2.9	644 ± 77.3	5.0 ± 0.6
FSM	2.3 ±0.2^{ab}	22.7 ± 2.7	595± 71.2^{b}	5.8 ± 0.7
Radiation(Rad)	2.1 ±0.2^{ab}	21.1 ± 2.5	507 ± 60.4^{ab}	4.9 ± 0.6^{b}
MNU	9.9 ± 0.8^{a}	19.5± 2.3^{a}	732 ± 87.4	6.6 ± 0.8^{a}
FSM+Rad	5.0 ± 0.6^{ab}	32.0 ± 3.8^{ab}	477 ± 56.9^{ab}	8.9±1.08^{ab}
FSM+Rad+FSM	2.52 ± 0.31^{abc}	25.7 ± 3.06^{b}	520 ± 74.67^{ab}	8.4±1.00^{abcd}
MNU+FSM	4.7 ±0.5^{ab}	20.6± 2.5	461 ± 55.1^{ab}	6.5 ± 0.79^{a}
MNU+Rad+FSM	8.1 ± 0.16^{ab}	27.9 ± 3.4^{b}	311 ± 37.2^{ab}	10.3±1.24^{ab}

^{a} significant compared to control, ^{b} significant compared to MNU.

3.2. Effect on lipid peroxidation and tumor necrosis factor alpha

Oral administration of FSM to MNU treated groups caused a significant increase in TNF-α, which was ameliorated via exposure to gamma radiation or FSM. Treatment with FSM accompanied with exposure to low dose of gamma irradiation markedly reduced TNF-α levels compared to control, fig.1.

Lipid peroxidation was significantly increased by MNU or gamma radiation. On the other hand, FSM significantly reduced MDA level caused by MNU, fig.2.

Fig (1): Effect of fermented soy milk and low dose of gamma radiation on TNF-α

Fig (2): Effect of fermented soy milk and low dose of gamma radiation on lipid peroxidation.

Effect on cell cycle

Cell cycle analysis of mammary gland via flow cytometryclearly shows that, FSM treatment caused significant alterations in cell cycle analysis as it caused cell cycle arrest at Go/1 appeared in increased cell population at Go/1 with significant decrease in cell population at S and G2/M phases compared to control. Rats of MNU group treated with FSM and gamma irradiation showed, amelioration in cell percentage of Go/1,S and G2/M phases compared to control and tumor groups.

Table II: The effect of fermented soy milk and or -irradiation on cell cycle analysis in the mammary gland tissue.

Groups	Go/G1%	S%	G2/M%
Control	15.2±1.8	37.2±4.4	5.6±0.7
FSM	37.8±4.5[ac]	14.3±1.7[ab]	2.04±0.3[ab]
Radiation(Rad)	13.7±1.6[b]	2.7±0.3[a]	0.38±0.1[a]
MNU	72.8±8.7[ab]	18±2.2[ab]	9.1±1.1[ab]
FSM+Rad	4.3±0.5[ab]	0.98±0.1[a]	0.42±0.04[a]
MNU+FSM	33.7±4.0[a]	3.2±0.4[a]	0.59±0.1[a]
FSM+MNU+FSM	56.90±6.81[abcd]	8.90±1.07[abd]	6.24±0.81abcd
MNU+Rad+FSM	14.7±1.7[b]	7.5±0.1[ab]	1.35±0.2[ab]

*Legends as in table 1

Effect on caspase-3 and apoptosis

Apoptosis and caspase-3 analysis by flow cytometryresults in fig.3. shows the inducing effect of FSM and gamma radiation on apoptosis along via caspase-3 mechanism. Combined treatment with FSM and gamma radiation markedly enhanced apoptotic cell number and caspase-3 mechanism.

Fig (3): Effect of fermented soy milk and low dose gamma radiation on of caspase-3 and apoptotic cell count %.

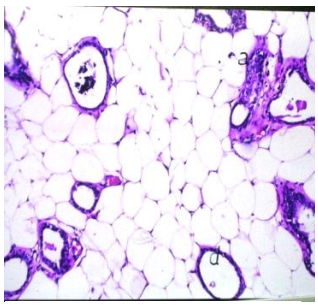
Effect on lipid profile:

Lipid profile shows the inducing effect of FSM and gamma radiation on lipids. Combined treatment with FSM and gamma radiation.

TableIII: The effect of fermented soy milk and or -irradiation on cell cycle analysis in the mammary gland tissue.

Groups	TG	Cholesterol	HDL	LDL
Control	90.00± 2.54[b]	98.18±3.81[d]	46.0 ± 3.16[b]	37.42± 3.11[d]
FSM	90 ± 3.80[b]	89 ± 4.12[ab]	45.00 ± 3.60[b]	27.08± 3.21[ab]
Radiation(Rad)	94.00 ± 4.52[b]	94.40 ± 3.84[d]	51.0 ± 5.70[ab]	32.46±3.95[ab]
MNU	99.20 ± 3.27[ad]	95.60± 4.13[d]	37.00 ± 2.23[a]	38.70± 1.61[d]
FSM+Rad	74.00±5.09[abd]	87.3 ± 2.54[ab]	45.00 ± 3.60[b]	21.5± 3.80[abd]
MNU+FSM	98.40 ± 3.84[ad]	78.36± 3.82[abd]	26 ± 3.00[ab]	32.14± 5.96[ab]
FSM+MNU+FSM	106.00 ±3.16[abd]	77.66± 4.12[abd]	26.0 ± 3.08[ab]	30.46± 4.05[ab]
MNU+Rad+FSM	70.0 ± 3.80[abd]	80.36 ± 4.76[abd]	36 ± 4.18[ad]	30.36±4.08[ab]

[a] significant compared to control, [b] significant compared to MNU.[d] significant compare to fermented soy milk (2cm).



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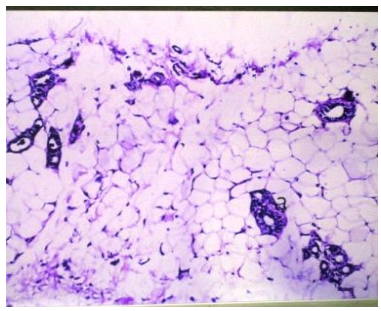

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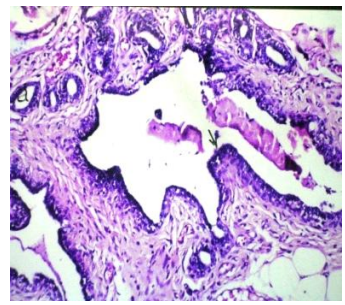

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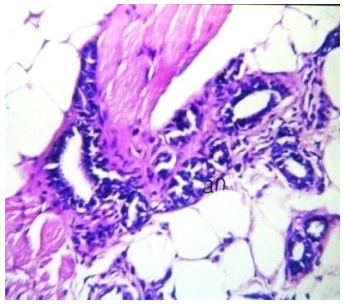

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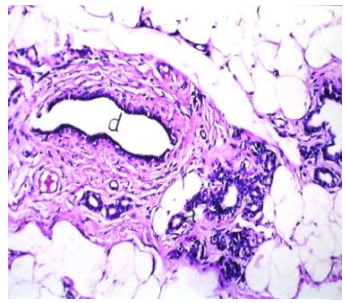

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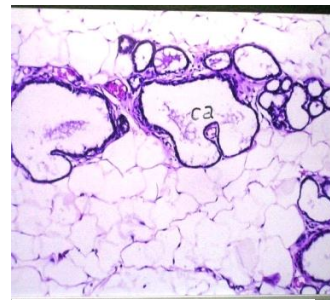

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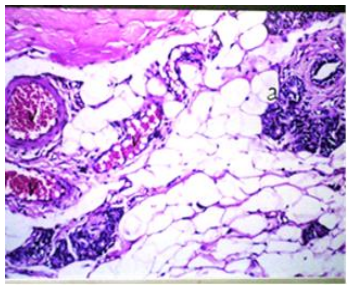

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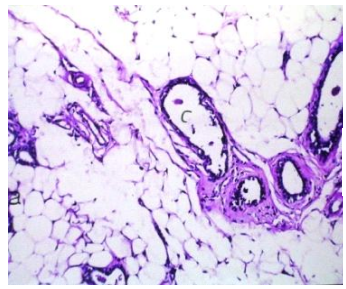

(H)

Fig.(1): Light microscopic photos of ratsmammary gland showing: (**a**) In control group histological structure of the lactiferous duct (d) and acini (a) embedded in adipose tissue. (**b**) In FSM treated group: no ultrastructural changes in thestructure of the lactiferous duct (d) and acini (a). (**c**) In irradiated group: no ultrastructural changes in the structure of the lactiferous duct (d) and acini (a). (**d**) In MNU group: showing anaplastic hyperchromatic lining epithelium of the acini (a) with lose of basement membrane (carcinoma). (**e**) In FSM+ irradiation group: showing normal histological structure of lactiferous duct (d) and acini (a). (**f**) In MNU+FSM group; showing mild systicdialation of the intact duct and acini. **(g)** In FSM+ MNU+FSM group: showing congestion in stromal blood vessels (v) with normal histological structure of the paranchyma (a). (h) In MNU+FSM+Radiation: showing normal histological structure of the duct (d) and acini (a).

Histopathological study

Histopathological study by light microscope of female rat mammary glands showed several marked changes with different treatments (fig.1). Mammary gland in the control group was distinguished with lactiferous duct and acini embedded inadipose tissue. Oral administration of FSM revealed normal histological structure, also, mammary gland of irradiated rats with or without FSM administration showed healthy histological structure with no structure alterations. Rats treated with MNU showed anaplastic hyperchromatic lining epithelium with lose of basement membrane (carcinoma). MNU group received FSM showed hyperplasia in lactiferous duct with polyformation and cystic dilation, while treatment with FSM for 15 days before MNU injection markedly ameliorated MNU effect. Female rats treated first with FSM and exposed to gamma radiation then injected with MNU showed normal histological structure.

Discussion

In a continuing effort to improve cancer therapy, it was found that ultra-low doses of radiation are capable of enhancing the efficacy of chemotherapy. The clinical results of this combined treatment approach have proven to be so effective it is now frequently employed for advanced abdominal and head and neck cancers. Combined chemotherapy and radiotherapy regimens have become the standard approach because they allow one to reduce toxicity while maintaining high overall efficacy, since antioxidants can reduce damage produced by both low and high doses of radiation. Antioxidant treatment before and after radiation exposure are essential for a maximal reduction in radiation damage. Prevention of immediate radiation-induced genotoxicity requires that an antioxidant be present at the time of irradiation (18).

Fermentation consists of modifying food by microorganisms (bacteria, molds, and yeasts) that grow and reproduce and consume part of the substrate and enrich it with the products of their metabolism. It is an ancient technology that remains one of the most practical methods for preserving foods and enhancing their nutritional qualities (19,20). Fermented soymilk, unlike fermented milk or yogurt drinks, contains nolactose or cholesterol and have the health benefits from both soy itself and the fermentation(21).

In the present study, MNU in female rats caused significant changes in antioxidant parameters: increase in GSH level accompanied with significant decrease in GPx, CAT and SOD activities, this effects were ameliorated with administration of FSM or combined treatment of FSM +radiation exposure. oxidative stress caused by MNU increased free radicals production result in significant increase in lipid peroxidation (22). Antioxidant enzymes are capable of eliminating reactive oxygen species and lipid peroxidation products, thereby protecting cells and tissues from oxidative damage. Superoxide dismutases convert superoxide radicals to molecular oxygen and H_2O_2, and catalase decomposes H_2O_2 to molecular oxygen and water. SOD, and GPx are decreased in tumor tissue (23). FSM administration was able to normalize SOD and GPx activities in MNU groups due to the presence of many antioxidantsas isoflavones, proteins, and saponins, alsoit contains many *lactobacillus* sp. which exerts potent antioxidant activity and free radical scavenging capability. *lactobacillus*sp. Possess several anti-oxidative mechanisms: catalase, glutathione-system-related compounds, and Mn-SOD, decreasing the risk of ROS accumulation also degrade the superoxide anion and hydrogen peroxide (24,25). N-methyl-N-nitrosourea

transforms mouse mammary epithelial cells to proneoplastic and neoplastic states in rat, however, malignant tumors appeared earlier and at a faster rate than the benign tumors (26).

Fermented soy and low dose of gamma radiation enhanced GSH levels, which protect vital organs from damage via free radicals through free-radical scavenging, restoration of the damaged molecules by hydrogen donation, reduction of peroxides, and maintenance of protein thiols in the reduced state (27). It was reported that, Soy(1)and exposure to low dose irradiation (0.5 Gy) significantly enhanced GSH content within 24 hrs post-irradiation(28).The presence of 3-hydroxyanthranilic acid (3-HAA) a by-product of soy fermentation in FSM and *Lactobacillus* markedly combat oxidative stress and reduced lipid oxidation *in vivo*(29). *Lactobacillus*, attenuate proliferation (30) and reduce NO levels (31).

Exposure to low doses of ionizing radiation may stimulate cellular detoxification and repair mechanisms leading to reduction of the DNA damage even below the spontaneous level and decreasing the probability of neoplastic transformation (32,33), such exposures may also enhance immune reactions of the organism and attenuate harmful effects of higher doses of radiation (34,35),significantly delayed the tumor growth, enhanced GSH content in the spleen within 24 hrs post-irradiation (28).

The inhibition of cytokine production or function serves as a key mechanism in the control of inflammation(36).In this study, FSM ameliorated the elevation in TNFα caused by MNU, is referred which may referred to the presence of genistein which reduce the production of TNF-α, IL-6, IL-1 via its effect on nitric oxide and COX-2 gene expression(37,38), also , the components of lactic acid bacteria or bifidobacterium cells and peptides formed during the fermentation, which have been reported to affect the production of cytokines (39,40).

Cell cycle analysis of female rat mammary tissue via flow cytometry showed disturbance in cell cycle in MNU group observed in all phases with accumulation of cell count at G1, this disturbance was significantly ameliorated by FSM treatment and combined treatment of FSM and gamma radiation decreasing MNU effect on cell cycle and apoptotic cell count compared to the control.

The presence of isoflavone, particularly genistein in soy, exerts its antioxidant effects to protect cells against reactive oxygen species by scavenging free radicals and reducing the expression of stress-response related genes. Genistein is a tyrosine kinase inhibitor; induce apoptosis in different types of cancers including breast cancers through both NF-κB dependent and independent pathways. It activates caspases, apoptosis and inhibits DNA-binding activity of NF-κB in various cancer cells.Furthermore, its pre-treatment abrogated the activation of NF-κB stimulated by H_2O_2or TNF-α (41).Although soy extract induced higher percentage of cells undergoing apoptosis than genistein or daidzein(42). Accompanied treatment of FSM and low dose of radiation induce more inhibitory effect on tumor cell, since low dose of gamma radiation also able to delay tumor growth in Ehrlich solid tumor bearing rats (28).

MNU has an impact on the expression of regulatory genes triggering apoptosis and directly development toxicity followed by accumulation of mutations either in somatic cells or blood cells (40).The proposed antiproliferative effects of FSM reflect the primary protective action on damaged cells. Induction of apoptosis may be considered in case of failure of reparative mechanisms lead to cell death and is also important for protection of the entire organism.

Carroll (1991)(46) and Potter (1995)(47) reviewed various hypotheses that are presented in this section. These include the amino acid composition of soy protein, an interruption of the intestinal absorption of bile acids and dietary cholesterol, direct effects on the hepatic metabolism of cholesterol, alteration of the hormone concentration involved in cholesterol metabolism, and the effects of components such as isoflavones, fibre and saponins.

Soy protein consumption may directly influence the hepatic metabolism of cholesterol by increasing the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, thereby inhibiting hepatic cholesterol synthesis. Lovati et al (1987)(47) reported a sevenfold increasein LDL receptor activity in humans, resulting in increased clearance of cholesterol from the blood in patients with raised serum cholesterol concentrations who consumed a soy protein (Cholsoy) diet compared to a standard low-lipid diet with animal protein.

However, the hypothesis of an activation of LDL receptors in liver cells is still controversial and more extensive studies are needed to ascertain the cholesterol-lowering mechanism of soy beans.

It has been proposed that variation in hormone secretion is responsible for the cholesterol-lowering effect of soy protein .the metabolic effects of hyperthyroidism are very similar to those observed

With soy protein feeding. That is, LDL receptor activity increases, HMG CoA reductase activity increases, bile acid excretion increases, and total and LDL cholesterol decrease (47). Some observers, as discussed by (46), suggest that changes in the ratio of serum glucagon to insulin inpatients on a soy protein diet may be important.

Isoflavones are known to have weak oestrogenic activity in biologic systems. Therefore it is more accepted that mechanism by which soy beans decrease serum cholesterol is via "oestrogenic" effects stimulated by the ingestion of isoflavones (47; 48). It is well known that mammalian estrogens have a significant impact on serum lipids, promoting decreases in LDL and increases in HDL cholesterol. Evidence for an effect of isoflavones on serum cholesterol concentrations has been demonstrated in rats, hamsters, nonhuman primates and humans (49,50,48, 51).

The three primate studies reported by Anthony et al., (1996)(48) demonstrated that soy protein rich in isoflavones favourably affected serum lipids, and that soy protein from which the estrogens had been extracted had a minimal effect. The authors concluded that soy isoflavones may account for 60% to 70% of the hypocholesterolemic effects of soy beans.

Moreover, whereby soy beans may decrease the risk of cardiovascular disease is to lower the susceptibility of LDL cholesterol to oxidation (52). Isoflavonoids have been reported to inhibit the oxidative modification of LDL by macrophages (53), enhance the resistance of LDL to oxidation and exhibit antioxidant activities in an aqueous phase (54). Genistein inhibits bovine aortic endothelial cell-mediated and human endothelial cell-mediated LDL oxidation, and protects vascular cells from damage by oxidised LDL (53) did not observe the antioxidative effect of genistein.

The histological observations indicate that FSM accompanied with low dose of gamma radiation has great efficiency as anti-inflammatory and antitumor treatment against MNU carcinogenesis. The ameliorative effects of FSM upon the structural alterations could be explained by the role of FSM in regulating vital cellular functions, including cell

proliferation and differentiation and its potent antioxidant activity and free radical scavenging capability.

This study demonstrates that soy antioxidants and microbial components accompanied with low dose of gamma radiation can reduce the harmful mutagenic and oxidative stress effect of MNU in inducing mammary tumors.

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PHYSICOCHEMICAL EVALUATION OF RAW MILK OBTAINED IN THE MOUNTAIN AREA

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Abstract

Introduction. The guaranty of authenticity for the milk products involves proofs of originality from both producer and processor, trying to respond to most of the actual standards. There has been shown a growing interest for the raw milk obtained in the mountain area and also traditional processing technologies which can represent parts of the quality and food safety systems. Purpose. This paper, focused mainly on the monitoring and investigation of the main physico-chemical paramenters (fat, protein, un-fat dry substance, density, freezing point, pH), of milk, aims the evaluation of the raw milk obtained in mountain area of „Valea Gurghiului", including traditional households and commercial farms. Material and methods. Research studies were carried in a high capacity, private, processing unit from the investigated area which uses both conventional and ecologic processing technologies. This unit has a processing capacity of 35000 liters raw milk, collected from small producers (50%), small farms (31.25%) and high capacity farms (18.75%) producing mainly ripened cheese products and also fresh dairy products (cottage cheese, ricotta, sweet cheese). Samples of raw milk were colected, framed in product standards, compliant with the requirements of current legislation and those established by the proccesing unit. After testing the raw milk, the data obtained was grouped in three categories, according to their provenience (A, B, C): small producers (n=650), small farms (n=11) and large farms (n=2). For each category of raw milk, samples were collected monthly (n=16). A number of 320 samples from each category were collected and tested, totalizing a number of 960 tests. Results. From the set of data obtained, we consider of high interest the adquate mean values for both national and European physico-chemical standards established for raw milk. The lowest fat content values were found in the raw milk from the small producers (3,73%), and seasonal influences revealed minimal values (3,71%), during spring and maximal (4,01%) in autumn. Un-fat dry substance was found with maximal values in the high capacity farms (8,58%), minimal values in autumn (8,46%) and maximal during winter (8,58) Conclusions. We consider as beeing essential, the influence of the mountain area on the raw milk composition. The correlation during autumn season, beetwen the increase of the fat content with the decrease of the un-fat dry substance and protein content can also be linked with the decrease of the density and freezing point.

Keywords: *raw milk, mountain area, milk products.*

Introduction

Milk and milk products occupy an important segment in human nutrition, being considered also a great source of essential elements due to the content of proteins, lipids, carbohydrates, vitamins and minerals. A great variety of milk products with various organoleptic, physicochemical, microbiologic characteristics are produced in order to satisfy the client's needs and preferences.

There are obtained a wide variety of cheese products, some produced in our country, which have a certain specificity, that requires a differentiation or a classification. Starting from this considerations we find necessary the classifying of dairy products in: conventional products, obtained with conventional recipes and technologies and traditional products, obtained in ecological conditions, including a certain geographical area (organic farming is practiced) and specific recipes.

This classification is required by the increasing demand of diversity of diary products, which has to be correlated with the increasing of quality and food safety standards.

All traditional products must have as a common characteristic, a high level of compozitional quality, hygienic and sanitary of the raw milk used as raw matter. (Ognean *et al.*, 2012).

Solutioning the numerous problems acumulated in the traditional products field, fully justify the need of new research within this segment of the food industry, focused on evaluation of the compositional parameters of raw milk obtained in mountain areas. (Rodica Someşan *et al*., 2013).

Material and methods

Studies conducted were focused on the monitoring and investigation of the main physico-chemical paramenters of raw milk (fat, protein, un-fat dry substance, density, freezing point, pH) proccesed in a private proccesing unit. The raw milk was obtained in the mountain area of Valea Gurghiului from indigenous cow breeds. Small producers, with average number of 3 animals/ house was the main source of the raw milk. In this households the milk is obtained mainly trough traditional milking, very few of them use mechanical milking. Small farms in the area have aroud 79 cows/unit and benefit of good hygienic conditions, equipped with automatic milking and cooling systems. Bigger farms in the area with 200 cows, offering excelent hygiene, separate milking chambers (equiped with automatic detection systems for mastitis). Prevailing in the area is the romanian breed „Bălţată Românească" with highly selected lines witch have a good adaptability to the local conditions. They show a very high level of disease resistance and seem to be very well adapted to the climatic and care conditions in the area „Valea Gurghiului". Milk cows feed is mainly represented of the green mass in the area during spring, summer, autumn and in the winter the predominant feed is fiber (hay), supplemented with concentrates (corn, grits).

The research began by identifying raw milk suppliers, with suitable product standards, in accordance with the requirements of legislation and requirements, set by the processing unit. Following the assessments, made in the activity area of the unit that we have studied, we identified the following three sources supplying raw milk, including: small producers (n = 650), microfarms (n = 11) and large farms (n = 2).

Milk from small producers was taken up in the 10 milk collecting points, which are organized and managed by the processing unit. Micro-farms and large farms use their own cooling tanks witch allowed receiving milk directly from the farm. The average amount of milk supplied daily from 3 sources was 24.6 liters / individual producer, 909 liters / microfarms and 3000 liters / large farm. The investigations were conducted over a period of 20 months (December 2011 - July 2013), during witch we tested the following three milk batches: batch A, raw milk from small producers, batch B, raw milk from microfarms and batch C, raw milk from large farms.

For each batch of milk samples were collected monthly (n = 16) and subjected to laboratory analysis. The number of tests performed totaled 960 samples, including 320 for each batch. Physico-chemical properties of each sample have been tested with the semiautomatic analyzer *EKOMILK M, Milkana KAM 98-2A,* in order to establish the folowing parameters: fat, dry substance, protein, freezing point, density, and pH. Furthermore the results obtained were verified in three points: fat (Gerber method), dry substance (in the drying oven) and density (with thermo-lacto densitimeter).The obtained data`s were

statistically processed by using MedCalc soft esepeciallly designed programe for biomedical research, and also GRAPH Pad Prisma.

Using summary statistic and frequency analysis we have been able to establish the graphical representations by using „box plot" diagrams. In this diagrams, each „box" represents values from the 25-75% quartile of the series, lines inside the „box" represent the group average value, while the lines that continue each „box" (verticaly or horizontaly) show the spreading of the values from the first and fourth quartile. We also used *Anova test* wich compares diferences between average values of more than two batches when the data`s show a Gaussian distribution. The *Anova test* compares the diferences between average values of three batches (producer, microfarm and farm), $p<0,05$ showing statistical differences between groups.

Results and discussions

Data obtained during physico-chemical testing of the raw milk, separetely on batches, revealed significant oscillations for some of the investigated parameters. Fat average values variations found for the three batches : 3,73 % for batch A, 3,85 % for batch B, 3,91 for batch C. The differences found were statistically significant (p=0.0001) (Fig. 1).

Regarding the evolution of mean protein values we have found the folowing variations for each batch: 3,28 % fot batch A, 3,29 % for batch B, 3,30 % for batch C. The differences found were statistically significant (p=0.0001). Sezonal comaprative analysis revealed minimum protein values of 3,26 % in autumn and maximum 3,31 % during winter also having statistically significant differences (p=0.0001)(Fig. 2).

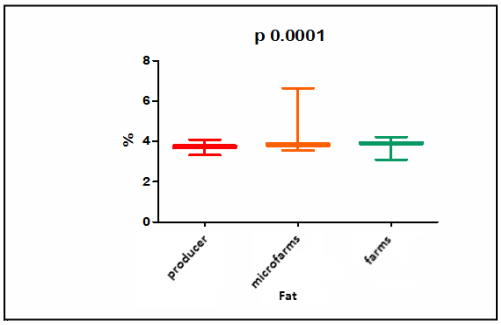

Fig. 1. Mean fat values (%) on raw milk batches

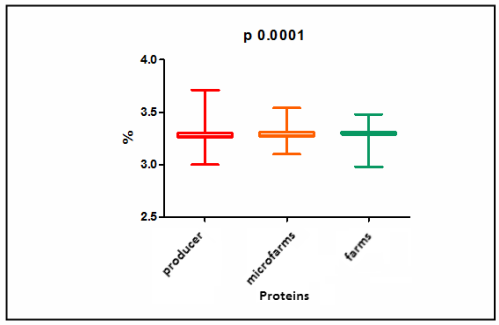

Fig. 2. Mean protein values (%) on raw milk batches

Seasonal comparative evolution of the fat content in the raw milk studied from the three supliers revealed minimum fat content (3,71%) during spring and maximum in autumn (4,01%), having statistically significant differences (p=0.0001)(Tab. 1)

Table 1. Seasonal statistical analysis of the raw milk fat content

Bonferroni's Multiple Comparison Test	Average differences	significant ? P < 0.05?	95% CI of diff
producer vs. micro-farms	-0,07917	Yes	-0.1190 to -0.03933
producer vs. farms	-0,1694	Yes	-0.2092 to -0.1295
micro-farm vs. farms	-0,09021	Yes	-0.1300 to -0.05037

Raw milk un-fat dry substance determination revealed statistically insignificant differcences (p=0.06), average values obtained: 8,54% (batch A), 8,55% (batch B) and 8,58% (batch C). Sesonal comparative evolution of this parameter indicated minimal values during autumn (8,46%) and maximum during winter (8,58%) showing statistically significant variations (p=0.0001) (Fig. 3).

Density determination (g/cm^3) also revealed statisticaly insignificant variations (p=0.49), average values of 1,029 for all studied batches (Fig. 4). Density sesonal evolution indicated statistically relevant variations (p=0.0001), minimal values during autumn (1,028 g/cm^3), and maximum values during spring (1,029 g/cm^3).

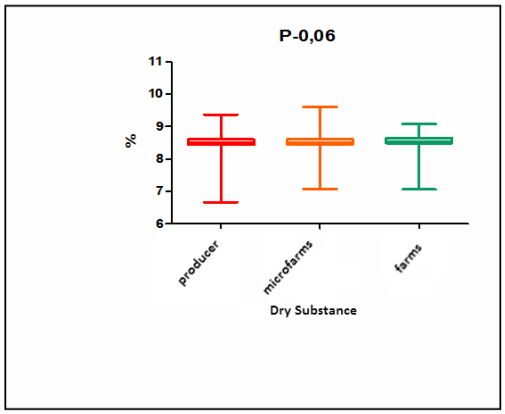

Fig. 3. Un-fat dry subsance mean values (%), on raw milk batches

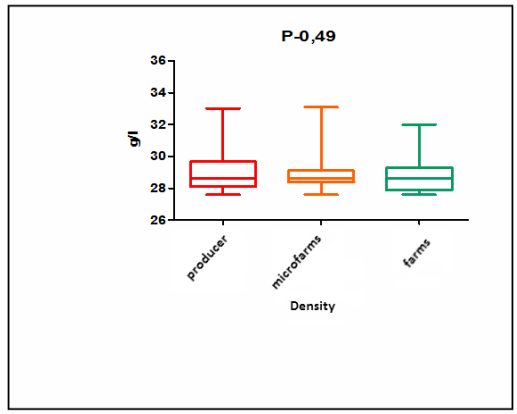

Fig 4. Density mean values (g/cm^3), on raw milk batches

Freezing point analysis (°C) revealed statistically insignificant variations (p=0.071), mean values of -0,566 for batches A and B and -0.565(°C) for batch C. (Fig. 5). Raw milk comparative seasonal analysis of the freezing point revealed highest values (-0,560 °C), during winter and the lowest (-0,571°C), in autumn. The diferences between the two seasons seems to have statistically sgnificant variations (p=0.01).

pH investigations revealed statistically significant variations (p=0.0001), maximum

values (6,69) were obtained from batch B (Fig 6). Seasonal comaprative studies of the raw milk pH indicated minimal values (6,67) during summer and maximal (6,69) during winter, having statistically significant variations (p=0.0001) for the two seasons.

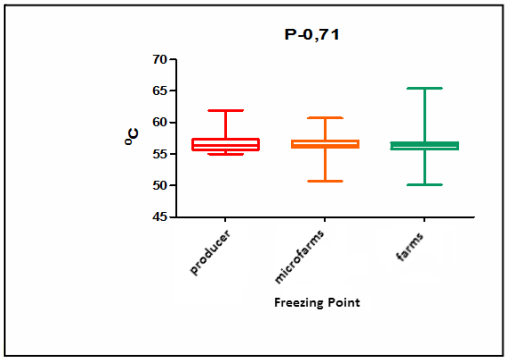

Fig. 5. Freezing point mean values (°C) on raw milk batches.

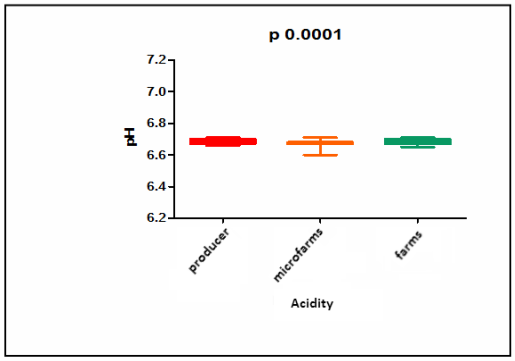

Fig. 6. pH mean values, on raw milk batches

Comparative analysis of the results obtained, within the three batches, revealed increases of the mean fat content for all three batches with 0,53-0,71% compared to the product standards. Compared to the 2012 national raported (National Institute of Statistics 2013) fat average value, are noteworthy the decreases with 0,08% for the milk from the small producers. Regarding the statistically semnificative variations (p=0.0001) encountered between the average fat levels of the raw milk from the three sources, we consider that they range within previous established intervals and other studies in the field (Delbès *et al.*, 2007).

Seasonal fat content evolution was, as aspected, with lower values (3,71 %) during spring and highest in autumn (4,01 %), diferences between the two seasons were statistically semnificatitive (p=0.0001). We consider that the diferences observed are due to the feed characterisitcs during autumn, when small producers (main raw milk source) add into the rations of the milk cows seasonal products (corn, beets, straw). Similar to our obsevation, other researchers in the field reported increased fat content of the raw milk, during the pasturing period in the mounatin area (Romanzin *et al.*, 2013).

Milk fat is a basic parameter for evaluating the quality of milk and setting the sales price, being one of the most important nutritional and economic indices. In this context, should be reviewed studies that have found positive correlations between high levels of the main milk components (fat, protein) and increased efficiency in milk products (Pretto et al., 2013).

At the batch comaparative analysis of proccesed milk protein content we give high importance to the increase found for the three baches with 0,08-0.1% to the product standard and 0.02-0.04% compared to the average content of protein reported nationally in 2012 (National Institute of Statistics, 2013)

Highest values of protein content (3,30%) were found in the milk from big farms similar to previous studies in the field, conducted by Delbès *et al.* (2007). Statisticaly significant diferences (p=0.001), found at the seasonal comparison of the batches, includes

minimal values (3,26%), during autumn and maximal (3,30%), during winter.

We consider that such differences, in the protein content of raw milk, may be due to different feeding mode , between the seasons as well as between batches. Compared with the evolution of fat content, it is noteworthy that if this parameter is at his maximum values in autumn , the protein content was minimal.

On the evolution of non-fat solids content, which according to the product standard should be minimum 8.5%, we find that raw milk from the 3 batches investigated was within limits, although there were insignificant statistical differences between them (p = 0.06). We note also that the non-fat solids content of raw milk had the lowest values, in autumn (8.46%) and higher in winter (8.58%), the differences between the seasons were statistically significant (p = 0.0001). This evolution shows that non-fat dry matter is influenced by seasonality, the highest values were obtained in the wet seasons and dry seasons minimum (Barbosa et al., 2013).

We find noteworthy density statistically insignificat variations (p=049), mean values obtained 1,029 for all batches. Comparative seasonal analysis revealed statistically signicant difereces (p=0.0001), minimal values in autumn (1,028 g/cm^3), maximal in spring (1,029 g/cm^3) .Comparing the seasonal results, fat and density indicators have indicated that they are inversely proportional (the fat is higher the density is lower and vice versa). If the fat peaks were obtained in fall, the maximum density was obtained in spring. Also, minimal fat values were obtained in spring and minimal density in autumn, as outlined in the specialty literature (Ognean *et al*., 2003).

Setting limits for freezing point of raw milk is very important because this parameter gives us clues to a possible falsification of the raw milk by adding water , but also on udder health (Ognean , 2002). According to the product standards under study, the limits are between -0.53 to 0.57 ^{o}C, with mean -0.555 ^{o}C. Compared results on batches for the freezing point, indicated statistically insignificant difference (p = 0.071) with the mean values of -0.566 for the samples A and B,and for the sample C -0565^{o}C; compared seasonal results showed the highest values (-0.560 ^{o}C) in winter and lowest (-0.571 ^{o}C) in fall, with statistically significant differences (p = 0.01).

Milk acidity according to the product standard is between 16-19^0 Thorner, meaning a pH between 6.55 to 6.75. Although there were found statistically significant differences between batches or seasons, pH indicator results show that the raw milk falls in standards and regulations in force. Also, to avoid receiving acidified milk collectors and drivers were trained to use the field test guidance with alcohol (59-61%), and where were positive results (precipitation of casein), milk was refused.

Taken as a whole, the results of physico-chemical testing of the milk samples studied, showed their inclusion in national and European standards for raw milk, compositional and microbiological indices as having a major impact on farmers (milk quality goods), processor (process and quality of dairy products) and consumer (quality and nutrition security), (Dobranié *et al.*, 2008).

Conclusion

- The level of fat content was lower for raw milk collected from small producers (3.73%) and during spring season (3.71%) compared to autumn (4.01%), with statistically significant differences (p = 0.0001) between batches and between seasons.

- Evolution of the average protein content reached the maximum (3.30%) in samples of milk from large farms in winter (3.31%), the differences between batches and seasons were statistically significant (p = 0.0001) .
- Evaluation of un-fat dry substance content showed maximum values for large farms (8.58%) and statistically significant differences (p = 0.0001) for the seasonal comparing, the minimum values in autumn (8.46%) and maximum in winter (8,58%).
- Freezing point values indicated average values between -0.566 and -0565 ^{o}C for the three investigated milk batches with lower value (-0.571 ^{o}C) in fall and statistically significant differences (p = 0.010) compared to other seasons.
- Density and pH of raw milk were within the standards set and established regulations in force for the product investigated, although statistically significant differences were found between samples and seasons.

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PATHOMORPHOLOGICAL STUDY IN RABBIT HAEMORRHAGIC DISEASE

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Abstract

Pathomorphological study in rabbit haemorrhagic disease - *Viral rabbit haemorrhagic disease occurs only in rabbits as both natural and experimental infection. Pathomorphological macroscopical lesions are of septicemic type with polyorganical involment having as a substrate vascular endothelial injury, followed by hemorrhagic syndrome and multiple organ dysfunction syndrome. Spontaneous asymptomatic death, as well as pathological changes haemorrhagic in character and predominantly affecting the lungs and liver support a viral etiological factor.*

Key words: *rabbit haemorrhagic disease, virus, haemorrhage, necrosis*

Introduction

Rabbit hemorrhagic disease is a viral disease which is highly contagious and often fatal to domestic and wild rabbits from *Oryctolagus cuniculus* species. The disease is caused by *Rabbit haemorrhage disease virus (RHDV)*, a member of the genus *Lagovirus* from the family *Caliciviridae*. The causative virus is highly resistant to inactivation and may persist in the frozen rabbit meat, carcasses in the process of decomposition in the environment for several months. Rabbits can be infected by oral, nasal or conjunctival way. Animals can remain contagious up to a month [1, 2].

It is possible that the rabbit hemorrhagic disease have occurred in avirulent caliciviruses that circulate asymptomatically in populations of European rabbits.

The first known outbreak occurred in China in 1984 and spread by angora rabbits imported from Europe. For the first time the rabbit haemorrhagic disease was described in 1984 Chinese scientists Liu C. and colleagues based on research done during an outbreak that was rapidly evolving and fatal, after a brief period of lethargy and fever [6]. By the late 1990s, outbreaks were reported in forty countries, and rabbit haemorrhagic disease became endemic in wild rabbit populations in Europe, Australia and New Zealand [4, 5, 7]. In other parts of the world including America periodic outbreaks in domestic rabbits were registered. Republic of Moldova is one of the European countries in which haemorrhagic disease has caused great damage. Currently attempts to control the spread of rabbit haemorrhagic disease are done by vaccinating against this disease. Haemorrhagic disease is not sufficiently studied in terms of morphological peculiarities and our study aims to eliminate partially these shortcomings.

Material and methods

Research on pathomorphologic hemorrhagic disease of the rabbits was carried out on 9 rabbit bodies aged mainly from 2 to 12 months, belonging to residents of Straseni town. Necropsy was performed in necropsy room of veterinary clinic of Straseni. The bodies were placed on the necropsy table in dorsal and lateral positions favorable to perform autopsy and then were firmly attached to the four extremities.

In order to execute a proper skinning a long skin incision from the lower thoracic midline, along linia alba bypassing the slice of watermelon navel, genitalia and breasts to the pubis. In still other four incisions were made that were running on the inside of each member, from longitudinal incision distal to the info-center.

Opening the abdominal cavity was done by performing a retroxyphoid buttonhole followed by placing two fingers which acted necropsy knife tip led the other hand. Severing the abdominal wall was performed on linia alba in the perineal region. This section was completed with two side sections executed from the same retroxyphoid buttonhole parallel to costal arch up near the lumbar transverse processes. After removing the two flaps of the abdominal wall sectioned a general examination of the abdominal cavity was made, following the topography of the abdominal organs, the situation diaphragm, peritoneum appearance and presence of pathological fluids.

Opening the thoracic cavity was made by cutting both walls charges. Then cage members were removed, and two costal incisions on either side of the cavity were performed, closer to the spine, starting from the last rib. Further the diaphragm was from the costal arch, sternopericardial ligament respectively.

After examination of the whole thoracic cavity, evisceration of the organs from oral cavity, cervical, thoracic and adominal regions was performed. It was gutted in a single piece tongue, pharynx, esophagus, larynx, trachea, lungs and heart. Then evisceration of the liver, spleen and omentum, the digestive tube in 3 pieces: stomach with the duodenum, jejunum with ileum, caecum, colon and rectum by applying three double bonds, suprarenal glands, kidney, ureters and the bladder was done. The autopsy concludes with the opening and examination of the nasal cavity, eyes, examining muscle, bones and joints.

In order to make histological investigation portions of the liver, kidney, heart, spleen, lymph nodes, brain were taken, then they were fixed in 10% formalin solution. The organ pieces were further washed with water, passed through the alcohol solution and embedded in paraffin. Histological sections were obtained, stained with hematoxylin-eosin.

Results and discussion

Pathological changes in haemorrhagic disease, despite some differences in the pathomorphological details, have a certain similarity in most rabbits.

The bodies of rabbits were well developed, with a satisfactory constitution. In some cases they had the lateral decubitus position with legs outstretched, neck and head turned to the left and back. In other cases the decubitus was dorsal, forelegs placed under the animal, posterior part was stretched in caudal direction and turned to the right. The coat usually was smooth or slightly disheveled, the hair was slightly glossy and maintained weakly in the follicle. Natural holes were clean and dry. In some rabbits the fur around the nasal passages was stained by bloody or serous secretions.

The mucosa of the nasal cavity was raked to bleeding, covered by aqueous bloody masses, in some cases with mucus. Laryngeal mucosa was swollen, hyperemiated, and in some cases covered by petechial hemorrhages.

The trachea was edematous and diffusely hyperemiated staining from pink to deep red. The strongest congestion was observed between tracheal rings. The lung consistency was crumbly, trying to fix with tweezers lungs, the organ breaks easily. On sectioning lungs a hemorrhagic-sparkling exudate was removed. Both under the pleura and the lung parenchyma depth point and petechial hemorrhages were emphasized.

The myocardium was brittle, of the brown colour, coronary vessels were filled with blood. Atria had from purple to black color, their walls being tense, filled with blood, bleeding was often observed in the epicardium. Bleeding was either petechial or in the form of spots or strips along the veins, or the entire surface of the heart. In the pericardial cavity pink colored (rarely - red) exsudate was observed, often in massive quantities. Under endocardium punctate hemorrhages were seen in some cases.

The liver damage was observed in all the animals. The liver was enlarged, soft, and brittle, colored from yellow-gray to dark brown and had a tensioned capsule. Usually the center was darker than the periphery. On the surface of liver incision dark blood drained. In some cases small petechial hemorrhages, were clearly visible.

The spleen was enlarged in volume 2-3 times (splenomegaly), filled with blood, the edges were well rounded after spleen incision edges did not match. The surface of spleen incision dense dark bloody liquid drained.

Mediastinal lymph nodes were enlarged in volume, had a soft consistency with a red-dark or red-gray colour. After incision of lymph nodes edges did not match, the cutting surface was convex where a bloody muddy fluid drained.

The haemorrhagic disease by frequency and degree of damage the trachea and lungs tops [5, 6]. During the histopathological study of the trachea venous congestion was observed in all cases, and circulatory disorders of the lungs was remarkable type of hyperemia, hemorrhage and edema in the surrounding blood vessels, bronchioles, bronchi, as well as proliferative processes in interstitial connective tissue. In all cases sero-hemorrhagic foci of bronchopneumonia were found.

A constant feature of rabbit hemorrhagic disease was liver damage characterized by active hepatitis of alterative type accompanied by vascular disorders like congestion and haemorrhage, dystrophy of hepatocytes, necrobiosis and necrosis.

Histological examination of the kidneys highlighted mostly vacular disorders (hyperemia and hemorrhage). These were located mainly in the medullar zone, rarely in the cortical area. From all the dystrophies hydropic and granular degeneration of distal and proximal tubuli prevailed. At the same time, in the kidney changes characteristic for acute serous, hemorrhagic, serous and haemorrhagic glomerulonephritis were detected. Other morphological changes in kidney included tubular necrosis, perivascular edema and edema of interstitial tissue.

In the heart localized hyperemia and bleeding in the thickness of the myocardium or endocardium was found. Simultaneously proliferative processes consisting of lymphoid cells, fibroblasts and histiocytes were reported, suggesting a viral endomyocarditis.

Mild degenerative changes in the muscle fibers with the formation of serous exudate accumulation areas was highlighted. Histopathological examination of the spleen revealed some changes in the blood system as venous stasis and hemorrhages of varying degrees, and blood clots. In most of the cases lymphofollicular hypoplasia and sometimes elements of follicular hyperplasia of white pulp were observed. Lymphoid and reticular cells were found in small quantities, scattered by groups of erythrocytes or divided into small groups.

The lymph nodes were affected by the disease in all cases. In mediastinal lymph nodes sero-hemorrhagic lymphadenitis was revealed, while in submandibular, subscapular, inguinal and mesenterial lymph nodes serous inflammation was observed. Parenchyma was swollen and congestioned.

Histological examination of the brain showed diffuse proliferation of glial cells, cerebral edema, perivascular and pericellular edema, perivaculare infiltration of lymphocytes, histiocytes and gliocytes. Plus to this, hyperchromatosis and pyknosis of neurons was found, and changes in the structure of neurofibers occurred parallelly to the changes of chromatophile substance. Moreover, after dispersing the tigroide substance neutrophils formed a microfibrillar network. When the tigroid substance was completely dissolved, the neurofibrillary structure of the neuron disappeared. Often The cells containing tigroid substance were often found in one of the poles. Changes occurring at the nucleus were manifested by its displacement to the periphery, kariorexis, kariolisis, kariopicnosis and vacularization.

Histopathological data we obtained support the pantropic nature of viral damage, polyorganical involment having as a substrate vascular endothelial injury, followed by hemorrhagic syndrome and multiple organ dysfunction syndrome, also mentioned by other researchers [7, 9].

Conclusions

1. Gross lesions in the rabbit haemorrhagic disease are of septicemic type with polyorganical involment having as a substrate vascular endothelial injury, followed by hemorrhagic syndrome and multiple organ dysfunction syndrome.
2. In rabbit haemorrhagic disease the changes of respiratory system prevailed and included haemorrhagic tracheitis, profuse hemorrhagic exudate in the trachea and bronchi, pulmonary congestion and edema and hemothorax.
3. Among other frequent histopathological lesions in rabbit haemorrhagic disease diffuse or focal necrosis of hepatocytes, disseminated vascular coagulation, represented by hyaline microthrombi in the capillaries and arterioles, foci of endomyocarditis, renal cortical infiltration and dystrophy of tubules, foci of encephalitis and demyelination, intranuclear inclusions in the brain were found.

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COMPARATIVE ONCOLOGY, THE COMPLEX STRUCTURE OF COMPARATIVE MEDICINE

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Abstract

The authors present comparative oncology in Romania from a historical point of view, mentioning its gained international relationships and also review the relationships that comparative oncology, both as a basic medical science (human and veterinary oncology) and as well as a science of environment medicine, uses to create an important resistance structure through comparative medicine, for the new medical concept "ONE HEALTH", launched by FEAM (Federation of European Academies of Medicine). The authors also approach the subject of the importance of comparative medicine and of the necessity of it to be studied furthermore.

***Key words**: Comparative oncology, comparative medicine, environment.*

Report on the occasion of the anniversary of "45 years of existence of Comparative Oncology in Romania"

It is a remarkable joy, as a Romanian and as a member of the Romanian Academy, to report those that follow in a logical link-up, having the quality of speaking fromfact reality and not from stories or writings that time has forgotten.

The notion of comparative medicine was first brought up in Munchen, in the year 1813, within a doctorate thesis on the topic of Comparative Anatomy, belonging to the Romanian Apostol Arsaky. This was quoted by Charles Darwin in 1859, on the occasion of presenting his thesis on species evolution. (Buda and Constantin Balaceanu Stolnici, 2012, Personal Note).

45 years ago (1968), together with the late Professor Dr. Octav Costachel, I have initiated within modern general oncology, for the first time in Romania, a new segment of science - comparative oncology-, a segment that links human oncology to veterinary oncology. Close to this date, similar projects were started in England, U.S.A. and Italy. In all of these years, numerous events and science doings were accomplished in our country.

Glanced upon as a biomedical science, comparative oncology was established at the beginning of 1983, in Paris, when the brilliant academician Radu Popescu presented his doctoral thesis that contained lymphoid leukemia elements in ephemeridae invertebrates.

Along with great personalities from Romania and abroad, over 20 science meetings were held, scientific papers were elaborated and published and within the Romanian school of Comparativ Oncology in Bucharest and Cluj-Napoca, specialized treaties were written.

Also, inside the faculty of Veterinary Medicine of Bucharest for the first time in our country the discipline of Comparative Oncology was created as well as in doctoral education the discipline of Veterinary Oncology and in post university education the discipline of Comparative Oncology.

In 1991 the "Romanian Society for Comparative Oncology" was established and in 2010 it became the "National Forum for Comparative Oncology'. Under its patronage, a specialized magazine published in English is elaborated- "The International Journal of Comparative Oncology".

"The National Forum for Comparative Oncology" is also a branch for the "Mediterranean Forum for Comparative Oncology", headquartered in Genoa, Italy. It was founded in 2009, by members from Romania, Italy and Spain. Its management was elected on a 7 year time basis (2009-2016) and has the following Romanian specialists within it:

- Nicolae Manolescu- vicepresident
- Corneliu Mateescu- general secretary
- Ioana Neagoe Berindan- member of the auditory board
- Gabriel Predoi- member of the science board

Activity base

Comparative oncology is involved in the four major areas of interest of general oncology:

1. Through the scientific activity it engages, comparative oncology permanently connects both nationally and internationally human and veterinary oncology, in regard to etiopathogenesis, diagnosis and therapy of cancer;

Within this activity, a particular role belongs to canine pets which play the role, inside the "family act", of bio sentinel for the cancer disease;

2. It identifies, monitors, nullifies and establishes the traceability of the main factors with cancer inducting potential, both biotic and a-biotic, from the environment(air, water, soil, plants and animals) and edibles of animal and non-animal origin, including animal food ratios;

This analysis refers to a number of around 40 substances with high cancer inducing potential listed on the W.H.O. and N.C.I. as very dangerous. This extremely important task covers the major part of primary cancer prophylaxis inn both humans and pets;

3. It engages in the quality control of "eco foods" as a result of ecological agriculture by accomplishing the specific analysis of identifying the cancer inducing agents;
4. It gets involved in specialized consulting of human and animal patients who lie in the terminal post treatment stages of the cancer disease, increasing their life quality.

All of these activities are manageable due to the setting up of the international frame of comparative oncology, made of:

- Veterinary oncology(farm animals, pets, exotic and wildlife);
- Plant oncology;
- Environment oncology(air, water, soil);
- Foods of animal and non-animal origin oncology.

All of the data gained is passed to the responsible structures for the human aspects of cancer for permanent monitoring of the degree of risk for the cancer inducting factors in every geographic area. For this, the following are necessary:

1. Infusing awareness in the human population of the risk factors of cancer, environment, behavioral and generic;

2. Promoting data regarding the cancer inducing factors (especially chemical carcinogens that are responsible, according to foresights, for 60%-90% for human cancers- see the list of the National Cancer Institute, U.S.A. of the chemic carcinogens/mutagens) and feeding this data to the institutions and interested people;

3. Elaborating offers for normative acts/laws concerning the monitoring and nullification of the chemical carcinogen pollution agents according to E.U. directives;

4. Bookkeeping new cancer diseasing cases, in both humans and animals, in species and in parallel, inside the same habitat;

5. Monitoring the incidence of human and animal cancer mortality;

6. Setting up human and animal cancer incidence/mortality maps;

7. Identifying the geographic areas with minimum/maximum values of cancer incidence and mortality;

8. Identifying areas with maximum values of mortality in humans and animals and establishing as much as possible a potential common etiology;

9. Identifying, in the selected areas, of local sub-zones, with elevated/decreased values related to the area average, for close investigation;

10. Performing multi-institute and multi-disciplinary research conventions of the presence of carcinogens in the selected areas;

11. Multi-disciplinary and multi-factorial studies on the presence of main carcinogens (chemical, physical and biological- cancer inducing viruses) in water, air, soil plants and foods;

12. Monitoring, through epidemiological studies and specific determinationsthat appeal to biotechnologies of people/individuals/populations (especially in humans but when demanded, probably in animals too) with high genetic risk for the cancer disease;

13. Bio-monitoring of people and animals in the investigated areas, following the biological effects of exposure to polluting agents, by:

- Identifying and dosing DNA adducts
- Identifying changes of gene expression(oncogenes, suppressor genes and others) as a result of exposure to high levels of carcinogens;
- As a final goal, bio-monitoring will allow the identification of cellular alterations at a pre-mutagen/pre-carcinogen level and the efficient therapeutic intervention at an early stage of cancer (slowing cell replication, stopping or even reversing it);

14. Identifying, including with biotechnology methods, of new main carcinogens and updating specific lists;

15. Identifying new technologies if reducing the concentrations of the main carcinogens, including the use of biotechnology techniques;

16. Prevention of cancer by identifying protection factors following experimental studies (anti-initiation, anti-promoting), that can be added in water, food or by direct administration, with effects on the level of biological transformation induced by environmental factors as well as implementing animal bio-sentinels in various life environments that have high risk in cancer development;

17. Extending studies at a national (national research programs, national cancer programs and others) and international (bi and multi-national programs) level;

18. Creating programs on a 4-5 year basis that have main subjects of eco-onco-therapy and eco-onco-prophylaxis of the environment to the purpose of reducing the incidence of the cancer disease in humans and animals;

19. All of this can finally lead to the decrease of the number of humans and animals that show anatomical and clinical signs specific to the cancer disease, drastically reducing current expenses that come from the general therapy of cancer. (Manoloescu N and collaborators (1)).

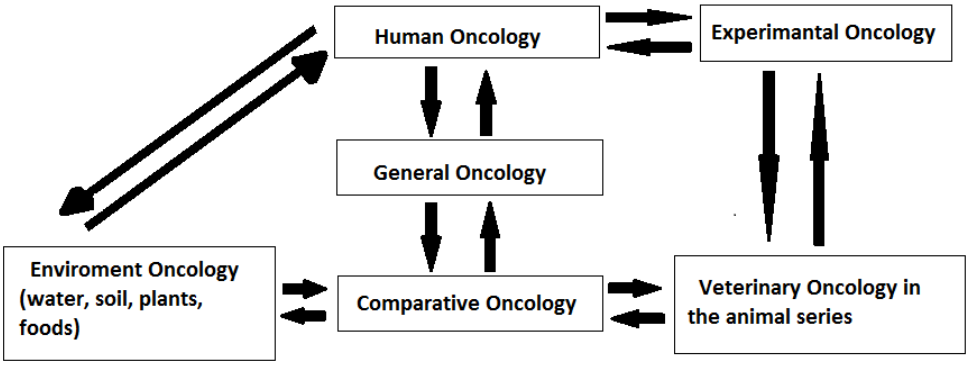

Fig. nr. 1- the relations of "Comparative Oncology"
(Emilia Balint and collaborators (2))

As a result of the above mentioned 45 year activity, dedicated to organizing and developing comparative oncology in Romania, in which the international component plays a particular role, the General Assembly of the French National Medicine Academy, section 5 reserved to veterinary medicine, has elected me, with a percentage of 95% of all votes as a foreign correspondent member. Because of this distinct honor I have received, Romania, the Romanian Academy and myself, as a patriot, wish to present to our readers the complete list with the 22 correspondent and associated foreign members- Romanian citizens (1890-2013):

1. Medicine: Daniel Danielopolu (1934), Basile Theodoresco (1957), Ion S. Pavel (1976), Stefan Milcu (1979);
2. Surgery: Ansatase Demosthene (1897), Thomas Jonnesco (1909), Constantin Angelesco (1935), Theodore Burghele (1963), Eugen Aburel (1968), Panait Sirbu (1976);
3. Anatomy-Physiology: Nikolai Kalendero (1890), Victor Babes (1892), Mihail Petrini-Galatz (1898), George-Jean Stoicesco (1900), Gheorghe Marinesco (1911);
4. Biologic sciences: Constantin Istrati (1901), Constantin Levaditi (1928), Johan Cantacuzene (1929), Emil Racovitza (1945), Georges Marinesco (1977);
5. Pharmacy: Radu Vladesco (1946);
6. Veterinary Sciences: Octavian Vladutiu (1966), Nicolae Manolescu (2013).

To them we owe the introduction of the 4 correspondent members born in Romania but that are currently foreign citizens:

- Herbert Tuchmann-Duplessis (1978)
- Henry Haimovici (1986)
- Franz Halberg (1990)
- Liane Deligdsch-Schor (2007)

Very recently Professor Dr. Irinel Popescu has been elected as a foreign correspondent member for the French National Medicine Academy, raising the total number of Romanian citizens that have been adopted by the French National Medicine Academy to 23,over a period of 123 years.

Conclusions

1. The urgent necessity to publish the guidelines with the "**RATING CLASSES**" and framing inside them the main chemical carcinogens, both from the environment (air, water, soil, plants and animals) and as well from animal and non-animal origin foods, including animal food ratios;
2. The final necessity to open the third battlefield against the terrible enemy of mankind that is the cancerous disease, to the level of the environment, due to new disciplines: eco-oncology, with its two aspects: eco-onco-therapy and eco-onco-prophylaxis of the environment;
3. Improving the life quality of humans and pets suffering from cancer;
4. Developing comparativ oncology epidemiology that, after monitoring the main carcinogens, create the "**TRACEABILITY MAP**" in the geographical regions of Romania;
5. By applying the first two conclusions, the cancerous disease in humans and animals, will know a substantial decrease in incidence, prevalence and mortality;

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LINGUAL STRUCTURE OF THE TONGUE OF THE COMMON KESTREL *(FALCO TINNUNCULUS)* IN RELATION TO FOOD INTAKE

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Abstract

This study evaluated the morphological and histomorphometrical characteristics of the tongue of the adult common Kestrel (Falco tinnunculus) (Aves : Falconiformes: Falconidae) in relation to food and feeding habitats. The aim of the present study was to investigate the structure of the tongue and to compare the present results with those reported in other avian species. Three adult birds were used in the investigation. Results showed that the tongue is characterized by a rectangular format with bifid apex. The entire dorsal surface of the tongue was covered by a thick stratified squamous epithelium which is thicker in the dorsal surface than that in the ventral one.. Conical papillae appeared in the dorsal lingual epithelium at the boundary between the root and body of the tongue. There is a lamina propria forming lamina propria ridges beneath the lingual epithelium.SEM revealed that the dorsal surface of the apex of the lingual epithelium has a carpet-shape structure. The lingual glands are formed from tubuloalveolar glands and present laterally and beneath the dorsal epithelium along the lingual body and lingual root. It was concluded that these features may adapt and support the functions of the tongue during food intake, especially in the common Kestrel which feeds mainly on small animal.

Key words*: Common Kestrel, Tongue, Epithelium, Scanning, Morphometry, Food.*

Introduction

Feeding mechanism is important to determines the adaptation and persistence of vertebrates to their environment (Darwish, 2012). The various shapes of the tongues have been studied by gross anatomical, light microscopy, scanning and transmission electron microscopy and by histochemical methods. Birds are spread out through the different habitats; the air, land, and around fresh and sea water. Many authors revealed that the tongue structure differ according to the type of food and method of food intake**.** Many authors described the various shapes of the tongues and lingual structures, especially, on the structure and topographical distribution and shape of lingual papillae as well as the tongue projections (McLelland, 1990; Vollmerhaus and Sinowatz, 1992; Koening and Liebig, 2001; Emura *et al*., 2008, 2009). The shape of the tongue is differ from bird to another bird according to food and feeding habits. For example, The tongue is basically triangular in shape in case of galliforms and passerine birds (Erdogan and Alan, 2012; Erdogan et al., 2012b), elongated and bulky in case of birds of prey (Emura et al., 2008a,b; Erdo˘gan et al., 2012a), elongated and oval, with many projections, in lamellirostrate birds (Iwasaki et al., 1997 ; Jackowiaket al., 2011) and relatively small with no functional projections, in ratite birds (Jackowiak and Ludwig, 2008; Crole and Soley, 2009a,b ; 2010; Santos et al., 2011; Tivane et al., 2011). The avian tongues are adapted for the collection, manipulation and swallowing of foods and also closely associated with food and feeding habit of the bird (Emura et al., 2008a,b; Parchami et al., 2010a,b). The microstructure of the tongue has been examined in many species of birds, e.g., in parrot, penguin, little tern, owl, white-tailed eagle, hen, quail, and bean goose (Homberger & Brush, 1986; Iwasaki, 1992; Iwasaki *et al*., 1997; Jackowiak &Godynicki, 2005; Jackowiak *et al*., 2006; Emura & Chen, 2008). Keratinization appeared as the most important charactaristic changes in the lingual

epithelium during the evolutionary adaptation of vertebrates from a wet to a dry habitat (Iwasaki,2002). The dorsal and ventral surfaces of the birds tongue are covered by a rather thick layer of stratified squamous epithelium, which is keratinized or non-keratinized, depending on the feeding habitat of the bird. The dorsal surfaces of most of the avian tongues are covered by keratinized layer (Iwasaki et al., 1997; Kobayashi et al.,1998; Samar et al., 2002; Jackowiak et al, 2010; Erdo˘gan et al.,2012b). Many authors found that keratinized layer of the lateral and lower surfaces of the tongue, are reduced in its thickness, especially, in domestic birds (Erdo˘gan et al.,2012b). In ratites, there is no keratinized layer on either the dorsal or the ventral surface of the tongue (Jackowiak and Ludwig, 2008;Crole and Soley, 2009b; Guimarães et al., 2009; Pasand et al., 2010;Santos et al., 2011). The dorsal epithelium is slightly thicker than the ventral epithelium (Jackowiak and Ludwig, 2008; Crole and Soley, 2009b). In some avian species such as white-tailed eagle (Jackowiak and Godynicki, 2005), parrot (Homberger and Brush, 1986), and nutcracker (Jackowiak et al., 2010) the keratinization of the ventral lingual epithelium lined the surface of tongue apex is extreme, as lingual nail and has a protective role against hard food. In birds, the lingual papillae play an important role in feeding mechanism. The lingual papillae in birds eat hard foods make as teeth (Iwasaki *et al*., 1997) and can keep food on the tongue's surface (Iwasaki, 1992). Morphological studies showed that salivary glands are absent in some birds such as the pelicanns but are present in others. They are poorly developed in birds that eat soft diet such as piscevorous species and well developed in granivorous, insectivorous and woodpeckers. Saliva in birds is primarily a lubricant or a sticky coat to the tongue to glue food and ease swallowing.

The present study was conducted to reveal the morphological, histological and morphometrical aspects of the tongue of the common kestrel (*Falco tinnunculus*) (carnivorous) in relation to food and feeding habits.

Materials and Methods

Experimental Animals:

The experimental animals of the present work included adult and healthy birds. Three adult common kestrel *(Falco tinnunculus)* (carnivorous) were used in the present study. Birds were killed and tongues were dissected free from the mandible, then lengths and widths of tongues were measured quickly. For routine light microscopy, two tongues from two birds were fixed in 10% buffered paraformaldehyde for histological investigation and one tongue from one bird was prepared for investigation by the scanning electron microscope.

Histological and morphometrical Studies:

The freshly removed tongues were washed under running tape water to remove any food debris and immediately fixed in 10% neutral formalin. The tissue was washed in tap water then dehydrated in ascending series of ethyl alcohols, cleaned in xylene and finally embedded in paraffin wax at 60°C. The transverse paraffin sections at 5-6µm in thick were prepared. For routine histological investigation, sections were stained with Haematoxylin and Eosin according to Carleton (1980).

For morphometrical study, Four slides of each region of the tongue of each individual (15 sections per slide) were measured. For each section, three measurements of thickness of the epithelium of different regions of the surfaces of the tongue were measured. Histological sections were studies by using a research microscope equipped with digital camera and connected to a PC based image analysis system. Sigma Scan Pro (version 4.0, Jendel

Scientific, SPSS Inc., Chicago, USA) was used for image analysis and morphometrical data acquisition.

For scanning electron microscopy, one tongue of each bird was fixed in 5% gluteraldehyde, washed in cocodylate buffer for one hour and post fixed in a buffered solution of 1% osmium tetroxide at 37°C for two hours. Then, specimens were followed by dehydration in ethanol; complete dehydration in amyleacetate for two days, dried in carbon dioxide at sputter coated with gold. The specimens were examined in a JEOL scanning electron microscope (JSM-5400LV).

For statistical analysis, univariate analysis of variance was used to test differences between thickness of different regions of the lingual epithelium. All values are gives as means ± standard deviation (S.D.). The values were considered significantly when $P<0.05$. All statistical analysis were performed using SPSS (version 9.0, 1998).

Results

Macroscopic observations: (Fig.1A)

The tongue of the adult common kestrel (*Falco tinnunculus*) has a rectangular format with bifid apex. It appears composed of two parts; the anterior part composed of body and the posterior one which formed the root of the tongue. the anterior part of the tongue is distinguished on the dorsal surface into two parts, the apex and the body. There is a shallow groove at the anterior half part of the lingual dorsal surface. From the morphometric point of view, the tongue is about 1.9 cm length and 0.8 cm width in its median part.

Microscopic observations:

The tongue is supported by paraglossum which is a cartilagenous, single and oval in shape through the apex and it is double at the body region of the tongue. Lingual muscle fibers were seen in a cross section of the tongue are transversely sectioned. The dorsal surface of the tongue was lined by a thick nonkeratinized stratified squamous epithelium, while the ventral surface exhibited a thin stratified squamous epithelium (Fig.1B). The connective tissue beneath the epithelium of the dorsal surface penetrated the epithelium in form of connective tissue of lamina propria forming lamina propria ridges but this was lacking in the ventral epithelial layer(Fig.1C).

The lingual glands are present laterally along the lingual body and lingual root. The lingual glands are formed from tubuloalveolar type beneath the dorsal lingual epithelium and lined with connective tissue(Fig.1C). The ducts of the lingual glands opened onto the dorsal surface of the tongue

Scanning Electron Microscopy:

SEM revealed that the dorsal surface of the apex of the lingual epithelium presents fine processes as a carpet-shape structure (Fig.1D). The epithelium of the lingual body is covered by flat and smooth layer with no papillae (Fig.1E). There are many openings of the lingual glands in the lingual body and lingual root (Fig.1E). Many conical papillae appeared in a conical shape with a sharp and directed caudally were present on the posterior dorsal surface of the lingual body and in each side on the body- root junction. (Fig.1F).

Morphometrical observations:

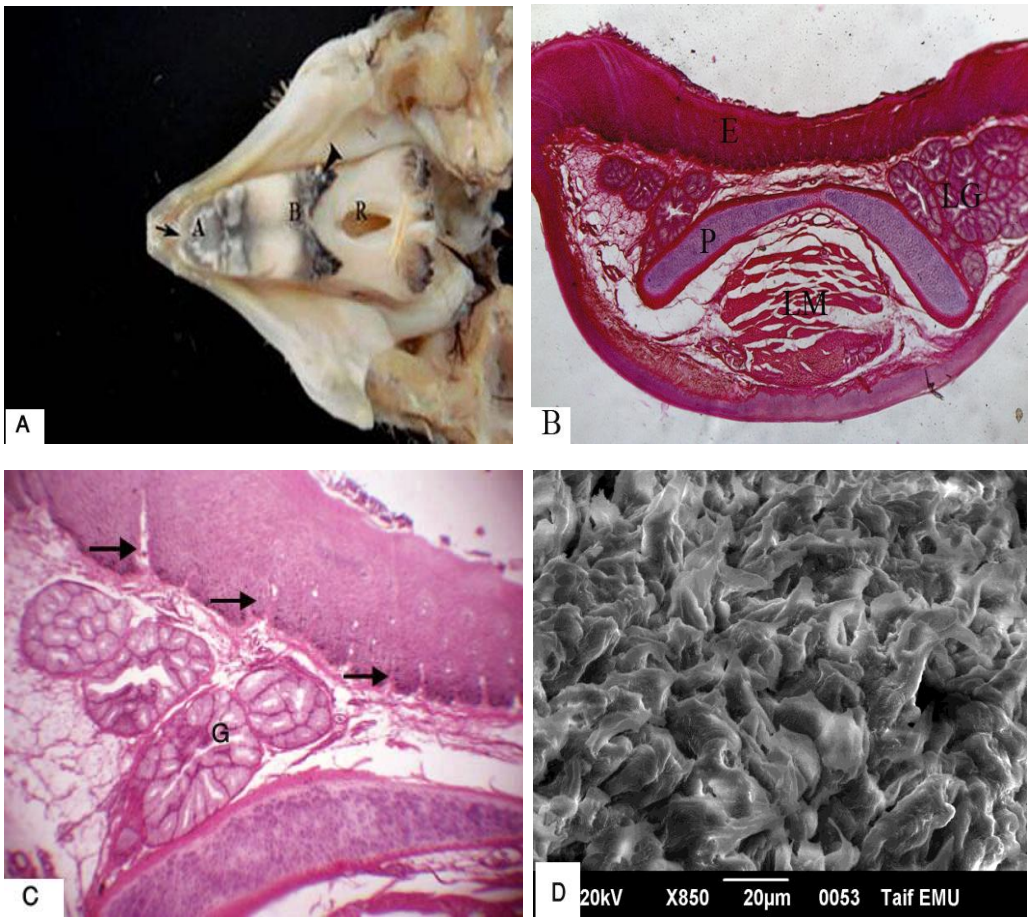
The thickness of the epithelium vary depending on the area of the tongue (Table1). At the whole dorsal surface of the tongue, epithelium thickness was (428 ± 39) μm, while the thickness of the epithelium of the lateral borders was (172 ± 23) μm. Ventral epithelium

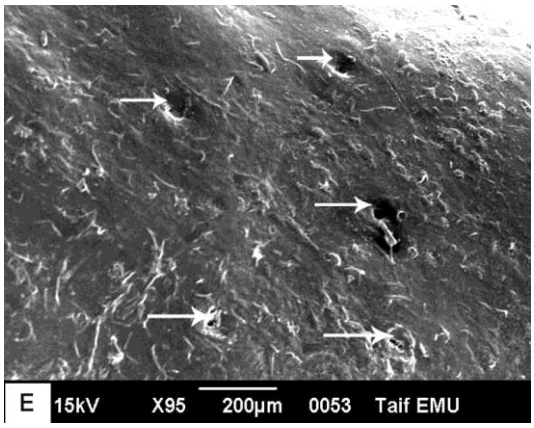
thickness was (132 ± 19) µm. There was a significant different ($P < 0.05$) in thickness between the three measured regions of lingual epithelium.

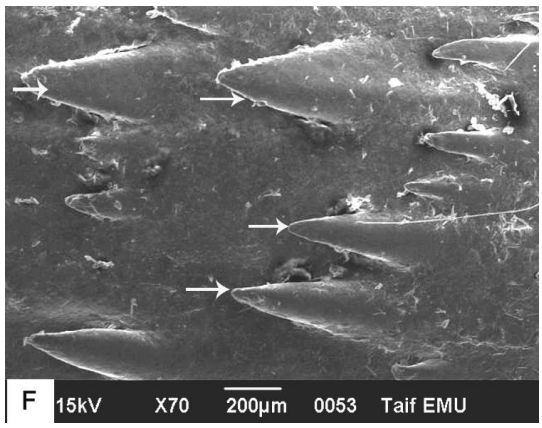
Table (1): Thickness (µm) of different regions of lingual epithelium of Common kestrel. (Values are means ± S.D.), N = 3 birds, 15 section for each region for all measurements)

Regions	Means ± Std
Whole dorsal surface	428* ± 39
Lateral borders	172* ± 23
Ventral surface	132* ± 19

* $P < 0.05$







Explanation of Figures

Fig.(1):

(A): Floor of the oropharynx of the Common kestrel, *Falco tinniunculus* showing the tongue apex (A), the body (B), the root (R) and caudally directed papillae (arrow head) between the body and the root. Scale: 1 mm.

(B): Photomicrograph of a T.S. of the of the tongue body of the Common kestrel showing the paraglossum (P), multilayered stratified squamous epithelium of the dorsal surface (E) and the ligual glands (LG). (H&E, X 40).

(C): Photomicrograph of a T.S. of the tongue body of the Common kestrel showing the ligual glands (G) and the lamina propria ridges beneath the epithelium (arrows). (H&E, X 400)

(D): Scanning electron micrograph of the dorsal surface of the lingual apex of the Common kestrel showing that lingual epithelium presents the carpet-shape structure.

(E): Scanning electron micrograph of the dorsal surface of the lingual body of the Common kestrel showing smooth lamellar aspect with no papillae. Note the openings of the lingual glands (arrows).

(F): Scanning electron micrograph of the dorsal surface of the posterior part of the lingual body of the Common kestrel showing a sharp conical papillae that appeared in a conical shape.

Discussion

Morphological and microscopic structure of the tongue of bird species may be directly associated with food type consumed and feeding habit of the bird (Campbell & Lack 1985; Vollmerhaus & Sinowatz, 1992; Parchami *et al.*, 2010). In the present study, the tip of the tongue was bifid. This is in accordance with the northern fur seal (Emura *et al.*, 2001) and beaver (Shindo *et al.*, 2006). Data obtained from the present study also showed that there is a groove in the apex of the tongue. The median groove is a characteristic feature found on the tongue of white tailed eagle, ducks and geese, whereas it is absent on the tongue of chickens and penguins (Jackowiak, and Godynicki, 2005).

The conical papillae are found between the body and root of the tongue and show considerable differences in their distribution and development among the different avian species. The papillae are well developed in birds such as White tailed eagle and owl which feed on fish or small animals and is absent in birds such as woodpecker and ostrich which feed on insects or plants (Jackowiak and Godynicki, 2005; Emura and Chen, 2008;

Jackowiak and Ludwig, 2008; Emura *et al*., 2009). Conical papillae of the tongue in the present study are distributed in a very wide area between the lingual body and lingual root and directed caudally. Caudal directed papillae facilitate the movement of food in only one direction towards the oesophagus and prevent regurgitation (Koolos, 1986). Presence of conical papillae between the body ant root of the tongue in the common kestrel are in agreements with (Iwasaki and Kobayashi, 1986) in a row and long-legged buzzard (Erdo˘gan et al., 2012a). (Iwasaki *et al.*, 1997) reported that the tongue of the goose has giant conical papillae located in a transverse row between the lingual body and the lingual radix.

In most birds, dorsal surface of the tongue is directly contact with food and may expose to injuries during feeding. Then, lingual epithelium is thicker than that of other region of the tongue as in case of the Common kestrel.

The degree of keratinization of the epithelium depended on the type of food intake; in herbivorous and granivorous birds it is appeared heavily cornified. Lesser degree of keratinization is found in water habitats birds (Iwasaki, S. (2002; Jackowiak and Ludwing, 2008). In the present work, The thick non-keratinized epitheliuml covering the dorsal surface of the tongue apex and body in the common kestrel and the presence of lamina propria ridges may adapted for mechanical protection of the organ against injuries during feeding. This finding is similar to the tongue of the emu (Crole and Soley, 2008),ostrich (Jackowiak and Ludwig, 2008) and Muscovy duck (Igwebuike and Anagor, 2013b) which do not show any lingual epithelial keratinization and thick the tongue epitheliun.

Lingual glands also show variation in birds. The glands are generally well developed in granivorous species, less developed in birds of prey, poorly developed in piscivores and absent in the Anhinga and Grea Cormorant (Whittow, G.C., 2000). In this study, Lingual glands are not well developed and restricted to the root of tongue with numerous openings through the dorsal surface of the tongue root. In some birds, the glands may be restricted to certain areas of the tongue (Kobayashi et al. 998; Al-Mansour & Jarrar 2004). There is a report that the cormorant lack lingual glands entirely (Jackowiak et al., 2006). In white-tailed eagle (Jackowiak and Godynicki,2005) and chukar partridge (Erdo˘gan et al., 2012b), the lingual glands have been classified as anterior and posterior glands. These finding are consistent with the results of McLelland (1979), King and McLelland, (1984) and Blanks (1993) who recorded that, the structure of lingual salivary glands is more developed and complex in birds that feed on dry food like seeds as compared to those having access to naturally well moist food.

In conclusion, this study has shown that the morphological and histomorphometrical features of the tongue of the Commom kestrel may be adaptive to the bird's mode of food acquisition and feeding habits.

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MORPHOLOGICAL ASPECTS OF CUMULUS-OOCYTE COMPLEXES IN DIFFERENT SPECIES

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Abstract
Quality of COC is an important factor that can influence the success of in vitro fertilization (IVF) technique. The presence of several layers of cumulus cells and clear, dense, homogeneous cytoplasm were criteria for a good quality oocyte in all system of classification for different species. This study was designed to evaluate the quality cumulus oocyte complexes (COCs) in different species based on their morphological features. Highest number of COCs was obtained from cat and bitch ovaries using slicing technique (429 COC, 759 COC) vs cow and gilts ovaries (500 COC, 300 COC) using puncture technique. Morphological classification based on 3 grade class revealed 29,6% class I in bovine; 31,66% class I in gilts; 17,03% class I in cat and 24,63% in bitch. The results suggest that classification of COCs by their morphological aspects is a fast and easy way to make a first evaluation on quality of COCs in order to continue IVM only with the highest grade of COCs.
Keywords: *cumulus oocyte complexes, in vitro fertilization (IVF)*

Introduction

In vitro production of embryos is an assisted reproduction technology (ART) used in a large number of animal species with the purpose of improving production potential, also for conservation the genetic material of endangered species, biomedical research (cloning, transgenic animals, stem cell research, establishment of oocyte banks) etc (6, 17).

In vitro maturation (IVM) is the first step for IVP technique and the most important because the success of *in vitro* embryos production depends on oocyte quality. Oocyte quality is defined as the competence to yield a blastocyst whitin an *in vitro* production system (2,13).

Quality of oocytes in all species is influenced by many factors: method of recovery (aspiration, puncture, slicing, slicing after aspiration, follicle dissection, *in vivo* by ovum pick up - OPU), follicle size, stage of follicular wave (follicular growth, follicular dominance), ciclicity status of the female, age, season, body condition, storage temperature of ovaries, transport time etc (6,12,15,18,19,20,22,23).

"Ideal oocyte" includes criteria such morphological aspects (cumulus cells layers, cytoplasmatic granularity, localization of granular area), biochemical aspects (proper lipid contents stored mainly as lipid droplets in the cytoplasm), molecular aspects (mRNA, proteins – necessarily for further embryo development), biological aspects (based on ovarian-follicular microenvironment, maternal signals mediated by granulosa and cumulus cells), metabolomics aspects (based on follicular fluid and culture media contents- glycolytic intermediates and amino acids that support oocyte growth), cellular aspects (intrinsic markers – mitochondrial status, glucose-6-phosphatase dehydrogenase 1 activity; extrinsic markers – apoptosis of follicular cells and level of TGF-beta superfamily in follicular fluid or serum)(11).

The commonly used classification of cumulus-oocyte complexes (COCs) quality is based on their morphology as observed on stereomicroscope: the number of cumulus cells layers and ooplasm characters. Why cumulus cells? Because these cells communicate with oocyte across zona pellucida through corona radiata cells which penetrate zona pellucida and form gap junctions with oolema, allowing metabolic transfer of molecules necessary for nutrition, growth and maturation (12).

According to these criteria, bovine COCs are classified in 3 or 4 grades/classes. The first two COCs classes have the same characteristics in both systems of classification.

Good quality/class I/grade A includes COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous.

Intermediate quality/class II/grade B includes COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation.

The difference between classification systems is in the last class. According to classification in 3 COC classes here are included *rejects/poor oocytes*: COCs with partially or fully expanded and dispersing cumulus, incomplete or nude oocytes, uneven cytoplasm granulation, intermixed very light and very dark areas, discolored cytoplasm, badly misshapen oocytes, small oocyte (3,4,8,15).

Considering the second system of classification, the last two classes are: *class III/grade C* with oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm and *class IV/ grade D* with oocytes completely denuded of cumulus cells and/or with irregular shrunken cytoplasm (12,20).

In swine, COCs are evaluated morphologically on the four-grade scale, similar to bovine species: grade I COCs – have homogeneous cytoplasm and a complete, compact cumulus oophorus; grade II COCs – homogeneous cytoplasm and an incomplete but compact cumulus oophorus with more than five cell layers, grade III COCs – heterogeneous cytoplasm and a greater than one-three cell layer cumulus oophorus and grade IV COCs – strongly heterogeneous cytoplasm and either partially or entirely absent cumulus (2,10).

Alvarez et al. (2009) proposed six subpopulations classification of COCs porcine according to morphological cumulus features: A1- with dense cumulus; A2 – with translucent cumulus, B1- with the corona radiata, B2- partly naked oocytes, C- naked oocytes, D- with dark cumulus and concluded that morphological features may be useful for selection of potentially competent oocytes with nuclear maturation (metaphase II) occurred in A1, A2, B1 and B2 classes, but higher cytoplasmatic maturation rate were observed in class A1.

Positive correlation was found between morphological aspects of porcine oocyte and pZP transcript contents - glycoproteins involved in gamete fusion (10); also with connexins and cyclin-dependent kinases (2).

Jaclowska et al. (2009) observed that in grade I oocytes pZP1, pZP2 and pZP3 mRNA contents were 2-4 time more increased compare to grades III and IV, and was no difference from grade II oocytes, meaning that oocyte morphology may be related to an increased fertilization ability of higher quality oocytes (10).

Increased level of mRNA molecules encoding connexins - transmembrane proteins that form gap junctions, which participate in communication between oocytes and complex of cumulus cells (Cx43; Cx45) and cyclin-dependent kinases proteins – involved in cell cycle progression (CDKN1, CDKN3, CDK5) in oocytes graded I as compared to other grades may suggest association between gamete morphology and their maturation ability (2). Similar,

Calder et al. (2003) observed in bovine that Cx43, FSHr, LHr mRNAs levels are dependent on oocyte quality, but also on time of maturation and that Cx43 may be an important mediator of positive oocyte maturation signals from cumulus to oocyte during IVM (3).

Assisted reproductive technologies were successfully used in canine and feline, with good results in cloning dog by nucleus transfer, transgenic dog, cat ovarian and embryo cryoconservation, cat somatic cell nuclear transfer. These met many purposes, such as studying human diseases (cardiomyopathies, muscular dystrophy, prostate cancer) due to fact that more than half of the approximately 400 known hereditary canine diseases have an equivalent human disease (17) and on the other hand in supporting perpetuation of endangered non-domestic felids (14).

Morphological classification of bitch and cat oocytes is based on the same criteria as for cow and sow, but usually for IVM , only grade I COCs – darkly pigmented ooplasm and complete surrounded by at least one layer of compact cumulus cells, are used (5,7) or when they are retrieved by laparoscopy from gonadotropin-treated female: oocytes surrounded by an expanded cumulus cell mass (preovulatory), partially or fully surrounded by compact corona radiata cells (7).

The aim of our paper was to quantify the quality of COCs retrieved from four different domestic species according to their morphological aspects.

Material and methods

Cow (n=104) and sow/pubertal gilts (n=78) ovaries were collected from slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution, at 35^0C within two hours. Canine (n=26) and feline (n=22) ovaries were obtained after routine ovariohysterectomy, at various stage of the estrous cycle, that were realized in the Clinique of Reproduction, Obstetrics and Veterinary Gynecology from Faculty of Veterinary Medicine, Timisoara. Carnivore COCs were recovered in the same day. Ovaries were washed 2-3 times with 0.9% NaCl solution and than was proceed in recovering the COCs in Dulbecco's phosphate buffered saline (PBS) solution, prepared in our the laboratory (6).

Different methods of oocyte recovery were used depending on species. Cow and sow ovaries were approached by puncture method: visible follicles 3-8 mm diameter at cow and 3-6 mm diameter at sow/gilts were suctioned with 18G needle attached to a 5 ml syringe. Follicular fluid obtained through suction was transferred to a 90 mm Petri dish. Bitch and cat COCs were recovered by slicing the ovaries with a sterile surgical blade into a 90 mm Petri dish. COCs were washed in PBS 3-4 steps before classified them. Classification of COCs based on morphological aspects was done with stereomicroscope [The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equiped through POSCCE 2669 programm] (Stemi 2000-C, ZEISS) with hot plate ($33,4^0$C). General criteria used for cumulus-oocyte complexes selection was based on 3 classes: good quality, intermediate and poor/reject oocytes as describe in introduction section.

Results and discussion

Morphological classification of COC from species included in our study is contained in table 1 and further details of morphological aspects of COC are shown in Fig. 1.

Table 1. Morphological classification of COC from different species

Specie	Harvesting method	Ovaries used	COC recovered	Mean no. of oocytes	CLASSIFICATION		
					CI	CII	CIII
Bovine	P	104	500	4,80	148	196	156
Swine	P	78	300	3.84	95	71	134
Canine	S	26	759	29,19	187	192	381
Feline	S	22	429	19,50	73	117	239

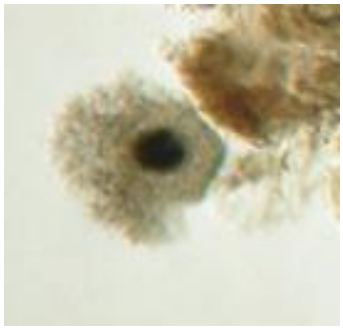
Mean oocyte recovery rate from bovine ovaries was 4,80 of which 1,42 (29,6%) were good quality; 1,88 (39,2%) intermediary quality and 1,49 (31,2%) poor quality; slightly higher than the results of Rao et al. (2012) in buffalo ovaries – 3,46; slightly lower than those of Wang et al (2007) – 5,6 in Holstein cows, and lower when compare with slicing method – 9,6 oocytes/ovary. Slicing and puncture methods are the most used harvesting technique in bovine reproduction biotechnologies.

Between maturation rate and quality of COCs is a positive relation in bovine species (12,20). Kakkasery et al. (2010) reported higher cumulus expansion in class A and B oocyte in comparison to class C indifferent of retrieval technique, except slicing (69,70% vs 83,08% by aspiration and 70,37% by puncture)(12). We obtained similar results in our laboratory: 80% expanded COCs at 24h in class I COCs, 75% in class II and 6,30% in class III (unpublished data).

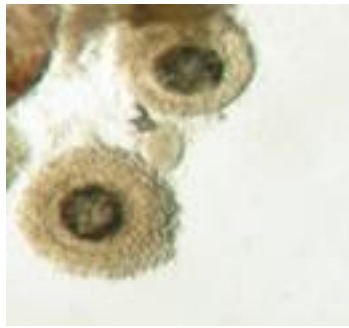
Mean oocyte recovery rate from sows and gilts ovaries was 3,84; from which 1,21 (31,66%) were of good quality; 0,91 (23,66%) of intermediary quality and 1,71 (44,66%) poor quality. The high number of COCs of poor quality is probably due to oocytes source (mostly oocytes collected from small follicles from gilts ovaries). It was shown that liquid from large follicles (5-6 mm in diameter) improve the developmental competence of oocytes via nuclear maturation and cumulus cells viability (9).

Mean oocyte recovery rate from cat ovaries was 19,50 of which 55,69% (mean=10,86) were poor quality and only 3,32 (17,03%) were good quality and 5,32 (27,28%) intermediary quality. These data indicates that although number of rejected COCs is high, due to the fact that the large number of COCs that we can obtain by slicing technique, this method is suitable and it is used in cat reproductive biotechnologies.

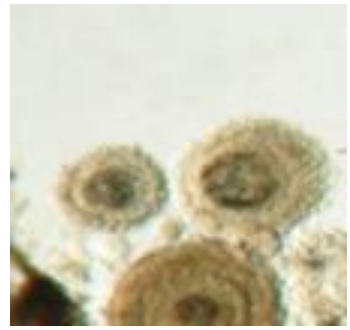
Increasing cat IVF success by supra-normal numbers of preovulatory oocytes can be reached by multiple gonadotropin hormone ovarian stimulations and laparoscopic oocyte retrivals. Pope et al. (2006) obtained from 21 retrievals in five fishing cats, 579 preovulatory oocytes (mean=27,6) and 348 embryos (mean=16,6)(14).



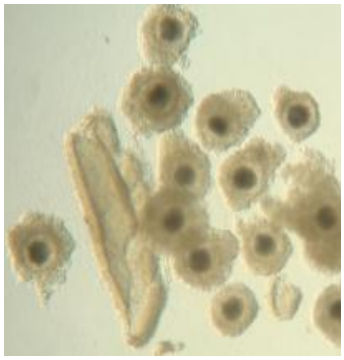
Cow COC good quality



Cow COC fair quality



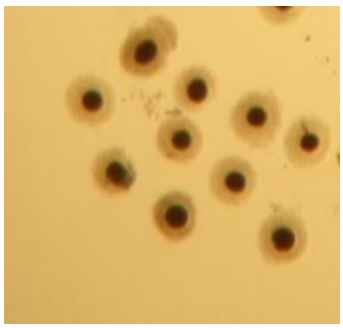
Cow COC poor quality



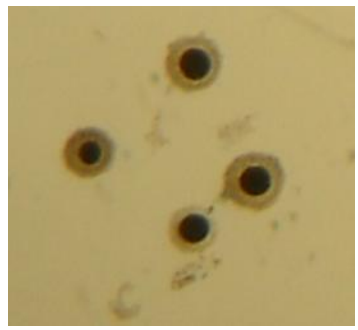
Sow COC good quality

Sow COC fair quality

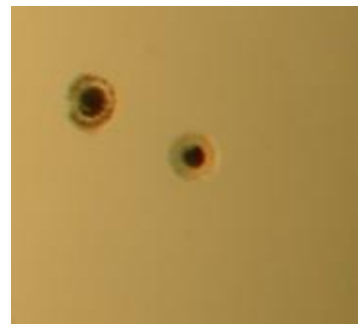
Sow COC poor quality



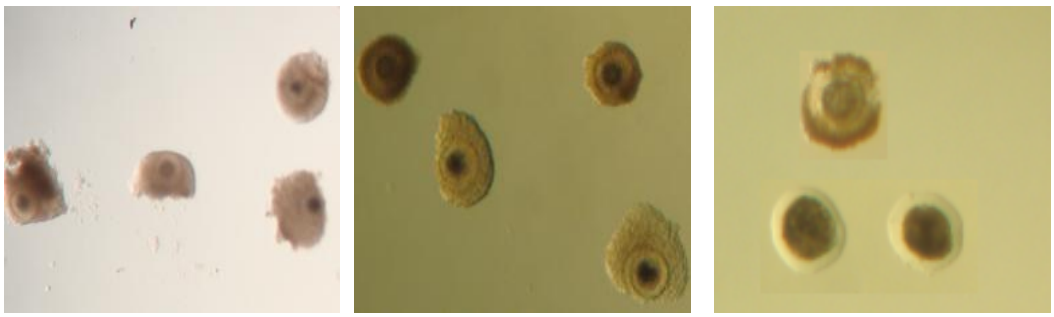
Bitch COC good quality



Bitch COC fair quality



Bitch COC poor quality



Cat COC good quality Cat COC fair quality Cat COC poor quality

Figure 1. Morphological aspects of different COC classes in 4 species (50X)

From bitch ovaries a total number of 749 COCs wtih 379 COCs (49,91%) were of good and intermediate quality. Evecen et al. (2009) obtained from 78 ovaries a number of 1138 COCs grade I. Usually in canine ART studies, only grade I COCs are used, so are few information regarding the other COCs classes (5). As in cat (16, 21), the transport temperature and the estrus cycle stage has a great influence on grade I COCs IVM rate. Evecen et al. (2009) concluded that oocytes harvested from follicular and luteal ovaries have a significantly higher maturation rates (MI + MII) than the oocytes from anestrual ovaries at 37^0C; transporting canine ovaries at 4^0C can improve in vitro maturation rates in oocytes harvested from anestrous ovaries (5).

Conclusions

- Morphological classification of cumulus-oocyte complexes (COCs) represents the first step for *in vitro* maturation
- Mean number of oocytes and quality of COCs are influenced by oocyte recovery method
- Our further research will focus on influence exerted by COCs quality on *in vitro* maturation, *in vitro* fecundation and *in vitro* culture in bovine.

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DIFFERENTIAL AND POSITIVE DIAGNOSIS CRITERIA BETWEEN LEUKEMIA AND LEUKEMOID REACTIONS

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Abstract
The authors review the defining elements of the differential and positive diagnosis between leukemoid reactions and Vera leukemia. These pathological entities hold an important role in the wide medical veterinary practice, partly because those who use the clinical blood morphologic cell exam can easily misdiagnose the situation. This paper can be used as a short guide for avoiding the eventual "mal-praxis" situations in veterinary medicine.
***Key Words**: Leukemia, leukemoid reactions, leukemia-like conditions.*

Introduction

The leukemoid reactions represent frequent hematologic syndromes that do not manifest their own clinical expression. They can be met in various conditions regarding the etiology, as a response of the body tissues responsible for red blood cells production, especially the bone marrow, to different exogenous or endogenous pathogens. These reactions disappear once the causing pathogen is eliminated from the body.

The main laboratory characteristics of these manifestations consist of quantity and quality alterations of the blood elements. The quality alterations refer to the dominance of a single cellular component in the blood screening, with an important degree of cellular atipism (granulocytes, lymphocytes or monocytes).

The leukemoid reactions are characterized by a hematologic picture associated with a consistent leukocytosis (sometimes over 50.000 elements/cubic millimeter) and also the presence of immature elements in the peripheral blood.

The most important and also the defining element for the leukemoid status is that of a transitory reaction, without any sign of the stabilization of this type of reaction, as it is the case of vera leukemia or malignant lymphomas with cellular discharge.

A thoroughgoing study of the leukemoid reactions is absolutely necessary due to the fact that such a diagnosis in veterinary medicine can sometimes shield the presence of a leucosis condition that often bring about severe social, economic and administrative consequences.

The latest book published in this field of expertise, "The Leukemia-like (leukemoid) syndrome in veterinary pathology", published by Dr. Emilia Balint, will be a reference point in the following, because it presents novel aspects regarding the leukemoid reactions as well as research data gathered for her PhD thesis, sustained in the year of 2000, under the coordination of Univ. Prof. Dr. Manolescu Nicolae. Also, the book presents over 160 literature references that demonstrate the contribution of the Romanian School of Hematology, led by Univ. Prof. Dr. Manolescu Nicolae. The debut was in 1976, with the paper entitled "The cellular morphology of the red blood cells producing marrow and of the

leukocyte concentrate in animals",proving afterwards, with the occasion of numerous papers, the importance of this subject for human and veterinary pathology. The book "The leukmoid reactions status in human and veterinary pathology" published by Dr. Emilia Balint is plea for hematology and for the initiation of specialists in clinical hematology, because, as the author of the book highlights, there is no disease in animals (regardless of etiology), that does not change the normal, quantity and quality values of the general blood tissue and the leukocyte territory.

Surely, in order to achieve this goal, the veterinary practitioner has two choices: either he can become a fine specialist in normal and pathological red blood cells producing mechanisms, either he can collaborate with a scholar in normal and pathological hematology, to whom he is to address permanently in order to gain, for every individual case, the hematology analysis data form that contains all of the constants, including those that refer to the "leukocyte status".

A chapter of the hematology data form will address generated and particular data of leukocytes, as well as the aspect of leukemoid reactions.

The question arises: "Why should we approach and know this chapter of leukemoid reactions within hematology sciences and how does it ease our daily practice?"

Because the lack of the information of the hematologic status can lead to a series of bad diagnoses, that in turn can bring upon the veterinary practitioner sanctions as „mal-praxis". This affirmation is based on the existence of two pathological features that can evolve in animals and that can each have a completely different and opposed diagnosis, prognostic and therapy.

These two features are:

- The leukemoid reaction – that evolves without significant signs and requires only a modest general treatment;
- Leukemia Veraor the malignant lymphoma with cellular discharge – with a detrimental evolution and that requires a complicated treatment.

Limiting our attention only to these two possibilities, that without a full blood work, can lead to severe diagnosis, prognostic and therapy errors. For instance, a healthy animal, that by some cause, is manifesting a temporary leukemoid reaction, is recommended for euthanasia, or the opposite situation, an animal that manifests a leukemia vera to be put on classic antibiotic or anti-inflammatory therapy. Through this paper we have tried to overcome these challenges for Veterinary Medicine.

The leukemoid reactions represent ample anatomic and clinic reactions that are usually temporary, with a possible range of events determined by multiform etiological causes.

Theoretically, within the leukemoid status, a series of anatomic and clinic syndromes can be defined, that have an important frequency of incidence in animals:

- Reactive blood status – known in the past as benign reticulocytes pathologies (reticulocytes lymphocytes pathologies);
- Pre-leukemia status – identifying the dysplastic states catalogued in:
- Lymphocyte dysplastic conditions
- Myelocytes dysplastic conditions
- Mastocytes dysplastic conditions
- Monocytes dysplastic conditions

Practically, all of these conditions that are components of an important chapter of the blood science (leukemoid reactions), are essential for correctly diagnosing an illness, in regard to the near to perfection similarities to the cellular blood malignant pathologies (leukemia and lymphoma).

The most important and defining element of the leukemoid status is that of being temporary, accepting no turnover of becoming a permanent situation, as is the case of leukemia Vera or malignant lymphomas with cellular discharge.

A more complex approach on the leukemoid reactions is absolutely necessary, because such a diagnosis in veterinary medicine can sometimes shield a leucosis condition that often bring about severe social, economic and administrative consequences.

The leukemoid reactions are usually confined to a single cellular line, being of sectorial type.

We can identify 3types of leukemoid reactions:

- Lymphocyte reaction
- Monocytes- Macrophages reaction
- Granulocytes reaction

In this veritable "Achilles' heel", our presentation desires to bring light upon a domain that has been insufficiently looked upon. For this aim, we have targeted a presentation free from data offered by advanced investigation and diagnosis technology, methods that are unfortunately unavailable to veterinarians from our country for now and the near future.

In the paper entitled „Hematology: basic principles and practice" Ronald Hoffman defines the term of leukemoid reactions, also known as temporary myeloproliferative disorders, as the increase of white blood cells count, as a physiological response to stress or infection, comparative to the malignant blood condition, known as leukemia.

Conventionally, a WBC elevation that rises above 50.000 WBC/mm3, with a significant increase of the early precursors of neutrophils, refers to a leukemoid reaction.
Within the peripheral blood samples we can observe myelocytes, metamyelocytes or even myeloblasts. Still, this is a combination of mature early precursors of neutrophils, in opposition to the immature forms, typically observed in the case of acute leukemia. In the case of leukemoid reaction, the examined bone marrow can appear to have a hypercellular characteristic, this being the only visible modification.

The leukemoid reactions generally are benign replies and do not present themselves as a significant danger, even though they can be a response in severe conditions. Still, the leukemoid reactions can resemble more severe conditions, like chronic myeloid leukemia, that can manifest with identical elements within the examined samples of peripheral blood.

Materials and methods

A number of 140 animals was investigated, as following

- 60 felines;
- 80 canines;

Samples were harvested from these animals on EDTA K2, 5%, 1 part blood thinner for 9 parts blood. Some situations obliged to lymphnode aspirate.

The samples were interpreted by ahematologic analyzer MS4, identifying the proportion of leukocyte populations. The eventual cellular atipism was identified using the cellular morphology technique (MGG stain) and that of leukocyte concentrate.

Hematologic aspects:

Leukemoid Reactions	**Leukemia vera**
Erythrocytes System	Erythrocytes System
Usually unchanged, except for the etiologies that constantly determine hyper-regenerative anemia	Collapsed in every case, with severe non-regenerative anemia
Platelets System	Platelets System
Unchanged	A quantity decrease - thrombocytopenia
Leukocyte System	Leukocyte System
Temporarily altered regarding quantity and quality – usually severe leukocytosis with cellular blasts at the periphery, but without cellular atipism, monstrosities or cellular mitosis	Quantity and quality modifications and in the absence of therapy gains a permanent feature. It can be observed at the relapse of the condition post-therapy or within the blastic crisis. Usually hyper-leukocytosis with the presence of predominant blasts, atipism and monstrosities, multiple and giant nucleoli that frequently show signs of mitosis. These modification can be observer in the peripheral zone, blood cells forming marrow and lymph nodes.

Cellular morphology differential diagnosis criteria between leukemoid reactions and leukemia Vera.

No. Crt.	**Criteria**	**"Leukemoid Reactions"**	**"Leukemia Vera"**
1.	x200 and x400 microscope general picture	Polymorphic aspect of the cell population in respect to nexus, shape, color, etc	Monomorphic aspect, one cell type dominates, with the exception of Chronic Myeloid Leukemia (CML)
2.	Cytoplasm	a) Polymorphic aspect in respect to color (intensely basophilic, light basophilic and acidophilus), with the presence of certain oxifile or basophilic granulations. b) Polymorphic aspect in respect to size, wide cytoplasmthat alternate narrow cytoplasm. c) Vacuoles present	a) Monomorphic aspect in respect to color, usually dominant, intensely basophilic or light basophilic b) Uniformity in size c) Rare presence of vacuoles, that combine with cellular monstrosities Granulations only appear in the case of CMLand KNL
3.	Nucleus	a) Nuclear shape and size polymorphism, with the absence of monstrosities or giant cells	a) Nuclear shape and size nuclear monomorphism. b) The chromatin has a

		mono-nucleated or multi-nucleated. b) The nuclear chromatin is extremely polymorphic, alternating cells with lax, spongy chromatin with those that have dense, homogenous chromatin, with a snowball appearance. c) Nucleus structures appear in patches. The nucleoli are low numbered and small-sized. d) The outline of the nucleus membrane is usually monomorphic, smooth, without de presence of specific bumps.	monomorphic arrangement, depending on the cellular proliferated type (either lax, spongy or dense). c) The nucleus structures are extremely frequent, the presence of giant nucleoli is definitive. d) The outline of the nucleus membrane usually presents polymorphism, with numerous specific surface bumps, in the case of T cell or histiomonocytes proliferation.
4.	Presence of cellular divisions	Only rare cases of cells in amitosis	High frequency, both under the form of typical and atypical mitoses and as well amitoses.

The hematologic status requires definitization with:

1) Clinical status

a) Usually, in the case of leukemoid reactions, the animal is received at the veterinary clinic showing signs of faintness, anorexia, thirst, reservation in movement, fever sometimes accompanied by important tachypnea and tachycardia with or without arrhythmias and rarely with the presence of hemoglobin in the urea. Usually, no clinical signs of parenchymal alterations, internal or external adenopathy or liver/spleen megaly are visible. The biochemical exam does not bring forward any crucial elements into the big picture.

b) In the case of leukemia Vera, the animal is received at the veterinary clinic showing signs of a chronic illness that dates back a couple of weeks and that has evolved with or without fever, with a capricious appetite, the animal most often having a certain degree of cachexia. Chronic suffering may occur, due to the invasion of one or more parenchyma by the metastases of the leukemia process. A rather important element is that of the presence of the anemia syndrome, usually accompanied by bleeding syndromes (gum bleeding, the presence of blood in urea, feces or in vomiting). If using an ultrasound exam, the presence of liver or spleen enlargement can be observed, usually accompanied by internal or external lymphadenopathy. The biochemical exam is not relevant, except for the situation in which the parenchyma is infiltrated by leukemia cells, leading thus to increased biochemical values for the invaded tissue

2) The evolution status

a) In the case of leukemoid reactions, usually, the blood cells aspect remits itself with the disappearance of the pathogen that has induced the illness and that has provoked.

b) In the case of leukemia vera, the anatomic and clinic cellular frame is partially remittent at the beginning, turning into a definitive one if proper chemotherapy is applied. The cellular blood frame can be repeated once the illness has relapsed.

A special situation that deserves an increased attention is that that connects to the apparition of leukemoid reactios of different cellular bases in the evolving course of some

blood parasites (Babesiosis) and of some infections with agents Rickettsia-like(hemobartonelosisand bartonelosis).

Conclusions

1. The differential diagnosis between the leukemoid reactions and the vera leukemia can be achieved in the best of conditions by combining the clinical aspects with those of the cellular hematology ones;
2. The cellular dominance within leukemoid reactions is different with every animal species;
3. The leukemoid proliferative cellular response is not identical in the same species (so it is an individual response) for the same etiological base of the leukemoid response;
4. A leukemoid response is catalogued only on its cellular quality feature (regardless of the number of cells). There is an important and constant difference between the data supplied by the electronic blood exam and by that of the blood smear;
5. Among the studied species, dogs and cats, there is an important distinction in regard to the dominant proliferative cellular base, which in dogs, in 50% of the cases is represented by neutrophils and in cats, in 40% of the cases, by lymphocytes;
6. The criteria for the differential diagnosis between the leukemoid reactions and vera leukemia that we have presented in our study, can be applied in the medical practice by every laboratory and are extremely cheap and safe to apply.

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EFFICACY OF *ARTEMISIA ANNUA* AGAINST *EIMERIA* SPP. INFECTION IN BROILER CHICKENS IN A BATTERY TRIAL

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Abstract

The goal of this study was to evaluate the efficacy of A. annua as essential oil in preventing Eimeria infection in broilers. Sixty broilers of fourteen days were randomly divided in 3 groups of 20 chickens as follows: negative control (NC) (uninfected, unmedicated), positive control (PC) (infected, unmedicated) and experimental group (EG) (infected, medicated). Experimental infection was done with $1x10^4$ oocysts of Eimeria spp. and treatment with 0.15 ml essential oil of A. annua/l water for 27 days. The chickens were kept in batteries, fed and watered ad libitum, and were exposed to continuous light. Essential oil of A. annua was obtained from a Romanian variety through a hydro-distillation process of plants vegetative parts, blooms and flowers. Efficacy of A. annua against Eimeria spp. infection was evaluated by: (i) oocysts shedded per gram of feces; (ii) lesional score; (iii) body weight gain; (iv) feed conversion ratio; and (v) mortality rate. The inoculum used for experimental infection consisted in E. tenella 35.0%, E. acervulina 32.5%, E. mitis 25.0%, and E. maxima 7.5%. All chicks survived in NC and EG, in PC 2 chickens died , at 7 and 10 days after challenge. Chickens treated with essential oil of A.annua produced with 97.1% less oocysts than chickens from PC, while the mean lesion score was lower only with 35.3% (EG = 0.86; PC = 1.33). Following the intestinal segment the highest reduction of lesion score in EG was registered in duodenum (1 and 2 in PC) and caeca (0.4, and 1 in PC), while in the jejunum was higher than in PC (1.2, and 1 in PC). Body weight gain and feed conversion ratio were higher in chickens medicated with A. annua and in chickens from NC at 7 days post-infection, but at 27 days post-infection chickens from PC presented a higher body weight gain and a lower feed conversion ratio. According to our results, A. annua Romanian variety as essential oil can be used with good results to treat coccidiosis mainly caused by E. tenella and E. acervulina in chickens, and not for prevention. Further analysis must be done.

Keywords*: coccidiosis, Eimeria, poultry, Artemisia.*

Introduction

Coccidiosis is one of the most important diseases of the poultry industry, caused by intracellular protozoan parasites of the genus *Eimeria.* It produces severe economic losses worldwide due to expensive and ineficient control measures leading to increased mortality, growth depression and poor feed conversion in broiler chickens. Combined, this losses exceed 3 billion US$ annually in the entire world (Dalloul and Lillehoj, 2006).

There are seven recognized species of *Eimeria* that affect poultry: *E. acervulina, E. maxima, E. tenella, E. necatrix, E. praecox, E. mitis* and *E. brunetti* (Williams et al., 2009). In broiler chickens, *E. acervulina, E. maxima,* and *E. tenella* are found frequently (Ogedengbe et al., 2011).

Control of coccidiosis is based on the use of in-feed anticoccidial drugs and rarely of live vaccines (Peek and Landman, 2011). The extensive use of prophylactic anticoccidial

drugs has led to the development of drug resistant strains of *Eimeria* against all products introduced (Chapman, 1997). Live vaccines can restore drug sensitivity and proved to be efficient in controlling the disease, but they are expensive and have adverse effects on early chick growth (Williams, 2002).

Therefore, there is an increasing need in discovering new alternatives for coccidiosis control. In the last decade, many different natural products such as mushrooms, and plant extracts have been tested for anticoccidial activity (Tewari and Maharana, 2011). Amongst them, *Artemisia annua*, a traditional Chinese medicine used for malaria treatment, was found to be effective against *E. tenella* and *E. acervulina* (Allen et. al, 1997). It is believed that acts by inducing oxidative stress (Allen et al., 1998). There are studies showing that artemisinin, a sesquiterpene lactone from *A. annua*, can significant decrease the number of oocysts shedded by chickens infected with *Eimeria* spp. (Arab et al., 2006; Naidoo et al., 2008).

In this study, we investigated the anticoccidial effects of *A. annua* as essential oil in broilers kept in batteries.

Materials and methods

The research has been conducted during 14th May – 12th June 2007 in the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca – Faculty of Veterinary Medicine, Department of Parasitology and Parasitic Diseases. We aimed to assses the efficacy of *A. annua* as essential oil (0.15 ml/l of water) in experimental infection with 1x10^4 *Eimeria* spp. sporulated oocysts, in broiler chickens kept in batteries.

Animals. One day old chickens (hybrid Ross 308) were purchased from S.C. Oprea Avicom S.R.L. hatchery (Venchi-Sighişoara, Mureş county), and housed in animal facilities until the experimental infection to avoid spontaneous infection with *Eimeria* spp. Chickens were fed with starter feed without anticoccidial feed aditives until the date of the experiment. Feed and water were provided *ad libitum*, and the lighting was continuous.

***Artemisia* product.** The essential oil of *A. annua* was obtained from a Romanian variety, from the green herb right after harvest, through hydro-distillation process using an artisanal machinery. The herb was cultivated in 2006, started from seeds in middle March, planted in early June, and harvested in early October, when plants were in late blooming - beginning of flowering stage.

Parasites. We used for experimental infection a suspension of mixed *Eimeria* species. The strains were obtained from died chicks in intensive broiler farms by scraping the intestinal content. Oocysts were propagated, isolated and sporulated in 2.5% potassium dichromate using standard procedures (Raether et al., 1995). The species were identified based on morphology of sporulated oocysts using microphotographs. We took microphotographs at Olympus BX61 microscope using 400x magnification with Olympus ODP72 camera, and then we sized, and analyzed 50 oocysts with CELL-F software.

Experimental design. At 13-days-old, chicks were subsequently randomly divided into 3 groups of 20 birds each as follows: negative control group (NC) - uninfected, unmedicated; positive control group (PC) – infected, unmedicated; experimental group (EG) – infected and medicated with essential oil of *A. annua* in water.

At 14-days-old chicks from PC and EG groups were infected with 1x10^4 *Eimeria* spp. sporulated oocysts in a volume of 1 mL, through gavage into the crop. The treatment in EG group started in the same day with the experimental infection.

The essential oil of *A. annua* was administered 0.15 ml/l water during the entire period of experiment, until 27 days after challenge. The essential oil was solubilised in water, by adding Tween 70, 0.75 ml/10 l of water.

Analytical procedures. Efficacy of *A. annua* essential oil was evaluated by recording the following: (i) clinical signs; (ii) mortality percentage; (iii) number of oocysts shedded/g of faeces (OPG) and the reduction percentage of OPG compared to the positive control; (iv) lesion score and the reduction percentage of the lesion score compared to the positive control; (vi) body weight gain, and (vii) feed conversion ratio (Holdsworth et al., 2004).

We used for oocysts counting McMaster method, and sodium chloride (sp.gr. 1.20) solution as flotation solution. Fecal samples were collected daily from 5 to 18 days post-challenge from each group. Lesion score was evaluated at 7 (5 chicks/group) and 27 days post-challenge using a score of 0–4 (Johnson and Reid, 1970). Body weight gain was assessed at 7 and 27 days pi, and the feed conversion ratio at the end of the experiment.

Statistical analysis. In order to identify the statistically significant differences, the data obtained were processed by student test (T) for comparison of the averages of two equal populations (Drugan et al., 2003), in Excel 2003. This statistical processing was applied for OPG, and lesion score. The normal distribution of data was verified before statistical processing (Drugan et al., 2003).

Results

Eimeria species

The oocysts suspension used for experimental infection contained 4 species based on oocysts morphology (Fig. 1): *E. tenella* (35%), *E. acervulina* (32,5%), *E. mitis* (25%), and *E. maxima* (7,5%) (Fig. 2). As we used 10^4 oocysts/chicken, one dose had 3500 oocysts of *E. tenella* , 3250 *E. acervulina*, 2500 *E. mitis*, and 750 *E. maxima* respectively.

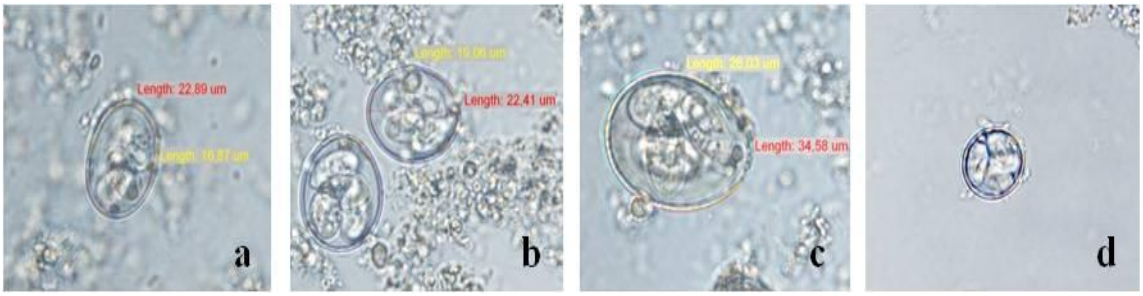

Fig. 1: Species of *Eimeria* in the inoculum based on morphology of the sporulated oocysts. *E. acervulina* (a), *E. tenella* (b), *E. maxima* (c) and *E. mitis* (d).

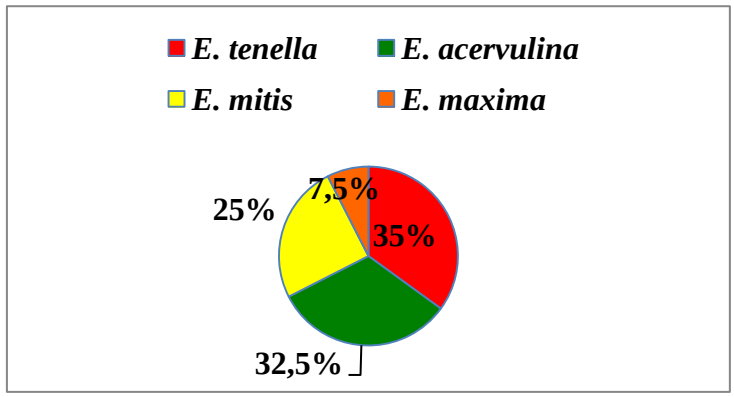

Fig. 2: The structure of eimerian population used for experimental infection

Clinical signs and mortality percentage

After experimental infection with 10.000 oocysts of *Eimeria* spp., clinical changes were visible in positive control group, at 3 days after challenge. These were represented by decreased appetite, polydipsia, and sleepiness. These clinical changes diminished and then dissapeared at 10 days after challenge. In the group treated with *Artemisia*, especially in the first part of the experiment, an increase in the daily feed consumption and a more pronounced state of liveliness of the chickens was noted.

The mortality rate was 0 in negative control, and experimental groups. In positive control group 2 deaths were recorded, the mortality rate being 10%. The first mortality case was recorded 7 days after challenge, and the second 10 days respectively. At necropsy were recorded typical lesions of infection with *E. acervulina* in the duodenum (lesion score 1, and 2), (Fig. 3) and hemorrhagic typhlitis (lesion score 3, and 4) (Fig. 3).

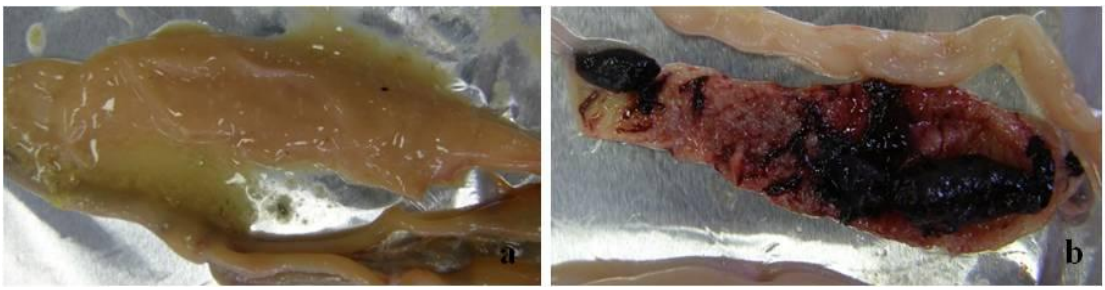

Fig. 3: Characteristic lesions in the duodenum and caecum in chickens from positive control group died 7-10 days postinfection. (a) White streaks oriented transversely across the intestine , lesion score 2. (b) Hemorrhagic typhlitis, lesion score 4.

OPG and lesion score

The dynamics of oocysts shedded per groups and days, beginning with day 5 after challenge is presented in fig. 4. The highest oocysts number was recorded in positive control group, with an average of 71980 oocysts/g of faeces. The chickens from the group treated with essential oil of *A. annua* have shedded an approximately equal number of oocysts (an average of 2079 ooccysts/g of faeces) to the one of the chickens from negative control group (an average of 2757 oocysts/g of faeces). In dynamics, the differences were observed in 5-9

days afer challenge, after that, in all 3 experimental groups, OPG was situated at the same level.

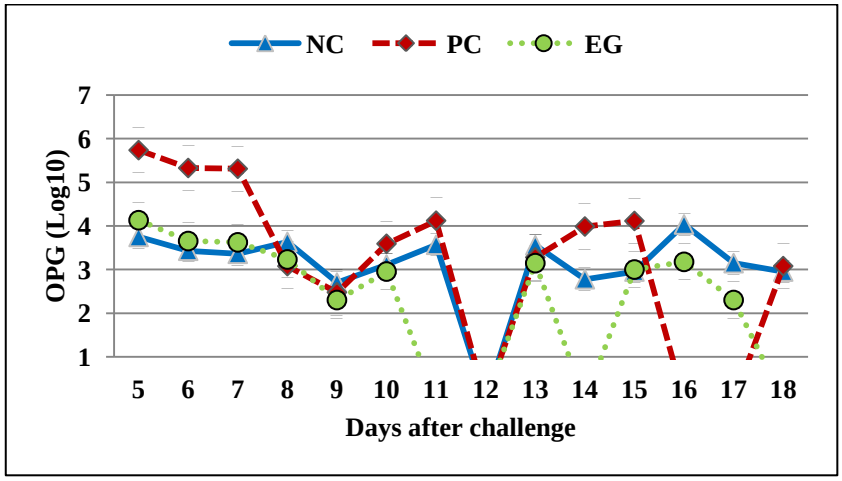

Fig. 4: Dynamics of OPG in experimental groups. NC = negative control; PC = positive control; EG = group treated with A. annua essential oil 0,15 ml/l of water.

The lesion score at 7 days after challenge was higher in positive control group (1.33), while no significant differences have been recorded between the 3 experimental groups at 27 days after challenge (Tab. 1). The lesion score in the duodenum and caecum was generally higher in positive control group, whilst in the jejunum it was higher for EG group (Fig. 5).

Tab. 1. Average of lesion score, body weight gain and feed conversion in experimental groups following treatment with *A. annua* and *Eimeria* infection

Experimental group	Lesion score		Weight gain (g/day)		Feed conversion (kg feed/kg spore)
	7 days a.c.	27 days a.c.	7 days a.c.	27 days a.c.	
NC	0,13	1,06	41,97	41,75	2,16
PC	1,33	0,9	31,42	50,87	2,19
EG	0,86	0,8	41,45	41,57	2,31

Legend: NC (negative control) – uninfected and unmedicated; PC (positive control) – infected and unmedicated; EG – infected and medicated with *A. annua* essential oil 0,15 ml/l of water

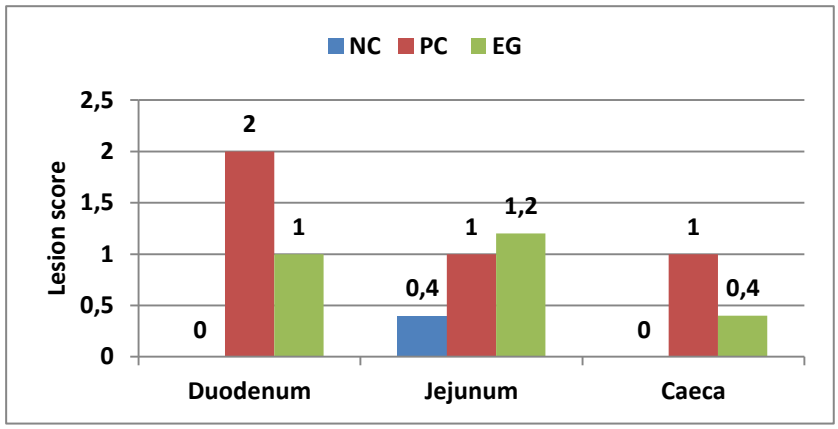

Fig. 5: Lesion score in experimental groups at 7 days after challenge in different parts of gut. NC -negative control; PC - positive control; EG - group treated with *A. annua* essential oil 0,15 ml/l of water

Body weight gain and feed conversion ratio

The body weight gain was different in the 2 evaluation periods (Tab. 1), being higher in NC group (41,97 g/day) and EG (41,45 g/day) at 7 days after challenge (PC = 31,42 g/day), whilst at 27 days after challenge a recovery in PC group (50.87 g/day) from the NC group (41,75 g/day) and EG (41,57 g/day) was observed. Also, the feed consumption (Tab. 1) was lower in positive control group (2,19 kg feed/kg spore) as against group treated with *A. annua* under the form of essential oil 3.75% in water (2,31 kg feed/kg spore).

Discussions

Coccidiosis remains one of the most costly diseases in poultry. Due to emergence of chimioresistant strains of *Eimeria*, eforts were directed towards finding inexpensive and efficient alternative treatments.

In this study we aimed to evaluate the prophylactic and therapeutic efficacy of *A. Annua* as essential oil in broiler coccidiosis. The results showed decreased OPG output, lesion score and mortality rate and higher body weight gain in experimental group, revealing the fact that chicken coccidiosis caused by *E. tenella* and *E. acervulina* can be treated with essential oil of *A. annua.*

A. annua is a traditional Chinese medicine used for malaria treatment. It is believed that the anticoccidial activity of this plant is based on artemisinin, it's active substance, which could be lethal to parasites by inducing oxidative stress (Allen and Fetterer, 2002). *A. annua* also contains flavonoids, which have high antioxidant activity, and may enhance the effect of artemisinin, or have anticoccidial effects on their own (Ferreira et. al., 2010)

Oh et al. (1995) are the first to report that *A. annua* has an anticoccidial effect by improving body weight gain, feed conversion rate and lesion score in broilers challenged with *E. tenella.* Two years later, Allen et al. (1997) demonstrated that dried leaf supplements of *A. annua* have a positive effect on lesion score in chickens infected with *E. tenella.* However, they found no protective effect against *E. acervulina* and *E. maxima*. According to lesion score in the jejunum, neither in our study, *A. annua* had no effect against *E. maxima.*

In our previous study (Drăgan et al., 2010), chickens treated with *A. annua* as oil and powder produced significantly reduced oocysts output and lesion score when compared to the *E. tenella*-infected group fed standard diet. The group treated with *A. annua* as powder had the highest body weight gain and the best feed conversion among the experimental groups.

Treatment with artemisinin alters the process of oocysts wall formation, as demonstrated by del Cacho et al. (2010). The consequence of this was death of developing oocysts and reduced sporulation rate. Through immunofluorescent studies the authors showed that artemisinin reduced sarcoplasmic–endoplasmic reticulum calcium ATPase (SERCA) expression in macrogametes. SERCA plays a role in calcium homeostasis so treatment with artemisinin may affect the secretion of wall-forming bodies, which is a calcium-dependent mechanism. This leads to abnormal oocyst wall formation and death of the developing oocysts (del Cacho et al., 2010).

Almeida et al. (2012a) found that during infection with *Eimeria spp.* in free-range broilers supplemented with *A. annua* dried leaves, the number of excreted oocysts significantly reduced, but no significant difference was noted in feed consumption between groups. In the present study, the feed consumption was lower in positive control group than the group treated with *A. annua*.

In a study made by Almeida et al. (2012b), the supplementation of *A. annua* in the diet (3% inclusion based on feed weight) influenced negatively the growth rates of the pullets and did not affect OPG. Ethanolic extract of *A. vulgaris* supplemented before appearance of clinical symptoms of disease showed a trend to reduce oocysts excretion in the late infection. The authors believed that the failure of suppressing oocysts excretion was due to the fact that pullets were challenged before a reasonable period of plant consumption.

Kaboutari et al. (2013) showed that a granulated formulation of *Artemisia* extract significantly reduced mortality, diarrhea, OPG output and lesion score in the treated groups, but no significant difference between the prophylactic and therapeutic groups was noticed.

As proven by several studies, *A. annua* is efficient against *Eimeria* in chickens, but further studies need to be done to establish the mechanism of action and the plant compound that has the best effect.

Conclusions

According to our results, *A. annua* Romanian variety as essential oil can be used with good results to treat coccidiosis mainly caused by *E. tenella* and *E. acervulina* in chickens, and not for prevention. Essential oil of *A. annua* Romanian variety had no effect against *E. maxima,* according to lesion score in the jejunum. Further analysis must be done.

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CLINICAL AND LABORATORY ASPECTS OF ASCITES SYNDROME IN DOGS

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Abstract

Clinical signs observed in dogs with ascites consecutive to chronic hepatitis (liver cirrhosis) were loss in weight, deficiency anemia with pale mucous membranes, increased abdominal volume and dyspnea. In ascites syndrome consecutive to chronic heart failure (CHF), to the clinical signs listed above were added respiratory distress emphasizing intercostal spaces, tiredness, dry, unproductive, torturous cough manifested especially at night and without pulmonary physical signs. The peritoneal fluid was serous, with noninflammatory character (protein concentration below 3 g / dl). In ascites consecutive of chronic hepatitis, blood counts indicated the presence of deficiency macrocytic, normochromic regenerative anemia syndrome explained by the scarcity of protein factors necessary for plastic erythrocyte synthesis (RBC = 4.9 million / ml , Hb = 12.6 g/dl , Ht = 39.7% , MCV = 81.0 μ^3 HEM = 25.71 pg, MCHC = 31.73 g/dL). Enzyme profile was elevated (AST = 232.3 IU/L, ALT = 102.1 IU/ L, ALP = 150 IU/L), which indicates the onset of hepatocytolysis pathophysiological syndrome consecutive to the hepatopathy. In ascites syndrome following CHF, the blood count characterized the deficiency hypochromic, microcytic anemia (RBC = 5.4 million/ml, Hb = 11.8 g/dl, Ht = 35.7%, MCV = 66.1 μ^3, MCH = 21.8 pg, MCHC = 33.0 g/dl), and the blood biochemical profile was within the limits of the average reference (TP = 5.7 g/dl, AST = 36.8 IU/L, ALT = 45, 2 IU/L, BUN = 25.0 mg/dl, CRTN = 1.4 mg/dl, Ca = 9.7 mg/dl, P = 4.9 mg/dl). White blood count indicates ascites syndrome of infectious origin when WBC is increased or overall insufficient functional hepatic function with synthesis disorder, in which case is associated with a downward trend in protein profile values (ALB = 2.9 g/dl, TP = 5.3 g/dl). Ultrasound examination revealed irregular echogenic liver structure with rounded edges and dilated blood vessels surrounded by ascitic fluid with hipoecogen appearance.

Keywords: *ascites, dog, hematology, biochemistry, ultrasound*

Materials and method

Research was done on dogs of different ages and breeds that presented with ascites syndrome manifestations. In some cases, besides the clinical examination, paraclinical tests were performed: blood count and biochemical parameters. Ultrasound and puncture were also used as aditional investigation tecniques.

Results and discussions

Clinical signs in ascites consecutive to chronic hepatitis (hepatic cirrhosis) were: loss of weight, mucous paleness, increase in abdominal volume (frog abdomena) and after puncture was performed, a serous like fluid was obtained, with noninflammatory characteristics (protein under 3g/dl) (picture 1).

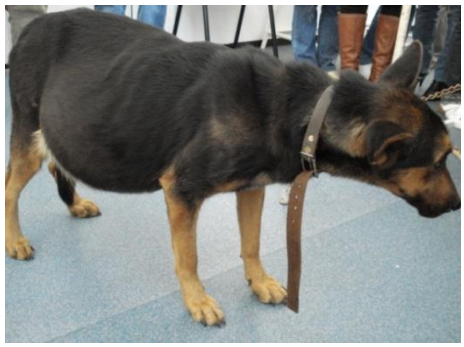
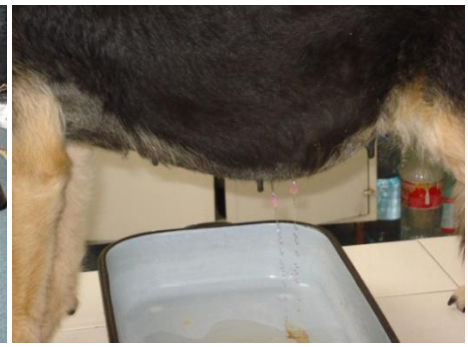



Fig. 1. Ascites consecutive to chronic hepatitis (loss of weight, , increase in abdominal volume, peritoneal noninflammatory fluid)

Blood count in dogs with ascites syndrome consecutive to chronic hepatitis is shown in tabel 1 and tabel 2.

Table 1. Blood count in dogs with ascites syndrome concutive to chronic hepatitis

	Hematological parameters				**White count**			
Parameter	RBC (mil/µl)	WBC (mil/µl)	Hb (g/dl)	Ht (%)	N(m) (%)	L (%)	E (%)	M (%)
Reference values (Merck)	5,5-8,5	6-17	12-18	37-55	60-70	12-30	2-10	3-10
Hepatic cirrhosis (Caniche, 7 years)	**4,9**	**12,7**	**12,6**	**39,7**	**62**	**14**	**2**	**8**
Ascites (Crossbreed, 5 years)	**5,1**	**17,9**	**12,0**	**38,6**	**64**	**13**	**2**	**4**

Tabel 2. Hematological parameters considered in dogs with ascites syndrome consecutive to chronic hepatitis

	Hematological parameters		
Parameter	VEM (μ^3)	HEM (pg)	CHEM (g/dL)
Reference values (Merck)	60-77	19,5-24,5	31-34
Hepatic cirrhosis (Caniche, 7 years)	**81,0**	**25,71**	**31,73**
Ascites (Crossbreed, 5 years)	**75,68**	**23,53**	**31,08**

In tabel 1 şi tabel 2 we can see that the red blood cell count was under the inferior limit of the reference values (NrE=4,9 mil/µl and NrE=5,1 mil/µl). Hb value was close the

inferior limit of the reference values (Hb=12,6 g/dl and Hb=12 g/dl). Ht was found within the boundaries of reference values (Ht=39,7% and 38,6%). Also, red blood cells medium volume aimed to the superior limit of reference values (VEM=81,0 μ^3 and VEM=75,68 μ^3). Red blood cell medium hemoglobin was within the limits of reference values (HEM=25,71 pg and HEM=23,53 pg), and CHEM was found close to the inferior limit (CHEM=31,73 g/dL and CHEM=31,08 g/dL).

The blood count values that were obtained indicate the onset of deficiency macrocytic, normochromic regenerative anemia syndrome explained by the lack of proteic plasmatic factors necessary for the synthesis of blood cells. This can come as a result of the failure of hepatic synthesis function (albumin synthesis) in dogs with ascites syndrome consecutive to chronic hepatitis.

After white count was performed, we observed that the white blood cells were situated within the reference values, (NrL=12,7 mil/µl) or sometimes aimes towards the superior limit (NrL=17,9 mil/µl). The other parameters of the white count were also within boundaries. This indicates the infectious onset of the ascites (when WBC is increased) or liver failure with involvement of the synthesis fuction.

Carential anemia appears due to numerous faults in metabolic synthesis and is characterized by low values of hemoglobin and hematocrit. This leads to loss of appetite wich sustaines the anemia, thus forming a vicious circle that worsens the disease.

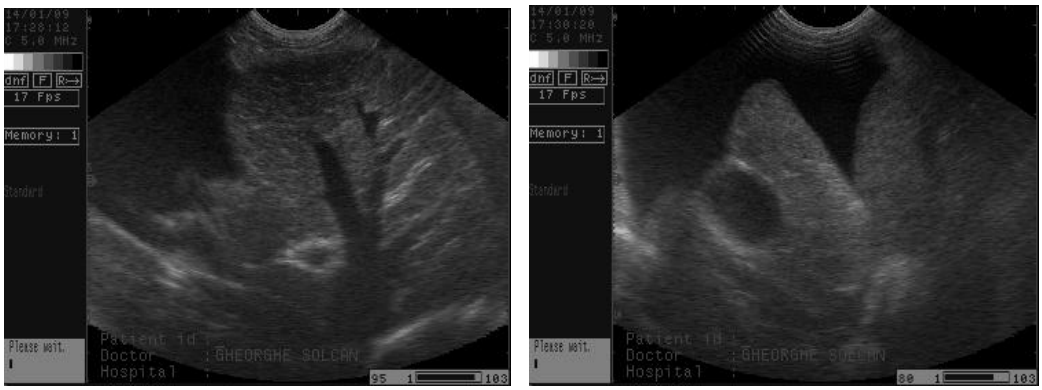
Biochemical profile in dogs with ascites syndrome consecutive to chronic hepatitis is shown in tabel 3.

Tabel 3. Biochemical profile in dogs with ascites syndrome consecutive to chronic hepatitis

Parameter	AST	ALT	ALP	TP	ALB	BUN	CRTN
Unit	UI/L	UI/L	UI/L	g/dl	g/dl	mg/dl	mg/dl
Reference values (Merck)	5-47	7-56	44-147	5,5-7,5	2,6-4,0	8,8-26	0,5-1,6
Obtained values	**232,3**	**102,1**	**150,0**	**5,3**	**2,9**	**23,4**	**1,4**

In tabel 3 we can see the increase of serum enzymes: AST=232,3 UI/L, ALT=102,1 UI/L, ALP=150 UI/L wich demonstrates the onset of the hepatocytolysis pathophysiological syndrome consecutive to liver suffering. The albumin obtained values aim towards the inferior limits of the reference values (ALB=2,9 g/dl), and the total protein value was under the inferior limit (TP=5,3 g/dl). Renal profile values were for blood non-protein nitrogen (BUN) 23,4 mg/dl, and for creatinin (CRTN) de 1,4 mg/dl; these high values that aim the superior limits of the reference values indicate the onset of a kidney failure that evolves besides the liver failure.

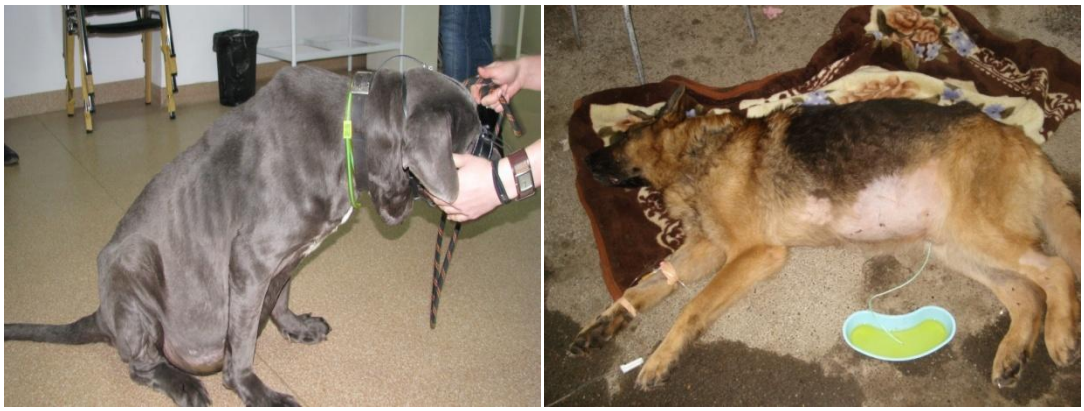
Ultrasounds in dogs with ascites syndrome consecutive to chronic hepatitis are shown in picture 2.



Picture 2. Ascites (hepatic cirrhosis)

In picture 2 we can see the irregular ecostructure of the liver, with round edges and enlarged blood vessels surrounded by hipoecogen ascitic fluid. The gallbladder and billiary vessels had hiperecogenic outlines (cholecystitis and angiocolitis) asociated with hepatic hypertrophy, congestion ascites. These detailes are chracteristic for chronic ascitogenic hepatitis (hepatic cirrhosis).

The clinical signs observed in dogs with *ascites syndrome consecutive to chronic heart failure* (CHF) were: respiratory distress emphasizing intercostal spaces –orthopnea or sitting dog position, fatigue with exertion, dry, unproductive, torturous cough manifested especially at night and without pulmonary physical signs, tachycardie cu tachypnea (picture 3).



Picture 3. Ascites consecutive to chronic heart failure
(orthopnea or sitting dog position, dyspnea, fatigue)

The hematological profile in a German Shepherd, 12 years with ascites syndrome consecutive to chronic heart failure (cardiac enlargemet) is shown in tabel 4 and tabel 5.

Tabel 4. Blood count in a dog with ascites syndrome consecutive to CHF (cardiac enlargement)

Parameter	**RBC**	**Hb**	**Ht**	**WBC**	**White count**				
					N	**E**	**B**	**M**	**L**
Mesure unit	mil/µl	g/dl	%	mii/µl	%	%	%	%	%
Reference values (Merck)	5,4-7,8	13-19	37-54	6-17	30-75	62-83	0-3	3-10	12-30
Obtained values	**5,4**	**11,8**	**35,7**	**17,1**	**83**	**61**	**1,3**	**4,0**	**13,0**

Tabel 5. Hematologicical parameters in a dog with ascites syndrome consecutive to CHF (cardiac enlargement)

Hematological parameter	**VEM**	**HEM**	**CHEM**
Mesure unit	μ^3	picograme (pg)	g/dl
Reference values	64,0-74,0	22,0-27,0	34,0-36,0
Obtained values	**66,1**	**21,8**	**33,0**

In tabel 4 and tabel 5 we can see that the blood count had medium values situated below the lower limit of the reference values or close to it: RBC=5,4 mil/µl, Hb=11,8 g/dl, Ht=35,7%. The hematological parameters also had medium values lower than the medium reference values: VEM=66,1 μ^3, HEM=21,8 pg, CHEM=33,0 g/dl. This data shows the existence of an carential, hypochromic, microcytic anemic syndrome.

The biochemical profile is shown in tabel 6.

Tabel 6. The bichemical profile in a dog with ascites syndrome consecutive to CHF (cardiac enlargement)

Parameter	**AST**	**ALT**	**TP**	**BUN**	**CRTN**	**Ca**	**P**
Mesure unit	UI/L	UI/L	g/dl	mg/dl	mg/dl	mg/dl	mg/dl
Reference values (Merck)	5-47	7-56	5,5-7,5	8,8-26	0,5-1,6	8,7-11,8	2,9-6,2
Obtained values	**36,8**	**45,2**	**5,7**	**25,0**	**1,4**	**9,7**	**4,9**

In tabel 6 we can see that obtained values for the biochemical parameters in a dog with CHF were within the boudaries of the reference values: TP=5,7 g/dl, AST=36,8 UI/L, ALT=45,2 UI/L, BUN=25,0 mg/dl, CRTN=1,4 mg/dl, Ca=9,7 mg/dl, P=4,9 mg/dl.

Conclusions

1. The clinical signs observed in dogs with ascites consecutive to chronic hepatitis (liver cirrhosis) were loss in weight, deficiency anemia with pale mucous membranes, increased abdominal volume and dyspnea. In ascites syndrome consecutive to chronic heart failure (CHF), to the clinical signs listed above were added respiratory distress emphasizing intercostal spaces, tiredness, dry, unproductive, torturous cough manifested especially at night and without pulmonary physical signs.
2. In ascites consecutive of chronic hepatitis, blood counts indicated the presence of deficiency macrocytic, normochromic regenerative anemia syndrome explained by the scarcity of protein factors necessary for plastic erythrocyte synthesis (RBC = 4.9 million / ml , Hb = 12.6 g/dl , Ht = 39.7% , MCV = 81.0 μ^3 HEM = 25.71 pg, MCHC = 31.73 g/dL). Enzyme profile was elevated (AST = 232.3 IU/L, ALT = 102.1 IU/ L, ALP = 150 IU/L), which indicates the onset of hepatocytolysis pathophysiological syndrome consecutive to the hepatopathy.
3. In ascites syndrome following CHF, the blood count characterized the deficiency hypochromic, microcytic anemia (RBC = 5.4 million/ml, Hb = 11.8 g/dl, Ht = 35.7%, MCV = 66.1 μ^3, MCH = 21.8 pg, MCHC = 33.0 g/dl), and the blood biochemical profile was within the limits of the average reference (TP = 5.7 g/dl, AST = 36.8 IU/L, ALT = 45, 2 IU/L, BUN = 25.0 mg/dl, CRTN = 1.4 mg/dl, Ca = 9.7 mg/dl, P = 4.9 mg/dl).
4. White blood count indicates ascites syndrome of infectious origin when WBC is increased or overall insufficient functional hepatic function with synthesis disorder, in which case is associated with a downward trend in protein profile values (ALB = 2.9 g/dl, TP = 5.3 g/dl).
5. Ultrasound examination revealed irregular echogenic liver structure with rounded edges and dilated blood vessels surrounded by ascitic fluid with hipoecogen appearance. These detailes are chracteristic for chronic ascitogenic hepatitis (hepatic cirrhosis). Cholecystitis and angiocolitis asociated with hepatic hypertrophy, congestion and ascites were also found.
6. The peritoneal fluid was serous, with noninflammatory character (protein concentration below 3 g / dl).

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THE INCIDENCE OF PERITONEAL FLUID COLLECTIONS IN DOGS

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Abstract

In relation to the incidence of other diseases, peritoneal fluid collections in dogs were diagnosed during the years 2011-2013 in only 23 out of 3781 patients presented for consultation, which represents 0.60 %. The recorded cases were as follows: ascites in 19 cases (82.6 %), peritonitis in only one case (4.4%), hemoperitoneum consequent to tumor disease or topographic intestinal disorders in 3 cases (13.0%). The incidence of peritoneal fluid collection syndrome in dogs was higher in the age group 7-14 years old - 13 cases (56.5 %) and lower in the age group under 2 years - 1 case (4.3%). In the 2-7 years category, peritoneal fluid collection syndrome was diagnosed in 9 cases (39.1 %), which means that the risk of peritoneal fluid collection syndrome secondary to liver disease or tumor increases with age. The main cause of the syndrome in dogs peritoneal fluid collection was congestive chronic heart failure - 9 cases (39.1 %), followed by liver failure and tumor disease with 5 cases each (21.7 %). In 4 cases (17.4 %) the etiology was different.

Keywords: *peritoneal fluid collections, dog, incidence*

Material and Method

Peritoneal fluid collections in dogs were ascites consequtive to congestive chronic heart failure (CHF) or chronic hepatitis (hepatic cirrhosis), peritoneal sero-sanguinolent fluid collection (hemoperitoneum) consequent to tumor disease or topographic intestinal disorders and peritoneal inflammatory fluid collection following peritonitis. For diagnostic we used the clinical examination, along with complementary examination methods like ultrasounds, X-rays, peritoneal puncture for fluid collection and diagnostic and therapeutic laparatomy.

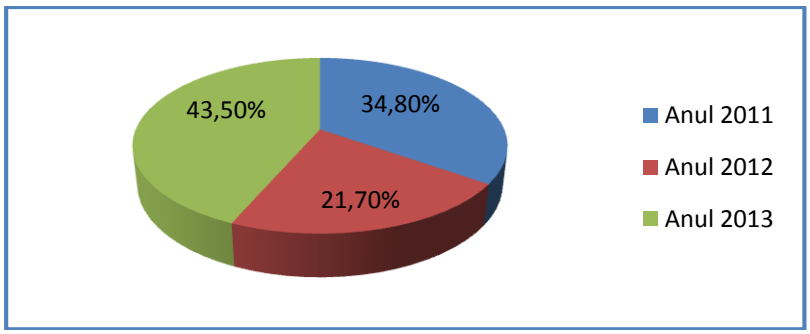
Based on the Consultation and Treatment Register of the Medical Clinic from the Veterinary Medicine Faculty of Iaşi, we studied the incidence of peritoneal fluid collections in dogs during the years 2011-2013, in relation to some variation factors like the nature of the fluid collection, the year, the morbidity among other diseases, the gender, age and breed of the animals as well as the primary condition.

Results and Discusions

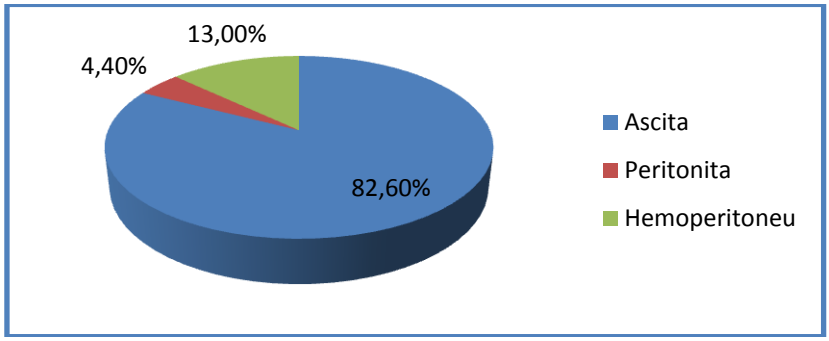
Peritoneal fluid collections in dogs diagnosed during the years 2011-2013 are shown in tabel 1, picture 1 and picture 2.

Tabel 1. The total anual incidence and the nature of peritoneal fluid collections in dogs

Year	Ascites		Peritonitis		Hemoperitoneum		Total	
	Nr.	%	Nr.	%	Nr.	%	Nr.	%
2011	6	31,6	1	100,0	1	33,3	**8**	**34,8**
2012	4	21,0	0	0	1	33,3	**5**	**21,7**
2013	9	47,4	0	0	1	33,3	**10**	**43,5**
Total	**19**	**82,6**	**1**	**4,4**	**3**	**13,0**	**23**	**100**



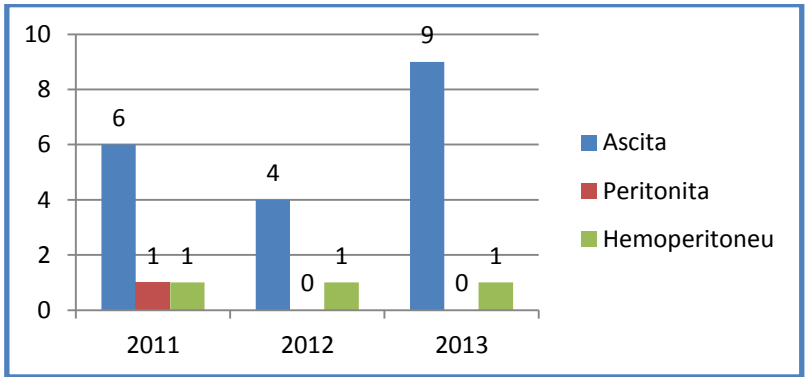
Picture 1. Diagram showing total anual incidence of peritoneal fluid collections in dogs



Picture 2. Diagram showing the incidence of the nature of peritoneal fluid collections in dogs

In tabel 1 and picture 1 we can see that the total incidence of peritoneal fluid collections in dogs during theese 3 years was of 8 cases (34,8%) in 2011, 5 cases (21,7%) in 2012 and 10 cases (43,5%) in 2013. Regarding the nature of peritoneal fluid collections (picture 2), ascites syndrome was diagnosed in 19 cases (82,6%), peritonitis in one case (4,4%) and hemoperitoneum consequent to tumor disease or topographic intestinal disorders in 3 cases (13,0%).

The diagram showing the anual incidence of the nature of peritoneal fluid collections is presented in picture 3.



Picture 3. Diagram showing the anual incidence of the nature of peritoneal fluid collections in dogs

From tabel 1 and picture 3 we can see that the majority of the ascites cases were diagnosed in 2013, 9 cases (47,4%), and the fewest in 2012, 4 cases (21,0%). In the year 2011, 6 cases were diagnosed (31,6%).

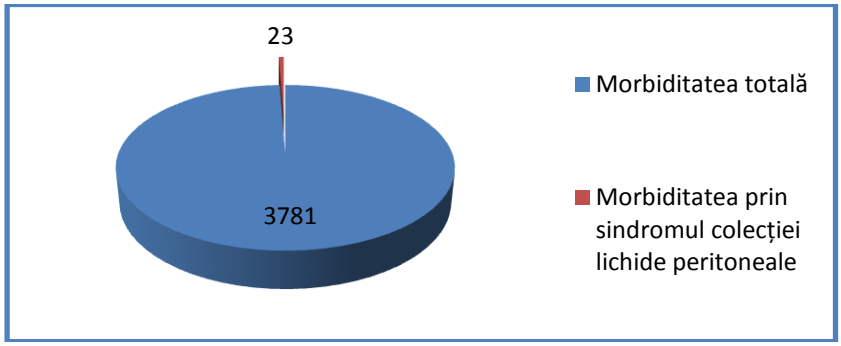
Peritonitis was diagnosed only once in 2011 in a dog with an intestinal topographic disorder (intestinal volvulus and intussusception).

Hemoperitoneum was found in 3 dogs with abdominal tumors between the years 2011-2013, one case for every year, representing 33.3%.

The incidence of peritoneal fluid collections in dogs in relation with the total morbidity is shown in tabel 2 and picture 4.

Tabel 2. The incidence of peritoneal fluid collections in dogs in relaton with the morbidity

Year	Cases	Peritoneal fluid collections	
		Nr	%
2011	1221	8	0,65
2012	1277	5	0,39
2013	1283	10	0,78
Total	3781	23	0,60



Picture 4. Diagram showing morbidity within the peritoneal fluid collection syndrome in dogs

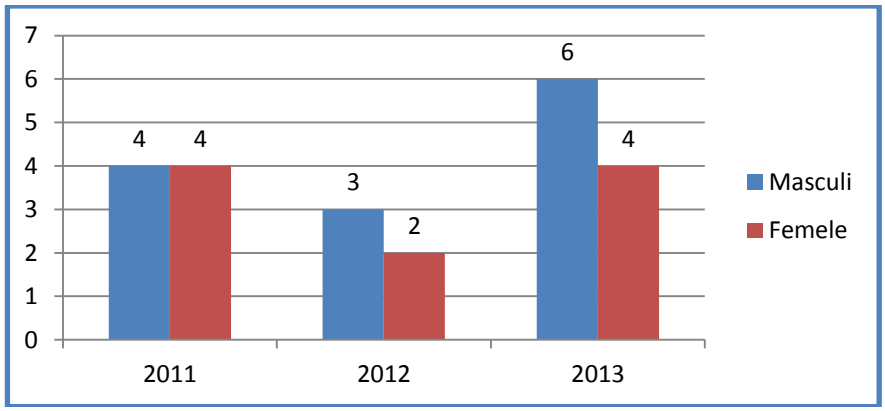
In tabel 2 we can see that in 2011 there were 8 cases diagnosed with peritoneal fluid collection out of 1221 cases presented for consultation, representing 0,65%. In 2012, 5 cases were diagnosed, representing 0,39%, and in 2013, 10 cases, representing 60% out of the 1283 animals presented for consultation.

Compared to the morbidity among other diseases, peritoneal fluid collections recorded a small total incidence value. They were diagnosed in only 23 cases out of 3781 patients presented for consultations, wich means 0,60% (picture 4).

The incidence of the peritoneal fluid collections in relation with the gender of the diagnosed animals is shown in tabel 3 şi picture 5.

Tabel 3. The incidence of peritoneal fluid collections in dogs in relation with gender

Year	Cases	Male		Female	
		Nr	%	Nr	%
2011	8	4	50,0	4	50,0
2012	5	3	60,0	2	40,0
2013	10	6	60,0	4	40,0
Total	**23**	**13**	**56,5**	**10**	**43,5**



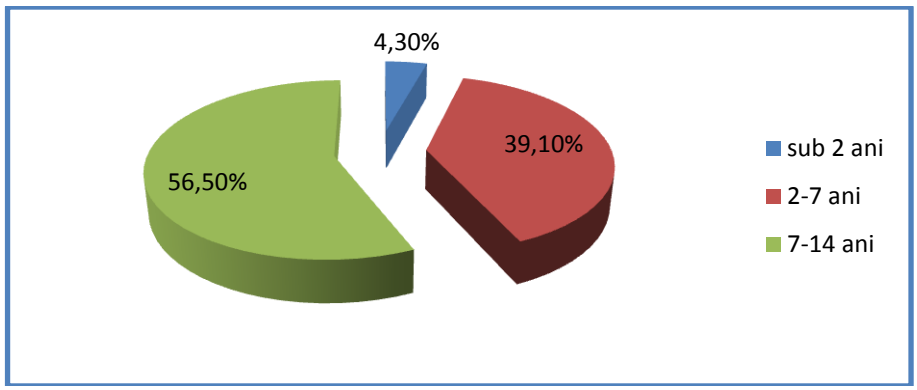
Picture 5. Diagram showing the incidence of peritoneal fluid collections in dogs in relation with gender

In tabel 3 and picture 5 we can see that out of 23 cases diagnosed with peritoneal fluid collection syndrome, 13 dogs were males (56,5%) and 10 were females (43,5%). The incidence of the peritoneal fluid collection syndrome was higher in males– 60%, between the years 2012 - 2013 and was equal for males and females in 2011 – 50%.

The incidence of peritoneal fluid collections in dogs in relation with age is shown in tabel 4 and picture 6.

Picture 4. The incidence of peritoneal fluid collections in dogs in relation with age

Vârsta	sub 2 ani	2 – 7 ani	7 – 14 ani	Total
Nr	1	9	13	23
%	4,3	39,1	56,5	



Picture 6. Diagram showing the incidence of peritoneal fluid collections in dogs in relation with age

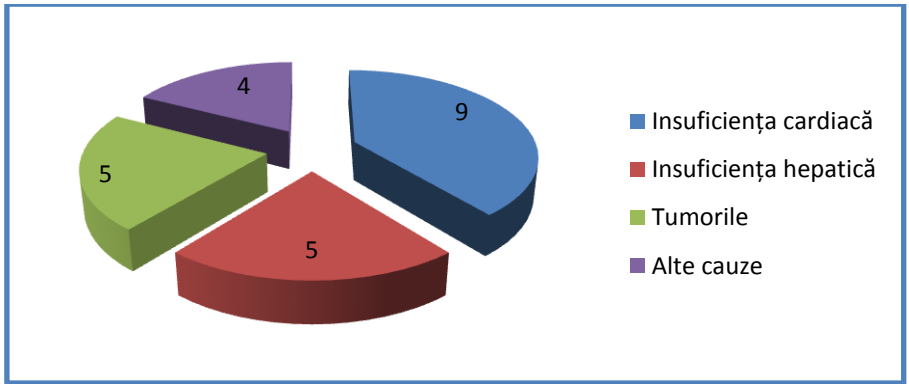
In tabel 4 and picture 6 we can see that the incidence of the peritoneal fluid collection syndrome in dogs was higher in the 7-14 years age category – 13 cases (56,5%) and lower under the age of 2 years – one case (4,3%). In the age category 2-7 years, the peritoneal fluid collection syndrome was diagnosed in 9 cases (39,1%). This illustrates that with age increase, the risk to develope the peritoneal fluid collection syndrome consequent to hepatic or tumor disease also increases.

In tabel 5 and picture 7 we present the cases diagnosed with the peritoneal fluid collection syndrome in relation with the breed, age and primary condition. (etiological diagnostic).

Tabel 5. The incidence of peritoneal fluid collection syndrome in relation with breed and primary condition

	Breed	Age (years)	Gender	Primary condition
Year 2011	German Pointer	8	F	Mezoteliosarcoma
	Crossbreed	13	M	Hepatomegaly, ascites
	Rotweiller	7	M	Ascites, hepatomegaly
	Crossbreed	9	M	Hepatic cirosys
	German shepherd	13	F	Hepatomegaly, splenomegaly, peritonitis
	Crossbreed	7	F	Congestive chronic heart failure
	German shepherd	11	F	Right heart enlargement
	Mastino Napoletano	5	M	Cardiac failure
Year 2012	Crossbreed	5	M	Ascites
	Pekinghese	15	F	Congestive chronic heart failure
	Rotweiller	6	M	Right heart enlargement
	Schnautzer	13	M	Liver and spleen tumor
	German shepherd	2	F	Right heart enlargement
Year	Crossbreed	13	M	Liver tumor and heart failure

2013	Caniche	10	M	Liver tumor
	Crossbreed	4	M	Ascites
	Romanian shepherd	6	F	Heart failure
	Caniche	7	F	Chronic hepatitis, ascites
	Pekinghese	14	M	Acute hepatitits
	Crossbreed	14	F	Congestive chronic heart failure
	Crossbreed	10	F	Congestive chronic heart failure
	German shepherd	12	M	Spleen tumor
	Pekinghese	6	M	Ascites



Picture 7. Diagram showing the incidence of peritoneal fluid collections in dogs in relation with the primary condition

In tabel 5 we can see a high incidence for the cases with peritoneal fluid collection was recorder among crossbreeds (8 cases), followed by the German Shepherd (4 cases) and the Pekinghese (3 cases). The main cause of the peritoneal fluid collection syndrome in dogs was congestive chronic heart failure, 9 cases (39,1%) followed by liver failure and tumor disease, each with 5 cases (21,7%). In other 4 situations (17,4%) the etiology was different (picture 7).

Conclusions

7. During the years 2011-2013 there were 19 cases diagnosed with ascites syndrome (82,6%), peritonitis in one case (4,4%) and hemoperitoneum consequent to tumor disease or topographic intestinal disorders in 3 cases (13.0%).
8. Compared to the morbidity among other diseases, peritoneal fluid collections were diagnosed in only 23 cases out of 3781 patients presented for consultations, wich means 0,60%
9. The incidence of the peritoneal fluid collection syndrome in dogs was higher in the 7-14 years age cathegory – 13 cases(56,5%) and lower under the age of 2 years – one case (4,3%). In the age category 2-7 years, the peritoneal fluid collection syndrome was diagnosed in 9 cases (39,1%), illustrating that with age increase, the risk to

develope the peritoneal fluid collection syndrome consequent to hepatic or tumor disease also increases.

10. The main cause of the peritoneal fluid collection syndrome in dogs was congestive chronic heart failure, 9 cases (39,1%) followed by liver failure and tumor disease, each with 5 cases (21,7%). In other 4 situations (17,4%) the ethiology was different.

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RESUMPTION OF REPRODUCTIVE ACTIVITY IN SOWS AFTER WEANING, USING *PG600*

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Abstract
In many countries in Europe, PMSG therapy is used to initiate a fertile estrus in sows with extended anestrous and gilts in which the sexual maturity delays to occur. This kind of therapy is used also as a prophylactic method, for reducing the weaning-estrus interval, inducing estrus in lactating sows or for obtaining poliovulation in cyclic sows. This study conducted on 144 post-weaning sows which were examined every day (for a period of 30 days) in order to detect estrus and to establish the right moment for insemination. Sows belonging to experimental group (LE), which did not show heat signs within 5 days from weaning were treated with 5 ml PG 600. In control group (LM), 87.5% of sows showed spontaneous estrus, while in experimental group the estrus was induced in 95,8% of the sows. We can observe an increase in the percentage of sows with heat in LE, which was with 8.3% higher than the one in LM. Estrus synchronization was evident in the first 10 days in LM and in first 5 days in LE. In LM, 76.4% of sows showed spontaneous estrus after weaning, while in LE (sows with anestrus), heat was induced in 75%. The aim of this study was to reinitiate the reproductive activity in sows with anestrus after weaning using hormonal therapy with PG600.
Keywords*: sows, anestrus, synchronization, PG600*

Introduction

Over the time, there were many attempts to control the reproductive function in sows by stimulating and synchronizing the estrus using various hormones that naturally coordinate the reproductive processes. The best results were obtained by using PMSG and HCG, which are easy to administrate and have effects on the follicular growth and dehiscence as well as in inducing estrus (5, 8, 11).

The lactating sows are receptive to gonadotropins' action, starting with day 15 post-partum, but gestation rate is lower, compared to artificial insemination after 25 days post-partum (6, 9).

By administrating PG600 to primiparous sows, reduced the weaning-estrus interval from 7,4 days to 5,7 days. Also, the more intense manifestation of heat allowed to establish the right moment for insemination more accurately, and to obtain a high percentage of fecundity (88%), compared to control group (79%) (2 - 4 7,).

Other publications show that, in multiparous sows, the treatment led to shortening of weaning-estrus interval, (from 13 days to 7.8 days) and to increased prolificacy by 0.8 piglets/parturition (5, 7, 9).

Knox (2001) studied the effect of administration of PG 600 to sows in the first days after weaning. The author has observed the initiation of heat in sows on day 4 after weaning, when 81% of treated sows were detected in estrus (7).

Material and method

The study was conducted on a number of 144 sows post-weaning which were examined daily (for 30 days) in order to detect heat and determine the optimal timing of

insemination. All the sows have been organized in two homogenous groups: control group (LM) and experimental group (LE), both composed of 72 sows.

Sows that formed the control group were at the time of weaning (day 0), while those from the experimental group were sows that did not show estrus until day 5 after weaning.

Table 1. Structure of experimental and control groups, depending on the moment of weaning and initiation of estrus

Group	**Number of sows**	**Observations**
Control group (L.M.)	72	Immediately after weaning, ziua 0
Experimental group (L.E.)	72	5 days after weaning
Total number of sows	144	-

Control group (LM) was formed of sows in which it was observed the natural (spontaneous) resumption of estrus after weaning, over a period of 30 days. The experimental group (LE) has been formed of sows weaned on the same day as LM sows, but in which estrus did not start in a period of 5 days. The sows in LE, which showed no estrus during this time were treated with 5 ml PG 600, in a single dose, subcutaneously at the base of the ear. The hormonal product used was the one produced by Intervet (The Netherlands) as a lyophilized form.Prior to use, 25 ml of solvent were added in the vial containing 5 doses of PG-600.

All the sows were surveyed for detection of heat until day 30 after weaning. The response to treatment was calculated as the ratio between the number of sows that initiated estrus within 5 days after inoculation of PG 600 and the total number of treated sows.

Sows which showed estrus spontaneously or after treatment were artificially inseminated according to the unit technology and further were fed and maintained according to the technological flow (classical insemination with 3.5 billion spermatozoa / 80 ml).

The sows included in the test groups were monitored twice a day, every day for 30 days in order to detect the initiation of heat phase. Females that exhibited estrus were directed to artificial insemination hall and then separated.

To highlight the occurrence of estrus, sows were grouped in three categories, by days. Thus, in control group (LM) the categories were: - sows in estrus in less than ten days; - from 11 to 16 days; - from 17 to 30 days. Also, it was calculated their total number up to 30 days.

In the group treated with PG 600 starting with 5^{th} day after weaning (LE), sows in heat were grouped by intervals, as follows: 0-5 days from therapy; 6 – 11 days and 12-25 days and alsotheir total by25 days from therapy (30 days after weaning) (Table 2).

Of the 72 studied sows in the control group, 63 were detected in estrus by the end of the studied period while 9 did not show any signs of estrus. In the experimental group, of 72 sows 69 initiated estrus and only 3 did not.

By percentage, in the control group (LM) 87.5% of sows showed spontaneous estrus while in the LE, induced estrus occurred in 95.8% of the sows. We can observe a high

increase in the percentage of sows that initiated estrus in the group treated with PG 600, compared to control group (8.3% higher).

The percentage of females that showed estrus signs was similar in both two groups for the first interval. Thus, in the control group, 76.4% of sows had spontaneous estrus after weaning, while in the experimental group (sows with anestrus) heat was induced in 75% of sows.

This synchronization of estrus within first 10 days for LM is due to resumption of hypothalamic and ovarian hormonal activity after lactation. On the other hand, in the LE, resumption of hormonal activity is induced by gonadotropins fromtherapy, which stimulated follicular growth, maturation and dehiscence, occurring within 5 days.

Table 2. Initiation of estrus in sows, after weaning, according to treatment and time

Group	**tiation of estrus after weaning (days)**							
	Number of sows	Between 0 and 10 days		Between 11 and 16 days		Between 17 and 30 days		Between 0 and 30 days	
		no	%	no	%	no	%	no	%
M	72	55	76,4	3	4,2	5	6,9	63	87,5
E	72	54	75,0	13	18,0	2	2,8	69	95,8
		within 0 - 5 days after PG 600		within 6 – 11 days after PG 600		within 12 – 25 days after PG 600		within 0 -25 days after PG 600	

Between 11th and 16th days from weaning, in LM, 4.2% of sows presented estrus; in LE, between days 6 and 11, 18% of the sows showed estrus. In the interval 17th to 30th day, 6.9% of LM sows initiated estrus, while in the interval 12th to 30th, 2.5% of LE sows were detected in estrus.

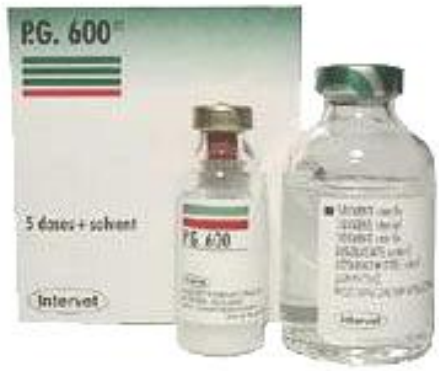

Figure 1. *PG 600,*used for stimulating estrus in sows

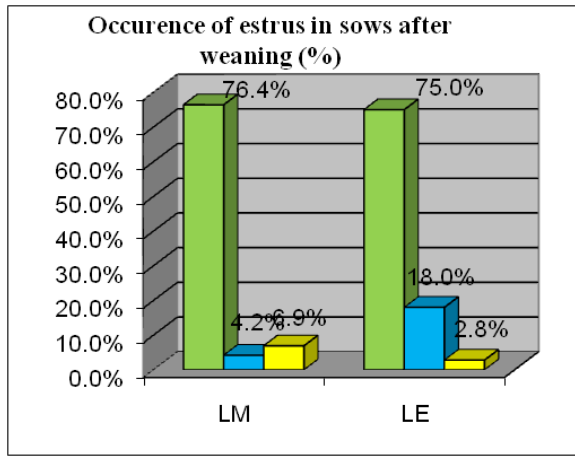

Figure 2. Percentage of estrus occurrence in sows, based on time intervals for each studied group

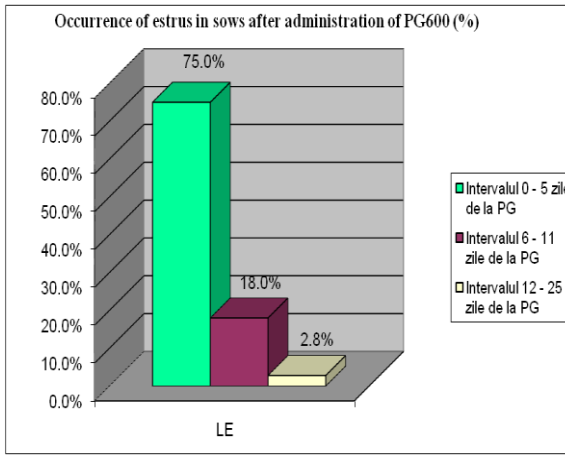

Figure 3. Grouping estrus initiation in sows treated with PG 600

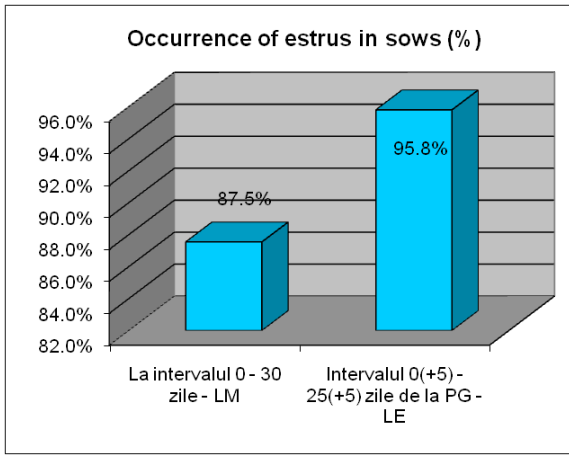

Figure 4. Comparative values of estrus initiation for each group, by time category

Acknowledgement

This study was conducted in the laboratoires of the University of Agriculture Science and Veterinary Medicine, Iasi, and was financed by Programme No. 5522 USAMV-Iasi.

Conclusions:

1) The initiation of heat in control group (LM) was detected in 87.5% of sows, which exhibited spontaneous heat, while in experimental group (LE), induced estrus occurred in 95.8% of sows. We can observe a high increase of percentage of sows with estrus, this percentage being with 8.3% higher in LE compared with LM.

2) Grouping estrus in sows was detected in the first 10 days in the control group and in the first 5 days in the experimental group. In the control group, 76.4% of sows showed spontaneous estrus after weaning while in the experimental group (sows with anestrus) heat was induced in 75% of sows.

3) Estrus groupingwithin 10 days in LM is due to hypothalamic and ovarian hormonalactivity resumption after lactation. On the other hand, in LE, hormonal activity resumption is induced by gonadotropins from therapy that have stimulated follicular growth, maturation and follicular dehiscence, estrus occurring within 5 days.

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RESEARCH CONCERNING THE INFLUENCE OF THE NON-IONIC CONTRAST AGENT ULTRAVIST 370 ON THE BLOOD IN CATS

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Abstract
Addressing the use of contrast agents is a major problem in cats, raising questions due to the fact that this species is sensitive to iodine, the main component of iodinated contrast agents of high osmolarity, which results in the loss of the animal after serious adverse reactions. Emergence of non-ionic contrast agents with low osmolality enables their use for radiological diagnostic in cat.This paper aims to track hematological and biochemical changes of blood following administration of contrast media Ultravist 370 in cats, and possible side effects that may occur. The contrast substance was tested on 5 cats both male and female. Blood tests (hematology and biochemical), both performed before and after administration of the contrast highlighting its degree of tolerance by the body. Several biochemical and hematological parameters were determined before Ultravist 370 administration and after 30 minutes, 60 minutes and 24 hours after non-ionic contrast substance administration, highlighting the modified parameters. Within 48 hours of substance administration, the values of the tested parameters came back within physiological limits
Keywords: *cat, blood, Ultravist 370.*

Intorduction

Radiologic contrast substances can produce adverse reaction that can vary from mild to severe modification that can endanger the patient's life (Thomas and Maddox, 2002). Complication that can occur after non-ionic contrast agents administration are determined by route of administration, chemical composition of contrast agents, patient's condition, way of handling the animals (Paithanpagare et al., 2008).

To avoid complication a crucial role has the correct selection of contrast medium, knowing that cats could develop allergic reaction to iod base contrast medium. The ideal contrast medium should have minimal neurotoxicity, should be pharmacologically inert, miscible with cerebrospinal fluids and radio opaque at an isotonic concentration (Wildmer and Blevins, 1991).

The reduced incidents of contrast-induced nephropathy is well known when using non-ionic, iso-osmolar contrast media agents (Baker et al., 2003; Soehardy, 2004; Azmus et al., 2005; Hernandez et al., 2009; Ye et al., 2012;).

In our study, we worked with Ultravist 370 (producer Schering AG - Germany), which is non-ionic contrast substance with low osmolality.

Material and method

The study was conducted on 5 adult cats, common breeds, males and females; the average weight was 5 Kg. The study monitored the changes of hematologic and biochemical values induced by non-ionic contrast media Ultravist 370 (producer Schering AG - Germany) administration.

The changes of major's physiological function were also monitored before and after non-ionic contrast substance administration. Before Ultravist 370 administration the cats underwent a 12 hours food diets, water ad libitum, the patients were examined clinically, blood was collected from every cat in order to determine the hematological and biochemical values and the major's physiological function were monitored. The non-ionic contrast media was slowly administered intravenously, in the cephalic vein or in the saphenous vein (fig. 1,). Blood samples were collected at 30 minutes, 60 minutes and 24 hours after non-ionic contrast media administration. We also monitored heart rate, body temperature and respiratory rate after non-ionic substances administration. The hematological analysis were performed on Diatron Clinical biochemistry Analyzer (Diatron LTD, USA) and the biochemical analyses were performed on device manufactured by Awareness Technology USA.

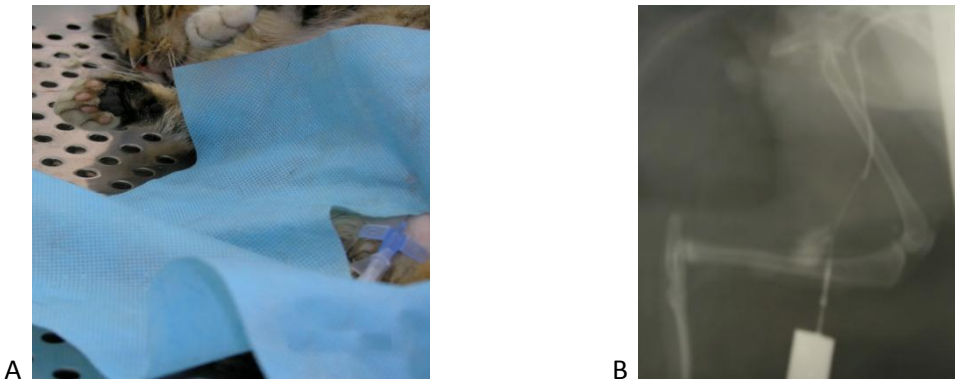


Fig. 1. Pregătirea venei safenă pentru administrarea substanţei de contrast, la pisică (A), expunere latero-laterală în timpul administrării de Ultravist 370 (vena safenă) (B)

Results and discussion

The secondary reaction after Ultravist 370 were: mild and transient chills, hypersalivation and tachycardia. Nervous system examination revealed no changes in behavioral state. Body temperature (BT) and respiratory rate (RR) before and after non-ionic contrast media administration were in normal ranges at any given time when the cats were examined.

For the biochemical analyses we have chose 13 parameters in order to reflect the status of liver and kidneys and for the hematological exam we have performed a blood elements analysis.

The references used for the obtained results were given by our university Clinical Laboratory Diagnostic.

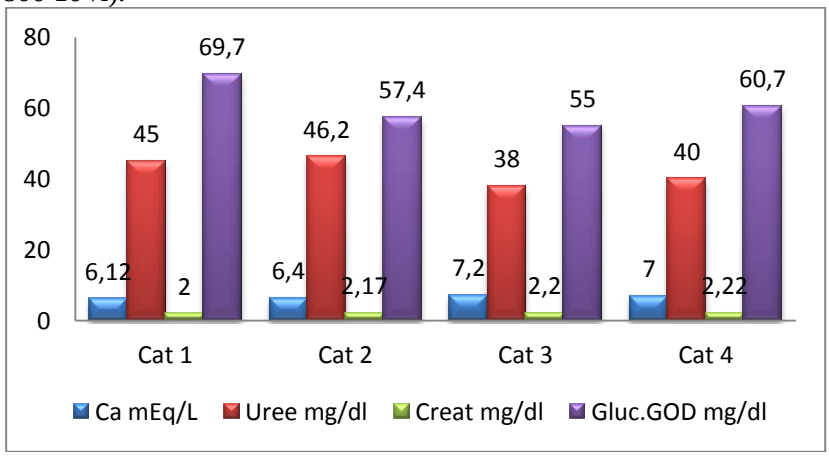
Before non-ionic contrast media Ultravist 370 administration (T_0) the biochemical values present a mild modification of alkaline phosphatase (ALKP) 70.2 ± 0.59 U/l (normal value ranging from 5 – 70 U/L) and gamma glutamic transferase (GGT) 13±1.16 U/L (normal value ranging from 2 – 12 U/l) (table 1). Blood elements analysis shows moderate thrombocytopenia (Platelet count - PLT 205.64±2.45 10^9/l - normal value ranging from 300-

800 10^9/l) and mild lymphocytosis (LYM 7.98±0.21 10^9/l - normal value ranging from 1.5-7 10^9/l).

Table 1. Average and standard deviation of determined biochemical value before Ultravist 370 administration

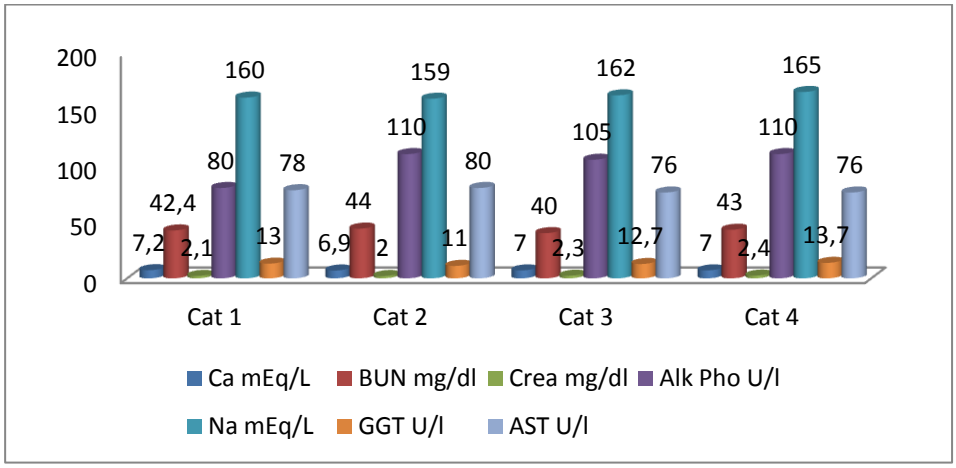
TEST	Result	UM	MIN	MAX
CHOL	190± 3.24	mg/dl	90	200
TRIGL	43±2.26	mg/dl	10	90
TOTAL CA	5.8±1.22	mEq/1	5.5	10.8
ALKP	70±0.59 ▲	U/I	5	70
GGT	13±1.16 ▲	U/I	2	12
BUN	29.5±0.25	mg/dl	10	30
GLUCOSIS	98±1.88	mg/dl	65	130
CREA	0.52±0.24	mg/dl	0.5	1.9
AST	23±1.12	U/l	10	50
ALT	21±3.41	U/l	10	60
TOTAL PROT.	6.9±0.47	g/dl	5.5	8
ALBUMIN	34±0.98	g/l	25	38
PHOSPHOR	3.9±0.34	mg/dl	4.0	7.5

At 30 minutes after non-ionic contrast substance administration (T_1), biochemical exam show an elevation of Creatinine at 2 ± 0.10 mg/dl and blood urea nitrogen (BUN) at 42.3±3.92 and a decreasing of Ca at 6.68 ± 0.50 mg/dl and Glucose (GLU) at 60.7±6.43 (graph 1). The remaining biochemical parameters show slightly elevated values that T_0, but they do not exceed the maximal values. The hematological exam show normal values, except mean corpuscular hemoglobin concentration (MCHC) 369.89 ± 2.14 g/l (normal value ranging from 300 – 360 g/l), thrombocytes (PLT) 153.73 ± 2.01 10^9/l (normal value ranging from 300 – 800 10^9/l) and minimum inhibitory dilution (MID) 3.48 ± 0.57 % (normal value ranging from 300 – 800 10^9/l).



Graph 1. The modified biochemical values on the 5 cats after 30 minutes of contrast media administration

After 60 minutes of non-ionic contrast substance administration (T_2), biochemical exam of the blood on the 5 examined cats show elevation of BUN 42.32 ± 1.17 mg/dl, Crea 2.2 ± 0.18 mg/dl, ALKP 101.25 ± 14.36 U/l, Na 161.5 ± 2.65 mEq/L, and GGT 12.6 ± 1.14 U/l and AST 77.5 ± 1.91 U/l, and a decreasing of Ca that was observed only in 2 of the 5 cats examined (graph 2). Rest of biochemical parameters being less elevated than T_1 but still in normal values. Hematological exam show a decreasing of red blood cell (RBC) 4.61 ± 1.06 10^{12}/l (normal value ranging from 5 – 10 10^{12}/l), hemoglobin (HGB) 68.85 ± 1.03 g/l (normal value ranging from 80 – 150 g/l), hematocrit (HCT) 20.24 ± 1.01% (normal value ranging from 24 – 45%), PLT 41.82 ± 1.28 %, and an increasing of MID 7.37 ± 1.1% , the rest of hematological values being in normal range.



Graph 2. The modified biochemical values on the 5 cats after 60 minutes of contrast media administration

After 24 hours of non-ionic contrast administration (T_3) biochemical examinations of blood show modification that were represented in table 2. The rest of biochemical parameters show decreasing in values related to T_2, parameters being in normal values. Hematological exam show a decreasing of red blood cell (RBC) 4.89 ± 0.23 10^{12}/l, HGB 79.90 ± 0.21 g/l, PLT 298.82 ± 1.02 %, the rest of hematological values being in normal range.

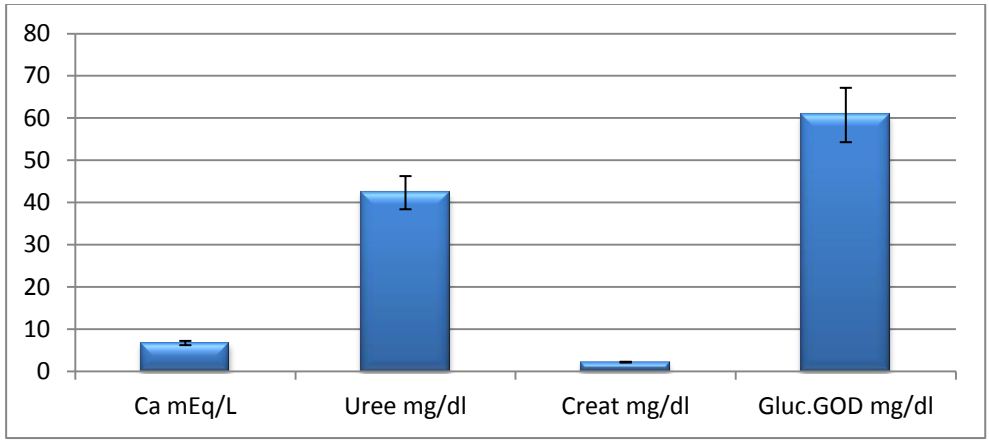
Table 2 Average and standard deviation ob biochemical values after 24 hours of Ultravist 370 administration

TEST	Min	Results	Max
CA mEq/l	5,5	4.8±0.2 ▼	10,8
NA mEq/l	145	143±1.23 ▼	158
ALKP U/l	5	105±2,14 ▲	70
GGT U/l	2	15±0,78 ▲	12
BUN mg/dl	10	50,2±1,04 ▲	30
CREA mg/dl	0,5	2,2±0,11 ▲	1,9
AST U/l	10	36±1,08	50

ALT U/l	10	72±2,19 ▲	60
Total Prot g/dl	5,5	8,9±0,32 ▲	8
Albumin g/dl	25	19±0,98 ▼	35
Phosphorus mg/dl	4	3,5±0,73 ▼	5

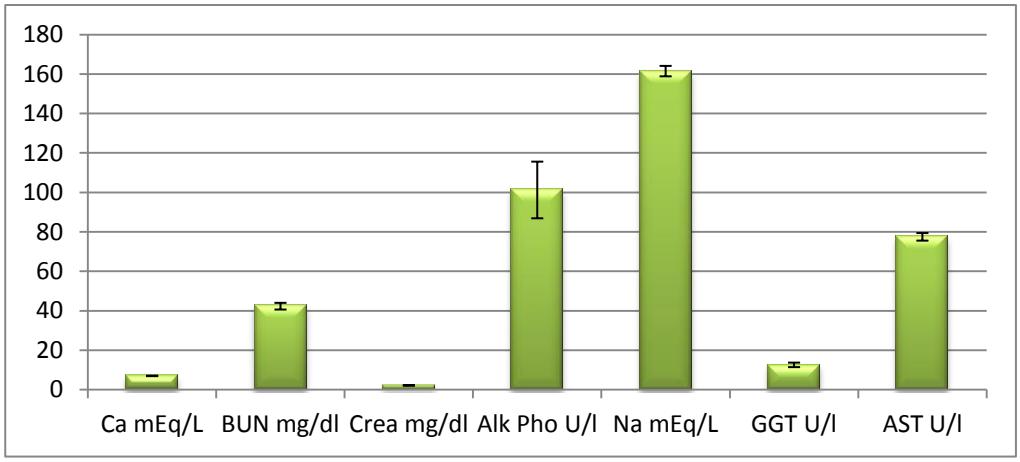
Before contrast substance administration, biochemical analyses show an increase of ALKP and GGT values that maintains until after 24 hours of administration.

At 30 minutes of Ultravist administration the increasing of Crea and BUN in correlation with increasing in MCHC value and decreasing of PLT show a possible dehydration and mild anemia most probably because of the low osmolarity contrast substance administration (graph 3). Decreasing of calcium and increasing of Crea and BUN values could indicate a low glomerular filtration rate or a spasm of ureters. Increasing BUN values could be correlated with stress induce by fasting prior intervention or by blood collecting procedures.



Graph 3. Average and standard deviation of modified biochemical values after 30 minutes of Ultravist 370 administration.

At 60 minutes after intravenous administration of Ultravist 370 the anemic state persist showing increased Crea, BUN and low RBC, HGB, HCT and PLT. The low glomerulary filtration is indicated by increased values of Crea and BUN. Increasing of ALKP, AST and GGT indicate a mild hepatic lipidosis due to lack of food prior intervention and a mild irritative - inflammatory process of the biliary ducts or could be associated with a acid-base imbalance. The ureteral spasm is presented only in 2 cats and is indicated by the low values of Ca. Pseudohypernatremia is due to dehydration (Vaden et al., 2009) (graph 4).



Graph 4. Average and standard deviation of modified biochemical values after 60 minutes of Ultravist 370 administration.

At 24 hours after Ultravist 370 administration the biochemical values are slightly modify compared to physiological values. At 24 hours is still present a mild anemia and dehydration shown by increased Crea, BUN and ALT and decreased Na, Albumin and Total Protein values, that are correlated also with low glomerulary filtration. The ALKP, GGT and ALT values are increased but tend to return to normal values, indicating an inflammatory hepatic process. After 48 hours of Ultravist 370 administration the values of biochemical constituents return to their normal values except glucose that could be stress related.

Conclusion

Non-ionic contrast substances, when properly administered, are not painful. Increased values of ALKP, Na at T_2 and T_3 correlated with BUN, ALT, AST valued indicate a mild dehydration and a stress factor related with handling and blood collection therefore these changes could be avoided if the cats will be tranquillized and the food diet reduced to 6 hours and water administered also after contrast substance administering. A long diet correlated with non-ionic contrast substance Ultravist 370 determine the increasing values of GGT, ALT, and ALKP after 24 hours and could produce hepatolipidosis and probably an irritation in the biliari ducts. Hematological values show mild anemia and dehydration after contrast media administration. The product tested had excellent neural tolerance and a minimal influence on the cardio-respiratory system.The effect on the liver and kidneys was minimal, no morphological or functional changes were noticed and the life of the subjects was not threatened.

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ASSESSMENT OF TWO BLOOD PARAMETERS RESPONSIBLE FOR BONE HEALING USING ULTRASOUND THERAPY

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Abstract

The healing of bone fractures is a complex multi step process. Several internal and external factors are responsible and can influence the quality and duration of the healing process. In the present study we took in observation two blood parameters , osteocalcin and alkaline phosphatase. We performed our study on 22 dogs of different breed and gender. The dogs were separated in two groups, the witness group and the group with fractured limbs which was treated using ultrasound therapy. We took blood from both groups using a specific protocol at day 1, 7, 15 and 30 post surgery. The difference between both groups was best observed at day 7 in both parameters. The bone biomarkers studied in this article can be used for evaluating the accelerated fracture healing process using ultrasound therapy.

Key words*: fracture, blood parameters*

Material and methods

Our study was conducted on 2 groups, clinical cases, a total of 20 dogs which had fractured limbs in different areas. The patients were split into 2 groups of 10 , a witness group and an experimental group. All patients had osteosinthesis surgery, each time applied specifically for the type of fracture (screws and plate, centromedular fixation). When we divided the patients in 2 groups we kept in mind their age, all dogs having being 2-5 years old. The time before the operation was in all cases under 24 h. The witness group recovered classically from the operation while the experimental group recovered with the help of ultrasound therapy. The witness group was treated post operation with antibiotics administered generally (Linco Spectin) and locally with Oxy Vet spray for 3 days.

The group treated with ultrasound had the following protocol: intensity 0,5 W/cm^2 , frequency 1 MH_Z with a 7-10 minute session per day for 10 days followed by a 5 day break and another 10 days of ultrasound therapy. Treatment was applied with the help of Misonic 12 ultrasound machine. For both groups, the evaluation method consisted of blood samples for the determination of alkaline phosphatase and osteocalcine.

Blood samples were taken in a sequential order: before surgery, 7, 15 and 30 days post operation, under normal circumstances, respecting all antiseptic measures. We considered this timeline to be of great importance in the forming of bone callus and the parameters are more relevant in this period.

Bone alkaline phosphatase is a sensible indicator for the bone metabolism. It can be found at high values during bone healing due to fractures, osteoblastic hyperactivity and the reshaping of bones. It was determined with the help of chemiluminescence (CLIA).

Osteocalcine is exclusively produced by osteoblasts, making it a relevant marker in the bone forming process (Minisola si col. 1997). Because of it's affinity towards calcium it could represent the site for hidroxiapathy crystals (Richard si col. 2007). Osteocalcine was determined with the help of electrochemiluminescence (ECLIA).

The statistic values from the obtained data (T student test and Bonferroni) was procured by the help of the programs Origin 8.5 and 7. The samples were analyzed two at a time from each specimen and an average was calculated.

We considered, the blood markers from bone metabolism was very important in the evaluation of the disease as well as the healing process (Allen, 2003; Brecur si col. 2004). Analyzing the variations was done so we could determine the statistical differences between the study groups. The average of every parameter was compared to the other parameters from the same group at different time intervals with the help of the T-student test, comparing between groups from the time when the blood was taken. P<0,05 (table. 1) was considered to be important.

Table. 1. Dynamic markers from blood taken into study from the study groups

Parametrii	**Ziua 1**		**Ziua 7**		**Ziua 15**		**Ziua 15**	
	M	**US**	**M**	**US**	**M**	**US**	**M**	**US**
Osteocalcina	4,10 ± 0,05	4,06 ±0,05	4,40 ±0,14	4,90 ±0,32	4,68 ±0,15	5,20 ±0,12	4,42 0,12	5,84 ±0,09
Bone alkaline Phosphatase (U/L)	9,20 ±0,04	9,32 ±0,04	9,18 ±0,04	10,10 ±0,04	9,00 ±0,03	10,66 ±0,04	9,02 ±0,03	11,12 ±0,03

Analyzing the witness group, osteocalcine presented significant statistical differences between day 0 and 7, P being 0,00006 and the average 0,386. Comparing the results from day 7 to day 15, they don't show major differences, P being 0,08259 and the average 0,116. Day 15 in comparison to day 30 has significant statistical differences, P being 0,00009 and the average 0,365 with a drop of osteocalcine at day 30 in comparison to day 15.

Table. 2. Statistic analyze of osteocalcine in both groups

Lot	**Ziua 0**	**Ziua7**	**Ziua 15**	**Ziua 30**
Lot Martor	4,10 ± 0,05	4,40 ± 0,14	4,68 ± 0,15	4,42 ± 0,12
	P=0,00006 0-7=0,386	P=0,08259 7-15= - 0,116		P=0,00009 15-30= + 0,365
Lot Ultrasunete	4,06 ± 0,05	4,90 ± 0,32	5,20 ± 0,12	5,84 ± 0,09
	P = 0,00000 0-7 = 0,879	P = 0,10532 7-15 = - 0,198		15-30

As far as alkaline phosphatase goes (table. 3) this parameter suffers modification as well. If we compare the first with the 7'th day, we have no significant statistical differences, P being 0 and the average 0,527. Day 7 in comparison with day 15 did not show any significant statistical differences, P being 0,92465 and the average 0,004.

Table. 3. Statistic analyze of alkaline phosphatase evolution at the groups taken into study

Lot	Ziua 0	Ziua 7	Ziua 15	Ziua 30
Lot Martor	9,20 ± 0,04	9,18 ± 0,04	9,00 ± 0,03	8,12 ± 0,02
	P = 0,00000 0-7 = 0,527	P = 0,92465 7-15 = 0,004		P = 0,00101 15-30 = 1,081
Lot Ultrasunete	9,12 ± 0,10	9,16 ± 0,11	10,00 ± 0,14	11,06 ± 0,09
	P = 0,16088 0-7 = - 0,089	P = 0,00000 7-15 = - 0,674		P = 0,00000 15-30 = - 0,410

Statistic differences were significant between day 15 and 30 where P was equal to 0,00001 and the difference between the average was 1,398 at day 30.

The group treated with ultrasound suffers modification on both parameters, in some moments, the parameters have statistic significance. Osteocalcine has high values at day 7 in comparison to the first day, the average being 0,879 and P is 0. No significant differences were reported between day 7 and 14, P being 0,10532. The average from day 15 was 0,201, higher than day 7. Day 15 to day 30 period was representative because P was 0 and the difference between the average was 0,639. Osteocalcine was risen until day 30 (table 2).

As far as alkaline phosphatase goes (table 3), the group treated with ultrasound at day 1 didn't show many differences in comparison to day 7, P being 0,16088. The difference between the average was 0,089.

Statistic differences were significant between day 7 and 14 where P was equal to 0 and the difference between the average was 0,674 and rising. Also, days 15-30 showed a growth with the average being 0,410. Some statistical differences existed between the period of taking the samples, P being 0. In comparison to the witness group, osteocalcine and alkaline phosphatase presented significant statistic differences in all 3 periods when samples were taken after the first day.

The biggest difference was registered at day 30. The difference between osteocalcine average from the two groups was 1,42 and P was 0. Alkaline phosphatase was 1,06 and P was 0.

Other authors had a significant rise in alkaline phosphatase between day 28-42. If we compare their result with ours, our result specifies this growth at the second week.

Conclusion

1. Bone biomarkers taken into our study (osteocalcine and alkaline phosphatase) evaluate the dynamic process of healing of fractures with the use of ultrasound.
2. The evolution of the blood parameters was superior for the study group in comparison to the witness group.
3. The evolution of osteocalcine in the study group was positive and rising from a determination step to another, the biggest difference between the two groups was at day 30 with a 1,42 difference for the study group and P was 0
4. Alkaline phosphatase has the same ascending evolution as osteocalcine until the end of the healing process but it will surpass osteocaline evolution by 2,94 with P being 0.

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OOCYTE RECOVERING BY OVUM PICK UP FROM HIGHLY ENDANGERED ROMANIAN GREY STEPPE COWS

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Abstract

The aim of this study was to collect oocytes by Ovum Pick-Up (OPU) on days 6 and 13-14 of cycle in Romanian Grey Steppe cows. A group of 10 cows was used to perform ovum pick-up on days 6 and 13-14 after estrus detection. The follicular wave was initiated by induced estrus and by puncturing the dominant follicle and all other follicles sized ≥3 mm at days 6 and 13-14 of the cycle. The dynamics of ovarian follicular growth were monitored by twice-daily ultrasonographic examinations. By using OPU biotechnology in Romanian Grey Steppe cows we made a number of 13 aspirations/session and 6.2 ± 1.5 aspirated follicles/cow/sessions. The mean number of recovery oocytes/cow/sessions was 4.20 ± 0.2. In conclusion the OPU biotechnology can be applied for the Romanian Grey Steppe cows for harvesting the oocytes and for conservation of genetic resources.

Key words*: Romanian Grey Steppe cows, OPU, Follicle, Oocyte.*

Introduction

Over the past 15 years, 300 out of 6000 breeds of all farm animal species identified by FAO have gone extinct (Scherf, 2000). It is argued in the World Watch List for Domestic Animal Diversity (DAD) that 1350 farm animal breeds currently face extinction. Romanian Grey Steppe, Hungarian Grey, Maremmana and Turkish Grey belong to the same Podolic group of cattle and show a similar external conformation. These breeds recently underwent a similar demographic reduction. Podolic cattle include a very ancient group of breeds, considered to be straight descendants from the Auroch (*Bos primigenius*). Podolic breeds are present in various European areas, and many of them are seriously endangered of extinction. From the FAO (DAD-IS) in 2003, the Romanian Grey Steppe was at risk of extinction (dad.fao.org).

The National Agency for Amelioration and Reproduction in Animal Husbandry (ANARZ – Romania) estimated in 2012 that populations (n = 150) of pure and half-breed Romanian Grey Steppe variety could still be found, with a different absorption level, isolated, in the North-East of Moldavia and the Danube Delta. In this context it is necessary to preserve its genetic resources by the increasing, reproducing and improving its population which has been reproductively isolated, reduced and removed from the sphere of influence of the factors that may modify the gene frequency so that they might remain unchanged (Government decision no. 822/2008).

In vivo oocyte collecting by transvaginal ultrasound-guided follicle aspiration (Ovum Pick-Up: OPU) can be a good alternative to increase the probability of preservation of this endangered cow breed. The combination of OPU with pellets frozen from 1970 (bull Sura Fort II from ANARZ- Bucharest) and the cryopreservation (Galli C et al., 2012) offer the opportunity to accelerate the genetic gain in the recovery of this breed of cows. This OPU-technique was first established in cattle by a Dutch team (Pieterse et al., 1988). These studies demonstrate that the repeated oocyte collecting by OPU could be performed without risks for

health and the reproductive activity. It is an alternative because it can be applied successfully irrespective of the reproductive status of the donor, *i.e.* in pregnant and acyclic animals, in those having patent tube or genital tract infections and in animals insensitive to superovulatory treatment. It is competitive because it can yield more transferable embryos per donor on a monthly basis (R. Boni, 2012).

In Romanian Grey Steppe cows, studies on follicular aspiration doesn't exist. In this study, OPU was performed at days 6 and 13 - 14 of cycle in 10 Romanian Grey Steppe cows for a total number of 20 transvaginal aspiration procedures.

Material and methods

Experimental animals

This study was performed at the dairy cows farm from S.C..D.C.B Dancu Iasi-Romania which has 59 Romanian Grey Steppe cows (Moldavian variety), included in a governmental program of genetic conservation (Government decision no. 822/2008). This group presents females with ages between 6 months and 21 years and two males aged between 2 and 3 years. The animals used were multiparous, free from reproductive abnormalities (n = 10), with ages between 4-5 years; they were kept on free stalls with a diet of 70 % corn silage and 30 % alfalfa hay. The mean body weight, age of cows was 450 ± 5.0 kg (ranging from 420 to 480 kg), 4.5 ± 0.13 yr (ranging from 4 to 5 yr) and body condition score 4.1 ± 0.2 (4 to 4.5 on a 0 to 5 point scale described by Lowman et al., 1976).

The cows used for the experiment (n = 10) were subjected to two sequential sessions of OPU in days 6 and 13-14 of the cycles.

The cows were synchronized in estrus by injecting 25 mg Dinoprost (PGF) (5 ml i.m. Dinolytic, Pfizer Animal Health Ma Eeig, Great Britain), a single administration after ultrasonography detecting of the corpus luteum (CL). All cows showed estrus in 2-3 days after PGF administration.

Characterization of follicular deviation

The ovaries were examined with a portable ultrasound device equipped with a trans-rectal linear-array 7.5-MHz transducer (Welld Wed-3000V, Shenzhen Well.D Medical Electronics Company Ltd., China). Evaluations were performed at the first beginning protocol OPU 1 of estrus detection for the experimental protocols and twice a day (every 12 h) after the start of the experiment. At each examination, the size and location of all follicles were recorded on a sketch (image stored) of each ovary. Ovaries were mapped by recording the diameters of three largest follicles at each examination.

Follicular aspiration and oocytes recovery

The animals were restrained in a suitably designed stanchion, which allowed minimal movement. They were prepared for OPU by being administered 100 µg/kg BW Xylazine HCL (Narcoxyl 2, MSD Animal Health, Holland) i.m., followed 10 min later by an epidural anesthesia of 7 ml of 2% Procainã clorhidrat (Procainã 2%, Romvac, Romania).

Transvaginal ultrasound guided oocyte collection was performed using an ultrasound scanner (Ultrasonic device SSDProSound2 convex real time scanner) with a convex-sector probe 7.5 Hz (Bold Medical, Romania), attached to an aspiration pump (Rocket medical) and ovum pick-up needle (COVA NEEDLE "type A", 17Gx500mm, Minitube GmbH) guidance system. For oocytes aspiration a vacuum of 100 mmHg was used. All the follicles were aspirated and a special attention was given to avoid partial aspiration or to miss a follicle. A

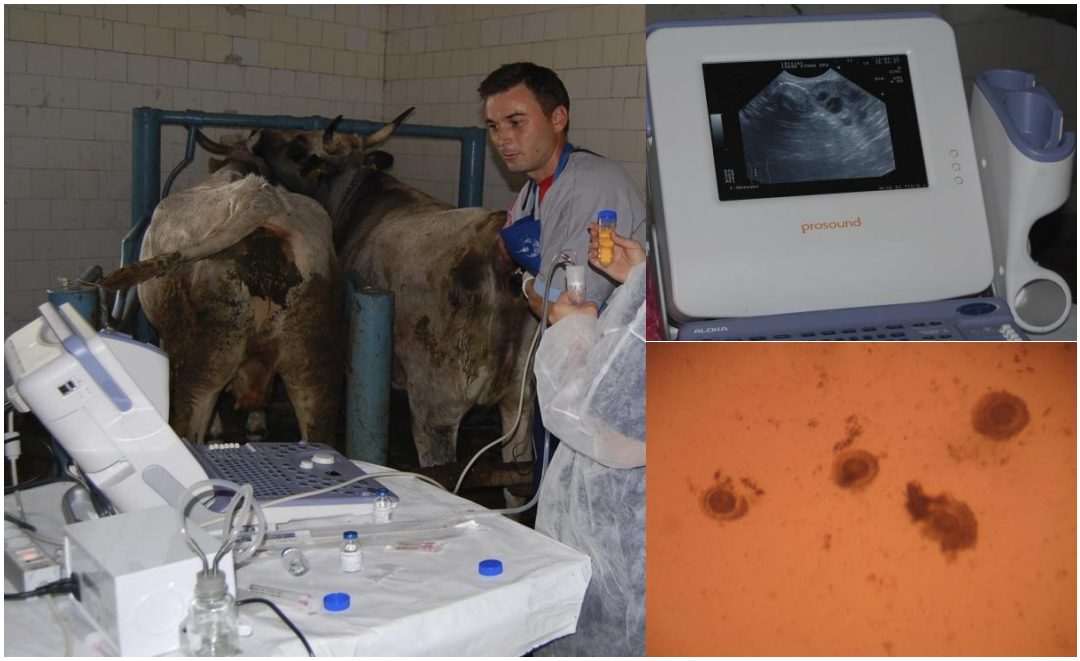
numbers of follicles with different diameters were recorded, and grouped according to their size.

The aspirated follicular fluid was collected in 50-ml tubes containing modified Dulbecco´s phosphate buffered saline (mDPBS), supplemented with 1% fetal calf serum (Gibco) and 125 IU/ml heparin (Sigma). Recovered COCs were morphologically classified into five categories according to those previously described by Chaubal et al. (2006), as follows: Grade A, >4 layers of cumulus cells; Grade B, three or four layers of cumulus cells; Grade C, one or two layers of cumulus cells; Grade D, denuded oocytes; and Grade E, oocytes with expanded cumulus. The values are presented in the text like mean ± standard error of mean.

Results and Discussions

Follicular aspiration and oocytes recovery

As per available knowledge, there is no date of ovarian follicular development, as the oocyte yields in Grey steppe cows. The results of OPU perform in 10 Romanian Grey Steppe cows are presented in table 1.



Img. 1. OPU biotechnique applied on Romanian Grey Steppe cow

Table 1. Total number and mean values (Mean ± SEM) of recovered COCs and classification of COCs collected in Romanian Grey Steppe cows

Protocol code*	**OPU**
Number of animals (n)	10
Characteristics	
Total number of aspirations/sessions	13
Number of aspiration sessions/cow	2
Mean number of aspirated follicles/cow/session	6.2 ± 1.5
Categories of follicles at aspiration/cow/session	
3-4 mm	3.2 ± 0.1
5-6 mm	1 ± 0.5
> 7 mm	2 ± 0.3
Mean number of oocytes/cow/session	4.20 ± 0.2
Recovery rate (%)	66.6 ± 1.9
Oocytes categories	
A/Total (%)	25/80 (31.25)
B/Total (%)	27/80 (33.75)
C/Total (%)	26/80 (32.5)
D/Total (%)	2/80 (2.5)
E/Total (%)	0

In this experiment we made a number of 13 OPU aspirations/sessions in two aspiration sessions/cow. The mean number of aspired follicles/cow/session was 6.2 ± 1.5 (mean ±S.E.M.). The follicle with the size between 3-4 mm was aspired in a highly number (3.2 ± 0.1) compared with the number of aspired follicle in 5-6 mm diameter (1 ± 0.5) and with the follicle larger than 7 mm diameter (2 ± 0.3). The average number of aspired oocytes/cow/session was 4.20 ± 0.2 which represent a recovery rate of 66.6 ± 1.9%.The distributions of oocytes according to morphological grades (percentage of total oocytes retrieved) was 31.25 % for grade A, 33.75 % for grade B, 32.5 % for grade C and 2.5 % for grade D.

Our research complements the reports made of the OPU technique applied on different breed of cow. Furthermore, results from super-stimulation of follicular growth with OPU techniques in Angus crossbreed cows (Chaubal et al., 2006; Chaubal et al., 2007), Holstein cows (De Roovera et al., 2005), Simmental heifers (Reis et al., 2001) and Nellore cattle (Monteiro et al., 2010) were reported.

Conclusions

In vivo oocytes recovery by Ovum Pick Up can be applied on Grey Steppe cows with positive results. Also, we observed a similar proportion in oocytes quality, in A, B and C grades, compared with D grade, which was generated in a small proportion. The E grade is missing from these two OPU sessions.

Future studies could explore the integration of OPU, associated with in vitro embryo production, in order to increase the population number and could also explore the integration of these biotechnologies into animal breeding programs using Romanian Grey Steppe (*B. taurus primigenius*) donors.

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THE USE OF SOME HEMATOLOGICAL AND BIOCHEMICAL ANALYSIS FOR TESTING THE TOLERANCE OF AN ANTIFUNGAL OINTMENT ON HEALTHY ANIMALS FROM TARGET SPECIES

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Abstract

The purpose of this study was to investigate the influence of an ointment, containing imidazole, on the evolution of some hematological and biochemical parameters in dogs and cats, that were subjected to the evaluation of tolerance to the active substance. For the investigations, three groups were used, each consisting of ten animals. The protocol was identical for both dogs and cats. The first group (n=10) was treated for 5 consecutive days by applying the ointment once every day, on intact healthy skin (the interdigital, axillary and inguinal areas) from the right side of the body. The second group (n=10) was treated the same way, but the ointment was applied twice a day; the third group (n=10) served as control, not being subjected to any treatment. After applying the ointment, clinical examinations were conducted to monitor the local and general reactions produced. These examinations took place at different time periods (after 1, 24, 48 and 120 hours from the first administration of the product), grading the results according to the Draize scale (1971) for erythema or edema. The final tests were conducted after 10 to 20 hours from the last administration of the product, consisting of a general examination of the subjects and hematological and biochemical investigations, respectively. Post-therapeutically, the mean value of the PCV was 46.12% for dogs and 36.89% for cats (37%-55%). The product did not induce post-therapeutic anemia as the red blood cell counts were 7.45 in dogs and 6,39 for cats, the recorded values being in physiological ranges (5,5-8,5 T/l). Regarding the leukocyte parameters, there were post-therapeutic individual variations, however with no pathological significance. The biochemical profile revealed minor differences between the groups of animals from the same species, outlining a protein, enzymatic and mineral profile, corresponding to a proper health status maintained throughout the whole period of observation. Analyzing the results from the tests, we can conclude that the two species proved a high level of tolerance to the tested antifungal ointment, thus it can be used without causing side effects to the general health status of the animals, especially to the hematological and biochemical profile.

Key-words: *antifungal ointment, hematology, metabolic profile, local tolerance*

Introduction

Establishing the tolerance of a medicinal product is important in order to determine the presence of adverse reactions when administering the drug. Local tolerance is tested for all the active substances from drugs that are applied on skin or mucosa.

The use of a metabolic profile to assess the health status and the tolerance of ointments represents a baseline for research of local and general tolerance tests (Cristina and Chiurciu, 2010).

The product used in this study was an anti-micotic ointment containing alfa-[2,4-(diclorophenil)-1h-imidazol-1-ethanol]. The active substance belongs to the imidazole group and can have antibacterial, antifungal, anti-protozoal and antihelmintic affects (Ross, 1997). Phenil-imidazoles are used in antifungal therapy, having a broad spectrum of action against

yeasts and funguses that can cause both systemic and superficial infections (The Merck Veterinary Manual, 2012).

Imidazoles modify the permeability of the cell membrane of the receptive species of fungi by blocking the ergosterole synthesis, the main sterol in the fungi cell. Various *in vitro* studies demonstrated that the miconazole owns its anti-bacterial and antifungal topical effects by inducing some changes in the lipid structure of the membrane (Van den Bossche et al., 2003). The antifungal imidazoles also have anti-bacterial effects, but are rarely used in this direction. Miconazole has a very broad antifungal action against most yeasts and mycosis of interest in the field of veterinary medicine. Some of the target species worth mentioning are: *Blastomyces dermatitis, Paracoccidioides brasiliensis, Histoplasma capsulatum, Candida spp, Coccidioides immitis, Cryptococcus neoformans* and *Aspergillus fumigatus* (Riviere and Papich, 2009); some strains of *Aspergillus* and *Madurella spp* have a decreased sensitivity for the action of miconazole (Van Cutsem, 1972; Georgopapadakou, 1987).

Materials and methods

This research has been carried out to test the tolerance of a veterinary product, containing imidazole (anti-micotic ointment), on animals from different races (cats and dogs), some coming from the Biobase belonging to the Faculty of Veterinary Medicine, Cluj Napoca, and others being kept as pets. The tests were carried out using various pharmacological, hematological, biochemical and clinical methods, appropriate for testing drug tolerance, at the same time, complying with the current legislation regarding the safety and tolerance assessment of drugs for veterinary use.

7 days prior to the beginning of the test, preliminary clinical and para-clinical tests (hematological and biochemical) were carried out to select the suitable animals, according to the inclusion criteria. Based on the results recorded in these tests, only the healthy animal were accepted in the study, thus resulting 3 groups of dogs and 3 groups of cats, each groups consisting of 10 animals. The minimum age was 2 months for dogs and 3 months for cats; the minimum weight was 1 kg for dogs and 300g for cats. The samples collected were analyzed in the laboratories of Physiology, Pharmacology and Microbiology from our Faculty.

According to the established work protocol, a single method was used, both for dogs and cats, having as the only variable the administered dose. This consisted in applying the ointment on the intact healthy skin (the interdigital, axillary and inguinal areas) from the right side of the body, for five consecutive days, once every day for the first groups (1A and 1B) and twice a day for the second groups (2A and 2B).

The third group (3A and 3B) served as control, not being subjected to any treatment, and included animals kept under observation in the same environmental and feeding conditions as the other experimental groups.

The animals had water and food at their discretion throughout the whole study period, having a daily schedule as follows:

- Day 0, before applying the product, included the behavioral surveillance of the animals (water and food consumption) and repeating the clinical exams;
- Days 1-5, were intended for the product administration, following the established protocol for each group;
- Day 6 included the final examination, at 10-20 hours after the last application of the ointment, consisting in the repetition of the clinical and hematological tests.

The animals used were monitored during the testing period, in order to identify and quantify the local reaction (erythematous or edematous type), using the intensity score, from 0 to 4, provided in the Draize scale (Draize et al., 1944). After the last administration of the antifungal ointment, all animals were kept in for clinical observation, for 10 days.

The hematological and biochemical tests were carried out using automatic analyzers (Abacus Junior Vet şi VetScan); the following parameters were determined: packed cell volume (PCV), hemoglobin (HGB), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), total (WBC) and differentiated leukocyte count; the main biochemical parameters determined were: total protein (TP), albumins (ALB), alanine aminotransferase (ALT), amylase (AMY) and globulins (GLOB) concentrations.

Results and discussions

The general analysis of the results from the clinical, hematological and biochemical parameters investigated, revealed variations of the mean results obtained. These were non-significantly influenced by the experimental variable and were within physiological limits for the species and the category investigated. These results were in accordance with the reference data and the results obtained in the control groups.

The analysis of the results obtained in dogs. In the case of the dog groups (control and those treated with the therapeutic dosage or the double dosage) no significant differences were recorded between the results obtained before- and after-treatment, for the main hematological and biochemical results, their variations being in accordance with those of the canine species.

The evolution of the red blood cells parameters was characterized with post-therapeutically average levels of the packed cell volume and hemoglobin situated in the physiological limits (37-55%, respectively 12-18 g/dl) (Merck Veterinary Manual, 2012).
The mean values for the packed cell volume (46,74%) and hemoglobin (16,34 g/dl) presented individual variations between 37,06% and 53,67%, and between 13,4 g/dl and 17,9g/d respectively, being very little influenced by the dose administered.

Thus, no tendency for anemia was noted post-therapeutically, the mean results of the total number of red blood cells being in the normal ranges (5,5-8,5 T/l), with fluctuations between a minimum of 5,71x1 T/l and a maximum of 7,75 T/l (Fig. 1).

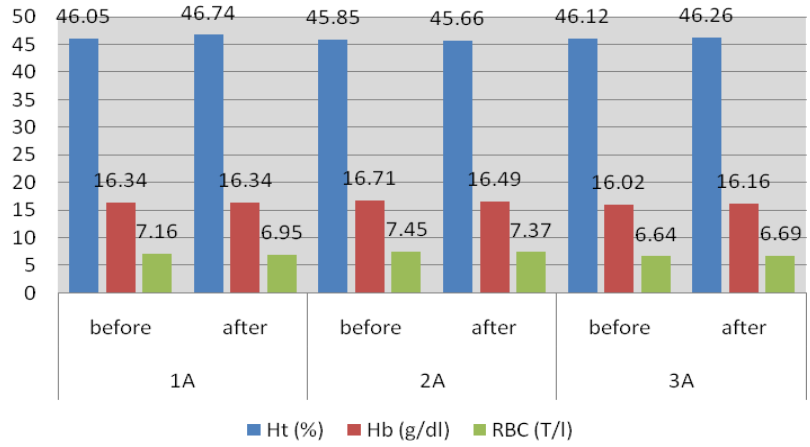

Fig. 1. The evolution of the mean red blood cell parameters, in dogs, before and after treatment (A1-therapuetic dosage, A2-double dosage, A3-control group)

A similar trend was also recorded for the red blood cells indices, which, compared to the normal ranges, showed non-significant fluctuations of the MCH (19.5-24.5 pg), MCV (60-77 fl) and MCHC (32-36 g/dl) (Fig. 2). The recorded variations revealed a high degree of erythrocyte homeostasis and thus of the main erythrocyte functions. The mean corpuscular hemoglobin (MCH) was characterized by small fluctuations, without being influenced by the number of administered doses of antifungal ointment, indicating a prevalence of large red blood cells.

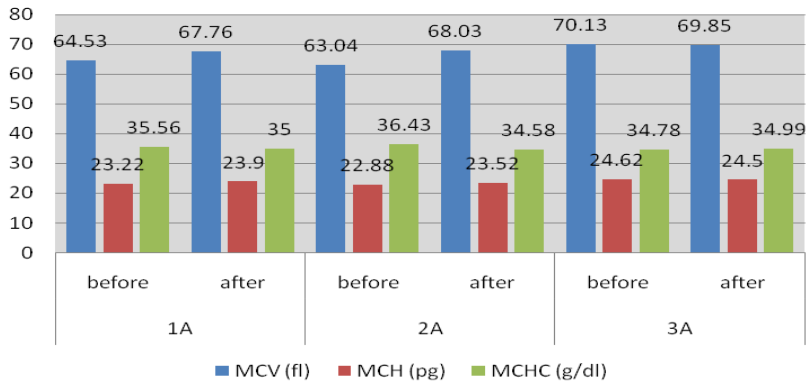

Fig. 2. The therapeutic dynamics of the mean red blood cell indices, before and after treatment in the dog groups (A1-curative dose, A2-double dose, A3-control group)

The dynamic of the leukocyte parameters revealed a good capacity of the body defense system. This leukocyte profile indicated further a high level of tolerance for the therapeutic and higher dose of the ointment, as well as the absence of adverse reactions over the leukopoiesis and the leukocyte functions in general, while also excluding possible sensitization adverse effects. In this matter, a special relevance was attributed to framing the variations of the main leukocyte parameters in the physiological ranges. The variations of the

total number of leukocytes can be reminded, ranging between 11.11-13.26 G/l reaching a maximum in the dog group treated with a double dosage (fig. 2). The evolution of the leukocytes population did not reveal important variations that exceeded the normal values (Fig. 3). Due to the fact that the results of the total number of leukocytes were in the physiological ranges of the species (6-17 G/L), inflammatory processes or sub-clinically infections were excluded.

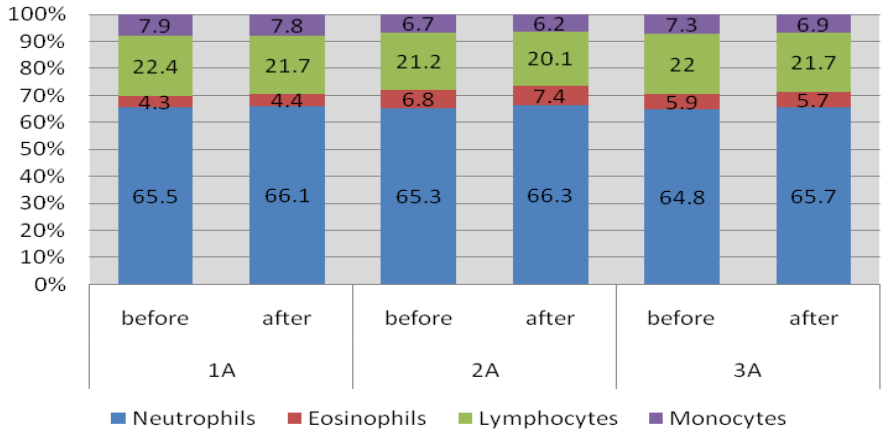

Fig. 3. The dynamics of the leukocyte population, before and after treatment, in the group of dogs A1-curative dose, A2-double dose, A3-control group)

The results obtained for the biochemical test, outline a protein, carbohydrate, enzymatic and mineral profile, that describe a good health status before and after treatment for the investigated animals, expressing also a high level of tolerance for the administered doses as well as the absence of adverse reactions expressed by metabolic dysfunctions. The protein profile of the tested dogs, was characterized by fluctuations of the serum protein concentration between 5,7 g/dl şi 6,4 g/dl, albumin, ranging between 3,2-3,4 g/dl and globulins, varying between 2,3-2,7 g/dl (fig. 4). At the evaluation of the enzyme profile normal variations were recorded for the aspartate aminotransferase, within the range 38,4 and 41,2 U/l, which corresponded to the normal evolution of the liver functions and of the creatine phosphokinase (Fig. 4). Regarding the evolution of the renal function, the recorded blood urea nitrogen values were in normal ranges (with variations between 16,4-19,6 mg/dl). In the general context of maintaining the health status of the animals, the evolution of some mineral components can be taken into account, such as: calcium (10,2-10,9 mg/dl), phosphorus (7,7-9,5 mg/dl) and magnesium (1,8-2,2 mmol/l).

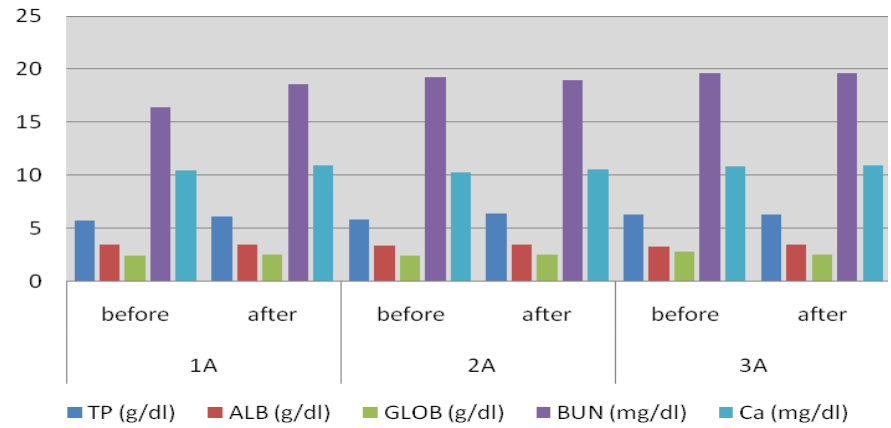

Fig. 4. The dynamics of the mean values of the metabolic profile, before and after treatment, in the group of dogs (A1-curative dose, A2-double dose, A3-control group)

The analysis of the results obtained in cats. Regarding the groups of cats used in the study, both for the control group and the two testing groups, the individual results and the mean values recorded before and after treatment, of the main hematological and biochemical parameters, were of no statistical importance, complying with the reference values for the species.

The evolution of the red blood cells parameters was characterized by mean post treatment levels for the packed cell volume and hemoglobin concentration, placed in physiological ranges (37-55%, respectively 12-18 g/dl) (Merck Veterinary Manual, 2012). The mean values obtained for the packed cell volume and hemoglobin concentration, presented individual fluctuations between 35.94%-38.47%, and 12.82 g/dl - 13.39 g/dl respectively, being only slightly influenced by the administered dose. Thus, no trend for anemia was observed after treatment, the mean values of the red blood cells being confined within the reference values (5-10 T/l), with variations ranging between 6.57 T/l and 6.84 T/l (Fig. 5).

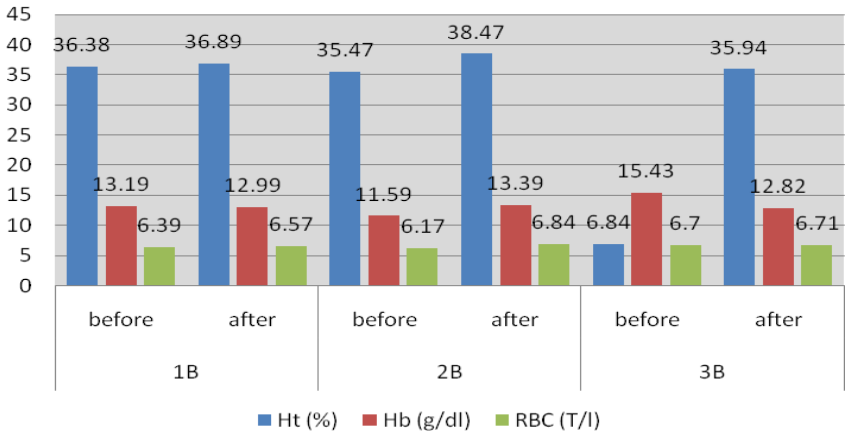

Fig. 5. The evolution of the mean red blood cell parameters, in cats, before and after treatment (A1-therapuetic dosage, A2-double dosage, A3-control group)

The mean red blood cell indices had a similar evolution, expressing minimal fluctuations compared to the physiological ranges of the MCH (61, 46 fl-63 fl), MCV (20, 64 pg-21,26 pg) and MCHC (32,28 g/dl-32,53 g/dl) (Fig. 5). The variations recorded, indicated normal erythrocyte functions. The MCV was characterized by minor variations during testing, not being influenced by the quantity of the applied ointment, showing a predominance of large erythrocytes.

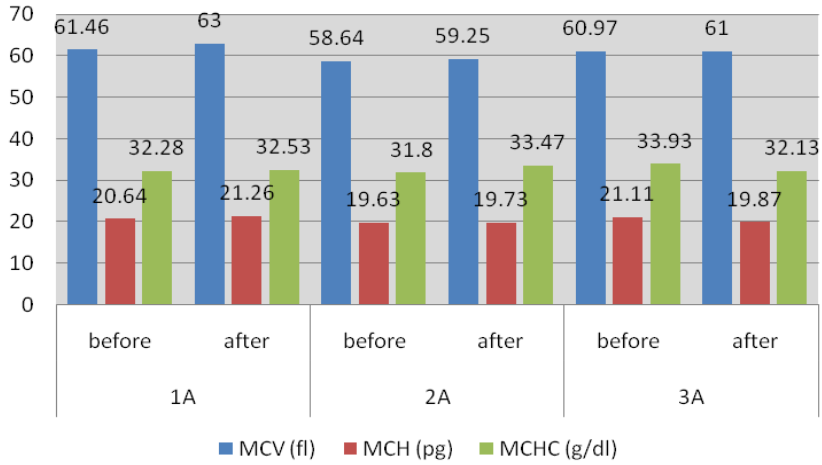

Fig.6 The therapeutic dynamics of the mean red blood cell indices, before and after treatment in the cats groups (A1-curative dose, A2-double dose, A3-control group)

The dynamics of the white blood cells parameters revealed the prevalence of a good defense mechanism. This profile also expressed a high level of tolerance regarding the doses used, as well as the absence of adverse effects on the leucopoiesis and the general leukocyte functions, while excluding the possible sensitization effects. In that regard, the results being in-between the normal range was of the outmost importance. Small fluctuations of the total number of leukocytes can be reminded, varying between 12.2-12.27 G/l, with higher values for the group treated with a double dose. The evolution of the leukocytes cell population did not reveal important variations which would pass the normal ranges (Fig.7). Due to the fact that the total number of white blood cells was within normal parameters (5.5-19.5 G/L), the possibility of existing inflammatory reactions, subclinical infections or other immune deficiency diseases can be excluded.

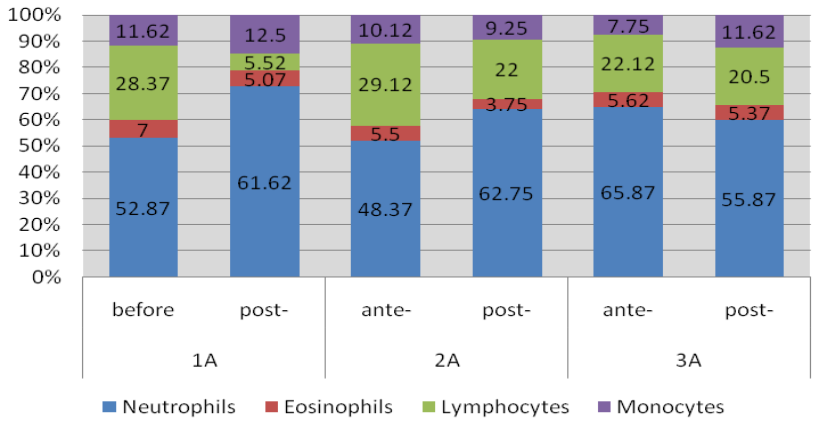

Fig.7 The dynamics of the leukocyte population, before and after treatment, in the group of cats (A1-curative dose, A2-double dose, A3-control group)

Regarding the biochemical tests, the recorded results outline a good metabolic profile, which indicate a proper health status, before and after treatment, for the examined animals. Likewise, it was noted an elevated level of tolerance for the dose administered for each group and the absence of adverse reactions characterized by metabolic dysfunctions. Thus, the metabolic profile of cats was characterized by small variations of the total protein concentration, between 6.22 g/dl and 6.57g/dl; 3,2-3,5 g/dl for the albumins and 2,54-2,83 g/dl for the globulin levels (Fig. 8). Regarding the evaluation of the renal function, the blood urea nitrogen was in the normal ranges (varying between 17,6-18 mg/dl). In the general context of animal health, some parameters regarding the ionic profile are important, some of these parameters being calcium (9.6-10.66 mg/dl), phosphorus (6.44-7.84 mg/dl) and magnesium (2.32-2.52 mmol/l).

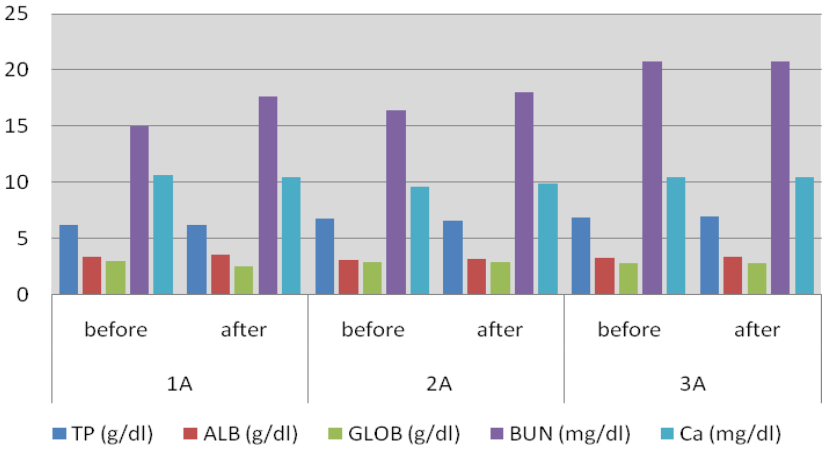

Fig. 8. The dynamics of the mean values of the metabolic profile, before and after treatment, in the group of cats (A1-curative dose, A2-double dose, A3-control group)

When detecting some possible systemic reactions, with or without endocrine involvement, a few clinical and laboratory results are relevant: maintaining the body temperature within normal ranges, keeping the general health status, the presence of a constant appetite, the distribution of the hematological and biochemical values within physiological limits, the lack of adverse symptoms evidenced clinically or as result from the observations of the owners.

After a thorough analysis of the results obtained in the clinical, hematological and biochemical tests, we consider that the administration of the antifungal ointment for dogs and cats, in the recommended dose or in double dose, did not provoke any adverse reactions

Conclusions

The experimental administration of the *Antifungal ointment for dogs and cats*, in therapeutic and double doses, on animals belonging to the targeted species was not followed by immediate (due to absorption) or delayed (due to product metabolism) adverse local or systemic reactions.

The results revealed large variations, with non-significant deviations from the physiological ranges, of the clinical, hematological and biochemical parameters, indicating a good local and general tolerance in the animals belonging to the targeted species, of the tested product. These results ensure a high level of safety in using this product for dermatological treatments.

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TOTAL URINARY BLADDER ABLATION IN DOG WITH URETHERO-COLIC IMPLANTATION. CASE STUDY.

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Abstract

Total ablation of the urinary bladder in dogs is an operation that has a high degree of difficulty and it is done in severe cases, acute blockage of the urinary transit, consecutive to urinary bladder neoplasia that cannot be removed surgically with the preservation of the uretheral opening into the bladder. In the Clinic of Obstetrics and Gynecology of the Faculty of Veterinary Medicine Bucharest, we have performed this surgery in a 12-year-old male dog, Teckel, named Moky. Using the electroscalpel, we dissected all the blood vessels, the ductus defferens and we have performed the circumferential excision of the bladder neck and proximal urethra with preservation of the neurovascular pedicles in order to remove the tumor involving the trigone and bladder neck that were causing urinary tract obstruction. Both urethers were dissected anterior to the kidneys and were placed into the colon using the Goodwin method, with the submucosal pattern. Before closing the abdomen and restoring all the anatomic layers, we had placed a drainage tube to collect all residual collection. The drainage tube was removed after 5 days. The defecation and the urination went form better to normal from day one to day 10 post surgery and the urination was conducted by the autonomy of the anal sphincter. We have used Ceftriaxone for the intravenous treatment, 25 mg/kg every 12 hours, for 3 days, then we continued with oral administration for another 7 days, raising the total daily dose to 35 mg/kg.

Key words: *canine, urinary, bladder, ablation, implantation.*

Total ablation of the urinary bladder in dogs is an operation that has a high degree of difficulty and it is done in severe cases, acute blockage of the urinary transit, consecutive to urinary bladder neoplasia that cannot be removed surgically with the preservation of the uretheral opening into the bladder.

A 12-year-old male dog, Teckel, named Moky was presented in the Clinic of Obstetrics and Gynecology of the Faculty of Veterinary Medicine Bucharest. Moky's owner first noticed blood in Moky's urine several days prior to his visit to the referring veterinarian, who prescribed a course of antibiotic therapy. Complete urinalysis results from that clinic revealed marked hematuria, without bacteriuria. Culture of a urine sample obtained via cystocentesis was negative for bacterical growth. Moky was referred to the Clinic of Obstetrics and Gynecology of the Faculty of Veterinary Medicine Bucharest for a second opinion and for an abdominal ultrasound check. Moky was current on all appropriate vaccinations and is on flea, tick, heartworm and intestinal parasite prophylaxis.

The differentials diagnoses at this time included urinary tract infection, pyelonephritis, urolithiasis, prostatitis and urinary tract neoplasia (primary or metastatic).

A biochemical panel was obtained to determine Moky's overall systemic health and to search for any concurrent disease process: Glucose 155 mg/dL, BUN 25 mg/dL, Creatinine 1.0 mg/dL, ALT 38 U/L, AST 44 U/L.

The abdominal ultrasound showed a large mass that occupied the entire urinary bladder with an important reaction at the urinary bladder's wall.

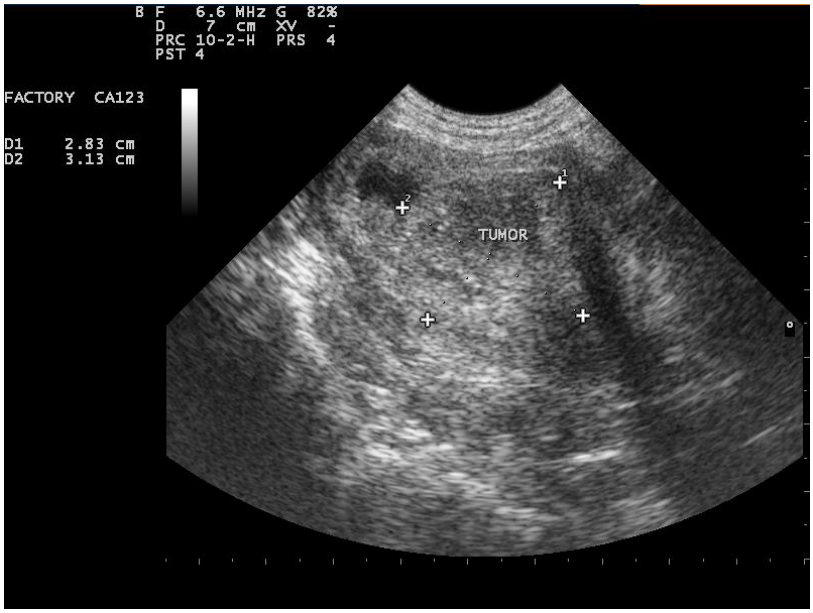

Image 1. Ultrasound aspect of the urinary neoplasia (orig.)

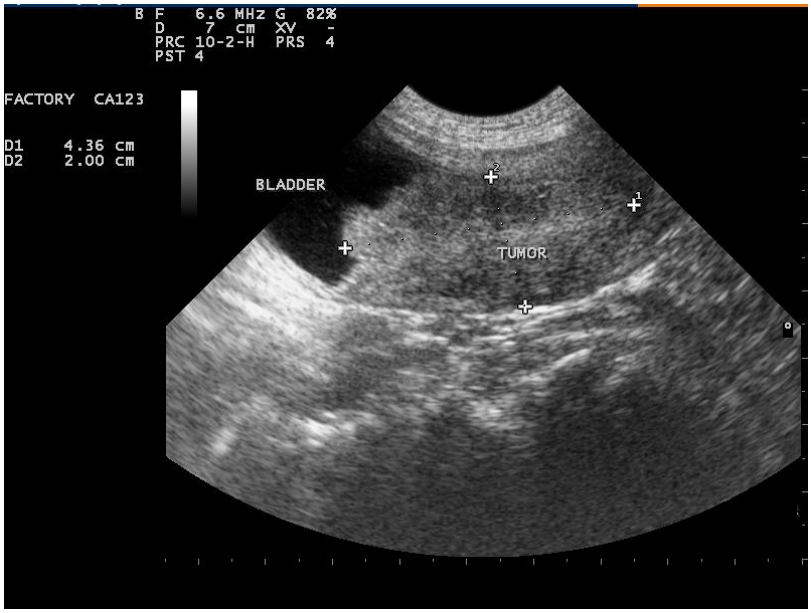

Image 2. Ultrasound aspect of the urinary neoplasia (orig.)

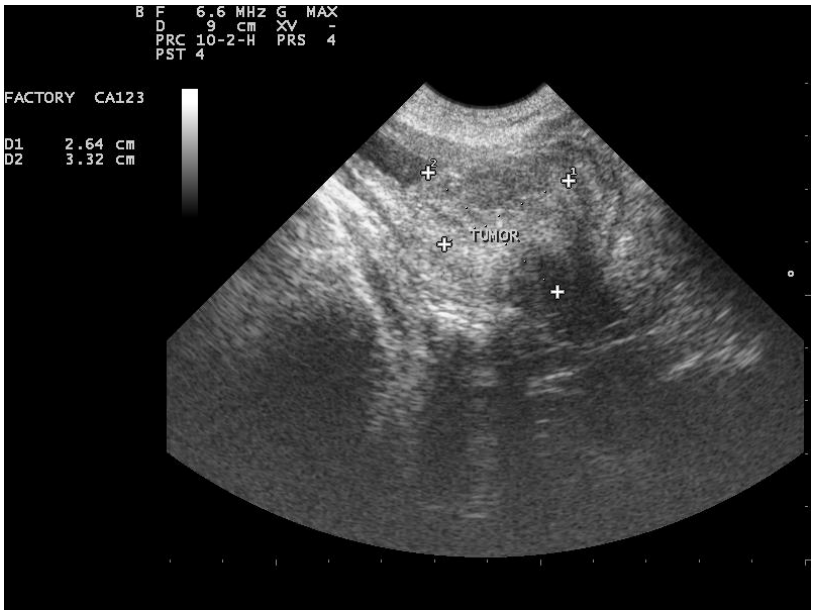

Image 3. Ultrasound aspect of the urinary neoplasia (orig.)

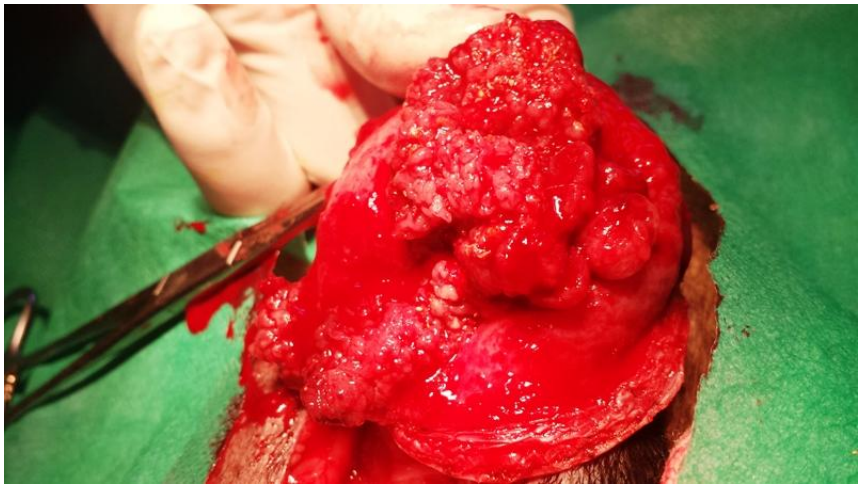

Image 4. Macroscopic, surgical aspect of the urinary neoplasia (orig.)

We have decided to perform a urine cytological exam. Urine was collected by catheterization of the urethra and the results showed a large number of big tumor cells. The pathologist suspected carcinoma.

Surgery of the urinary bladder was elected as the only option. After classic laparotomy, identification and isolation of the urinary bladder, using the electroscalpel, we dissected all the blood vessels and we have performed the circumferential excision of the bladder neck and proximal urethra with preservation of the neurovascular pedicles in order to remove the tumor involving the trigone and bladder neck that were causing urinary tract obstruction.

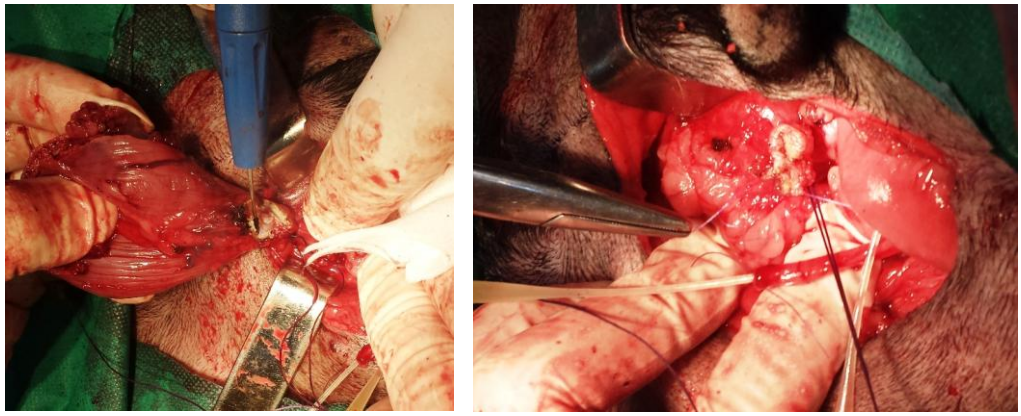

Image 5. Circumferential excision of the bladder neck and proximal urethra with preservation of the neurovascular pedicles (orig.)

Both urethers were dissected from the bladder's wall and isolated anterior to the kidneys. They were placed into the colon using the Goodwin method, with the submucosal pattern.

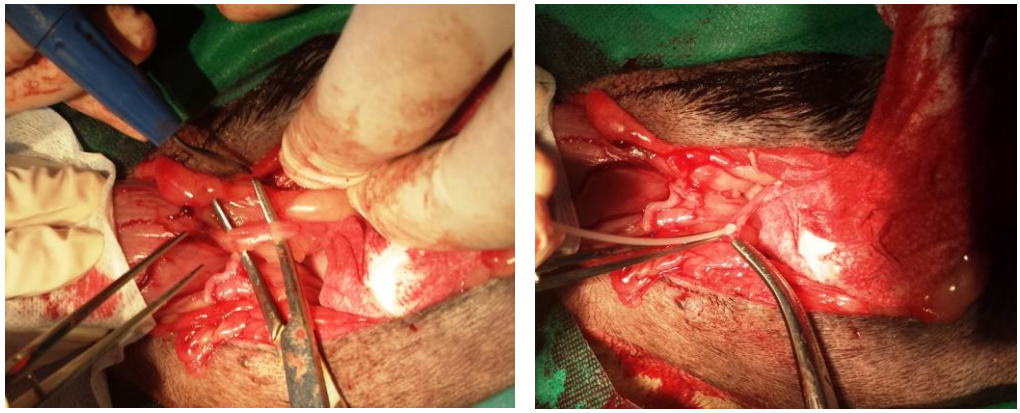

Image 6. Left. Identification of the urethers.
Right. Catheterization of the urethers (orig.)

Each urether was catheterized using a 3 Fr catheter from the opening to the renal pelvis and the opening was sutured with 4/0 PDS.

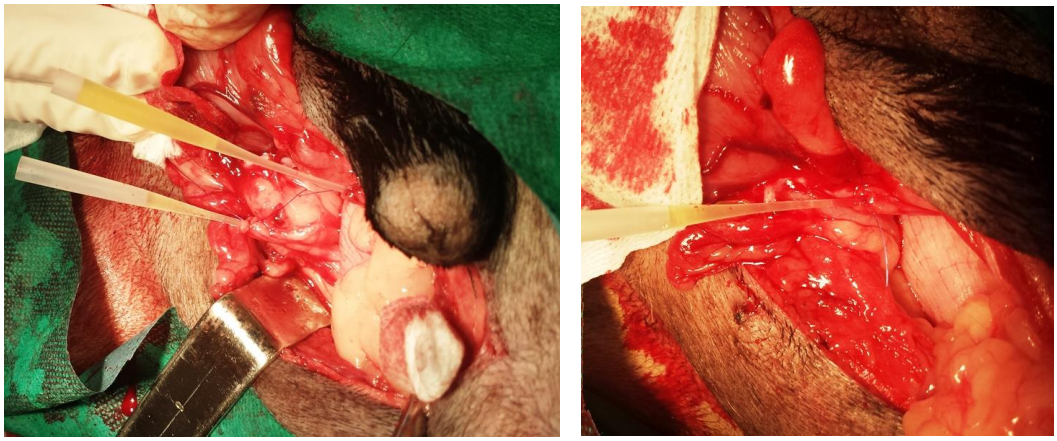

Image 7. 4/0 PDS ligature of the catheter in the urethers (orig.)

A two cm longitudinal incision was performed using the scalpel on the ventral wall of the descendent colon, interesting all the layers.

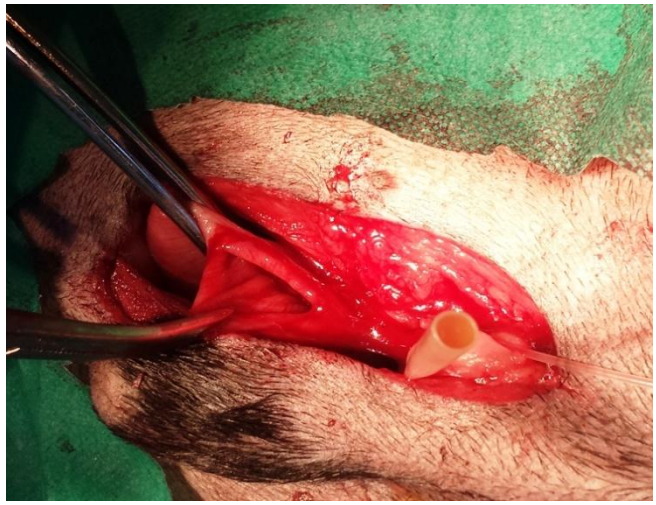

Image 8. Two cm longitudinal incision of the colon ventral wall (orig.)

Being able to visualize the colon mucosa, we performed two stab incisions near the longitudinal opening, interesting the serous and going into the submucosa, oriented posterior. The urethers were then introduced into the two stab incisions and their openings were splinted and fixed at the colic mucosa using two single appositional 4/0 PDS sutures.

At the exterior of the colon, using 4/0 PDS we performed a sero-serous suture for each urether.

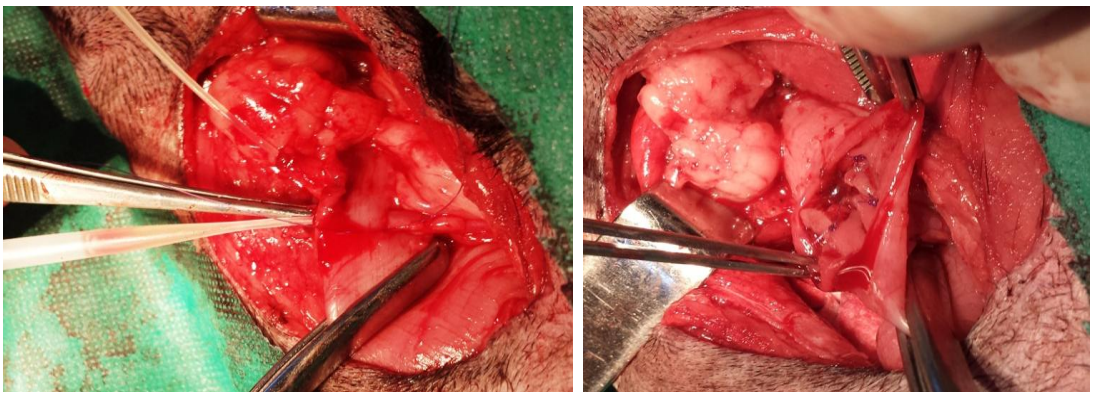

Image 9. Left. Sero-serous suture for each urether.
Right. Urether fixation at the colic mucosa (orig.)

The opening of the colon was closed using 4/0 PDS simple continuous appositional suture in two layers.

Before closing the abdomen and restoring all the anatomic layers, we had placed a drainage tube to collect all residual collection. The drainage tube was removed after 5 days.

The defecation and the urination went form better to normal from day one to day 10 post surgery and the urination was conducted by the autonomy of the anal sphincter.

We have used Ceftriaxone for the intravenous treatment, 25 mg/kg every 12 hours, for 3 days, then we continued with oral administration for another 7 days, raising the total daily dose to 35 mg/kg.

We performed a ultrasound check 30 days after surgery and the renal pelvis presented normal aspect in both kidneys.

Conclusion

In dogs with invasive bladder tumors causing life-threatening urinary tract obstruction, resection of the bladder and proximal urethra with urethero-colic implantation should be considered as a promising surgical alternative to urinary diversion.

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ENDOPARASITE PREVALENCE IN CAPTIVE BIRDS OF PREY

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Abstract
The study was conducted from January 2013 to March 2014 on 87 birds of prey (18 species) aged between 2 months and 20 years from two stockfarms in Timis county (Romania) and one stockfarm in Waldreishs (Austria).We did the study into several aspects of birds of prey: identification of species, conditions of keeping and looking after them, feeding habits, possible sources of infestation, faecal collection and examination. Following the faecal examination by Willis, Mc.Master and polivalent methods, we have obtained a prevalence of 8% parasites such as: Caryospora sp. oocysts, ascaria (Porrocaecum sp.) and capilaria (Capillaria sp.) eggs.
Keywords*: birds of prey, endoparasites, prevalence.*

The avian parasites were found in birds of prey long time ago and consequently, studies were carried out regarding the way of infestation distribution, clinical signs and treatment procedures (5, 7, 8).

Birds of prey around the world were diagnosed with protozoa, helminthes and blood parasites. Unfortunately, there is little research on parasites in birds of prey in Romania. For this reason, the purpose of this study is to identify the species of birds of prey, their habitat conditions, feeding behavior, possible sources of infestation, and to determine the endoparasite prevalence by coprological examination of cage birds of prey from Austria and Romania.

Materials and methods

The study was conducted for 1 year on 87 birds of prey aged between 2 months and 20 years from 2 stockfarms in Romania and one stockfarm on Austria: groups 1, 2 and 3 (tables 1, 2, 3).

This study deals with:

- Identification of birds of prey species
- Feeding behavior
- Possible sources of infestation
- Examination of faeces samples

The faeces were examined in the Parasitology Clinic of the Faculty of Veterinary Parasitology using the following methods:

- Willis
- Mc. Master
- Polivalent

Table 1. Group 1 – Stockfarm from Lugoj (Romania)

Species	**Number**	**Positive**	**Parasite**
Saker Falcon(Falco cherrug)	2	1	Protozoa(*Caryospora sp*.)
Harris Hawk (*Parabuteo unincitus*)	3	1	Ascarides (*Porrocaecum sp.)*
Common Buzzard (*Buteo buteo*)	2	1	Capillaria (*Capillaria sp*.)
Nothern Goshawk (*Accipiter gentillis*)	5	-	-
Euarasian Sparrowhawk(*Accipiter nissus*)	1	1	Protozoa (*Caryospora sp*.)
Common Kestrel (*Falco tinnunculus*)	2	-	-
Eurasian Eagle Owl (*Bubo bubo*)	2	-	-

Table 2. Group 2 – Stockfarm from Timisoara (Romania)

Species	**Number**	**Positive**	**Parasite**
Nothern Goshawk (*Accipiter gentillis*)	3	1	Ascarides(*Porrocaecum sp)*
Euarasian Sparrowhawk (*Accipiter nissus*)	1	-	-
Hen Harrier (*Circus cyaneus*)	1	-	-
Red-footed Falcon (*Falco vespertinus*)	1	-	-

Table 3. Group 3 – Stockfarm from Waldreichs (Austria)

Species	**Number**	**Positive**	**Parasite**
Saker Falcon (*Falco cherrug*)	16	1	Protozoa (*Caryospora sp*.)
Harris Hawk (*Parabuteo unincitus*)	3	-	-
Golden Eagle (*Aquila chrisaetos*)	15	-	-
Nothern Goshawk (*Accipiter gentillis*)	3	1	Ascarides(*Porrocaecum sp)*
Common Kestrel (*Falco tinnunculus*)	1	-	-
Eurasian Eagle Owl (*Bubo bubo*)	2	-	-
Cinereous Vulture (*Aegypius monachus*)	2	-	-
White-tailed Eagle (Halietus albicilla)	2	-	-
Eastern Imperial Eagle (*Aquila heliaca*)	2	-	-
Red Kite (*Milvus milvus*)	3	-	-
Peregrine Falcon (*Falco peregrinus*)	5	-	-
Gyrfalcon (*Falco rusticolus*)	1	-	-
Ferruginous Hawk (*Buteo regalis*)	2	-	-

Results and discussions

Following the study on 87 birds from three stockfarms in Romania and Austria we obtained the following results:

- The number of birds of prey species was 18 (fig. 1, 2, 3, 4)
- The birds were raised in lofts or in the garden on supports
- The endoparasite incidence was 8%

In group1 the endoparasite incidence was 23%. We identified *Caryospora* oocytes in Saker falcons (*Falco Cherrug*) and in Eurasian Sparrowhawks, roundworm eggs in Harris Hawks and *Cappilaria* eggs in the Common buzzard (*Buteo buteo*).

Only one bird of prey, that is Northern Goshawk, was found infested with roundworm eggs in group 2 following the coprological examination.

Group 3 was 1.2% infested. We identified roundworm eggs in one Northern Goshawk (1/64) and *Caryospora* oocytes in one Saker falcon (1/64). The protozoa oocytes (*Caryospora*), the roundworm eggs (*Protocaecum sp.*) and the C*apillaria* eggs were identified by Willis method (fig. 5, 6, 7).

We did not identify any trematode eggs by the polivalent method and therefore the endoparasite incidence was 0.

When using Mc. Master method we identified:

- Average infestation with *Caryospora* oocytes (n=200 eggs / faeces) in one Saker Falcon from Waldreicks stockfarm;
- Huge infestation (n=3200 eggs/gram of faeces) was recorded in one Saker Falcon from Lugoj stockfarm.

The habitat conditions (raising in spacious lofts or in the garden on supports), the feeding on chicks, quail, pigeons, hens, guinea pigs (defrosted or fresh, eviscerated), the training and the hunting seasons under control reduced the risks of parasite infestation of the captive birds of prey in the three stockfarms under study.When comparing our results with those reported by foreign researchers, it is obvious that birds of prey are frequently infested with *Caryospora*, especially in Falconiformes which causes enteric diseases (4, 6, 7). Although this parasite infests mainly the young, it was found in adult birds, too (1, 3, 5).

In our study the birds of prey diagnosed with *Caryospora* were 3 years old (Lugoj stockfarm) and 2 months, respectively (Waldreicks stockfarm).

The parasitism with roundworm and *Capillaria* had a high incidence in hawks and singing birds (2, 3, 5).

Following the coprological examination we have also found the parasitism with roundworm and *Capillaria* in the Northern Goshawk, Harris Hawk and the Common Buzzard.

Fig. 1. *Accipiter gentillis* - female

Fig. 2. *Falco cherrug*

Fig. 3. *Parabuteo unincitus*

Fig. 4. *Buteo buteo*

Fig. 5. Roundworm eggs (*Porrocaecum sp.)*

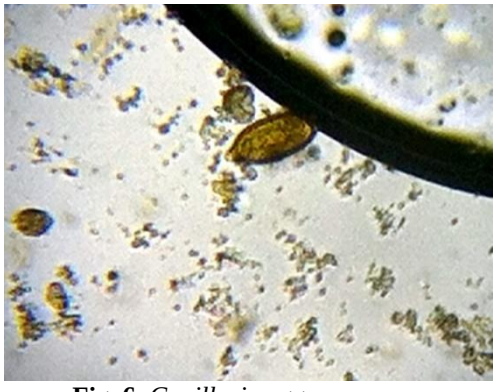

Fig. 6. *Capillaria* eggs

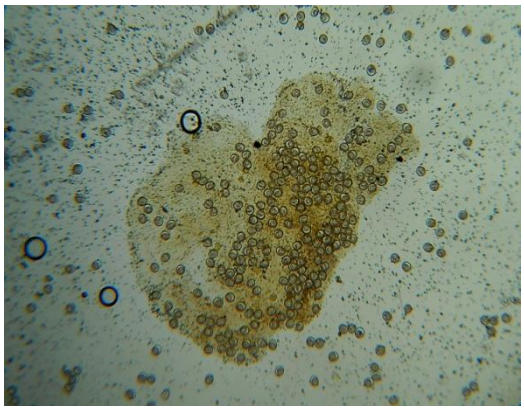

Fig. 7. *Protozoa* oocystes (*Caryospora sp*.)

Concluzii

The study conducted on 87 birds of prey from 2 stockfarms in Romania and one from Austria revealed an endoparasite incidence of 8% (8/87).

The parasites identified coprologically were: *Caryospora sp*., oocysts, roundworm eggs (*Porrocaecum sp.*) and capillaria eggs (*Capillaria sp*.).

Out of 18 species of birds of prey aged between 2 months and 20 years, we diagnosed endoparasites coprologically in the Saker Falcon (protozoa), Northern Goshawk (roundworms), Eurasian Sparrowhawk (protozoa), Harris Hawk (roundworms) and Common Buzzard (capillaria).

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CURCUMIN ROLE DURING HEPATIC TOXICITY IN WISTAR RATS: PRELIMINARY STUDY

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Abstract

In this study we examined the effect of curcumin on paracetamol overdose induced liver toxicity in rats. Forty male Wistar rats were allocated into 4 groups. The first group served as control and received corn oil; second group received curcumin (400 mg/kg BW daily) dissolved in corn oil; third group received a single dose of paracetamol (500 mg/kg BW); fourth group was served as protective group and received curcumin plus paracetamol. Animals received the respective administrations orally for 7 successive days for first, second and third group, while paracetamol was administered on the 6th day for third and fourth group. Animals were slaughtered and blood and liver tissues were collected for various biochemical and gene expression. Serum analysis revealed an alteration in GPT, GOT, Urea, albumin and lipid profiles that incluse LDL, TG, cholesterol and HDL. Moreover, a decrease in antioxidant activity of liver was reported and was normalized in curcumin protective group. Moreover, curcumin improved atherogenic index and Cholesterol ration that are increased during hepatic toxicity. Curcumin increased super oxide dismutase (SOD) and glutathione peroxidase expression while paracetamol decreased them. Co-administration of curcumin with paracetamol normalized SOD and GPx expression compared to paracetamol administered rats. Molecular gene expression revealed that paracetamol administered rats showed decrease in the expression of acute phase proteins that are represented by alpha-2 macroglobulin (α-2M). Curcumin administration ameliorated the alteration in the expression of α-2M. It can be concluded that curcumin has a protective effect on paracetamol induced hepatic toxicity in rats.

Keywords: *Curcumin; Liver toxicity; Paracetamol; Antioxidants; gene expression; acute phase proteins; Wistar rats.*

Introduction

Curcumin (Turmeric) is widely used in therapeutic preparations against variable diseases (Chattopadhyay et al., 2004). Moreover it is used as food spice, additive, flavoring, preservative and as coloring agent in foods and textiles (Basnet and Skalko-Basnet, 2011). Curcumin has several activities among which is the antioxidant (Al-Jassabi et al., 2012), antimicrobial (Tajbakhsh et al., 2008), anti-inflammatory (Bereswill et al., 2010), antiviral (Kutluay et al., 2008), anti-carcinogenic activity (Das and Vinayak, 2012) and anti-diabetic activity (Aziz et al., 2013). Recently, it has been shown that curcumin and its constituents have hepatoprotective properties (Jobin et al., 1999; Somanawat et al., 2013). Curcumin has a protective effect against liver damage in animals induced by a variety of hepatotoxic substances such as carbon tetrachloride (Morsy et al., 2012). Moreover, curcumin has silymarin like actions (Girish et al., 2009). It has been shown that curcumin has antiapoptotic activity *in vitr*o and *in viv*o through its action on hepatic injury (Li et al., 2013).

Liver functions include removal and inactivation of toxic substance and drugs to be excreted in urine. Hepatic toxicity is attributed primarily to the changes in oxidative stress and alteration in acute phase proteins (Flora et al., 2013). The imbalance between production of free radicals and reactive metabolites is named oxidative stress that alleviated by antioxidant systems (Evans et al., 2002). Oxidative stress leads to the damage of important biomolecules and organs (Durackova, 2010). This damage, is probably associated with DNA,

protein, and lipid damage, and is the cause for liver diseases such as chronic liver injury, hepatic inflammation, fibrosis and hepatocellular carcinoma (Tanikawa and Torimura, 2006; Vera-Ramirez et al., 2013).

Paracetamol (P); acetaminophen or N-acetyl-p-aminophenol (APAP); is a widely analgesic medication in many countries. An overdose of paracetamol is the reason for liver and renal toxicity and probably death (Zhao et al., 1998). The exact mechanism of such toxicity is not clear but most studies focused on paracetamol effect on antioxidants levels in blood and tissue activity (Li et al., 2013) together with liver and kidney function (Somanawat et al., 2013). High dose of paracetamol causes glutathione depletion, apoptosis and cell death (Sun et al., 2002).

The aim of the current study is to examine the protective effect of curcumin against hepatic toxicity induced by paracetamol in Wistar rats based on biochemical and molecular study. The expression of antioxidant genes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and acute phase protein □-2 macroglobulin,□□-2M) were examined using semi-quantitative RT-PCR analysis.

Materials and methods

Reagents and Kits

Acetaminophen, ethidium bromide and agarose were purchased from Sigma-Aldrich Co., MO, USA. The Wistar albino rats were purchased from King Fahd center for Scientific Research, King Abdel-Aziz University, Jeddah, Saudi Arabia. Serologic kits for GPT, GOT, albumin and urea were purchased from Bio-diagnostic Co., Dokki, Giza, Egypt. The deoxyribonucleic acid (DNA) ladder was purchased from MBI, Fermentas, USA. Qiazol for RNA extraction was purchased from QIAGEN Inc., Valencia, CA.

Animals and Experimental Design

All animal procedures were approved by the Ethical Committee Office of the dean of scientific affairs of Taif University, Saudi Arabia. Forty male Sprague Dawley rats, weighing 200–280 g were used for this study. The animals were kept a 12-h light-dark cycle and gained access to food and water ad libitum. Rats were randomly divided into 4 groups (10 rats per group and 5 rats per cage) as follows:

Group1, Control (C) served as negative control. **Group2**, curcumin (CUR) received curcumin dissolved in corn oil in a dose of 400 mg/kg bw daily for 7 days. **Group 3**, Paracetamol received single intra-gastric dose of paracetamol (500 mg /kg B W), 24 h before sampling. **Group 4**, protective group received curcumin dissolved in corn oil (400 mg/kg BW) daily for 7 days and on the 6^{th} day after curcumin administration, paracetamol (500 mg /kg BW intra-gastric) was administered. The dose of paracetamol used in this study, was determined based on study of Li et al. (2013).

Sampling

Twenty four hours after administration of tested chemicals, all animals were anesthetized; blood and tissues were collected after rats sacrificing by diethylether inhalation. Serum was extracted after blood centrifugation for 10 min at 4000 *xg*. For gene expression, liver tissues were kept in TriZol reagent for RNA extraction and in 10% neutral formalin for histopathological and immunohistochemistry.

Determination of liver antioxidant activity

For Catalase activity, one gram of liver tissues was homogenized in 5ml of cold buffer of 50mM potassium phosphate buffer (PBS, pH 7.4), containing 1mM EDTA and 1mL/l Triton X-100. After centrifugation at 4000 *xg* for15 minutes at 4ºC, the supernatant was removed and stored frozen at -80ºC until the time of analysis of catalase (U/g tissue). For malondialdehyde (MDA) measurements, one gram of liver tissues was homogenized in 5ml of cold buffer of 50mM potassium phosphate buffer (pH 7.4). After centrifugation at 4000 *xg* for15 minutes at 4ºC, the supernatant was removed and stored frozen at -80ºC until the time of analysis of MDA (nmol/g tissue) The activities of catalase and MDA were determined by ELISA reader (Absorbance Microplate Reader ELx 800TM BioTek®, USA). Results were calculated according to the manufacture instructions.

Gene expression analysis

RNA Extraction

Total RNA was extracted from liver tissue samples (approximately 100 mg per sample) of experimental rats. Liver samples were flash frozen in liquid nitrogen and subsequently stored at -70°C in 1 ml Qiazol (QIAGEN Inc., Valencia, CA). Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then, 0.3 ml chloroform was added to the homogenate. The mixtures were shaken for 30 seconds followed by centrifugation at 4°C and 16,400 *x g* for 15 min. The supernatant was transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shacked for 15 seconds and centrifuged at 4°C and 16,400 *x g* for 15 min. The RNA pellets were washed with 70% ethanol, briefly dries up, and then dissolved in Diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically at 260 nm. The RNA integrity was confirmed in 1.5% agarose stained with ethedium bromide. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.

cDNA Synthesis and Semi-quantitative PCR Analysis

For cDNA synthesis, mixture of 3 µg total RNA and 0.5 ng oligo dT primer (Qiagen Valencia,CA, USA) in a total volume of 11 µl sterilized DEPC water was incubated in the Bio-Rad T100^{TM} Thermal cycle at 65°C for 10 min for denaturation. Then, 2 µl of 10X RT-buffer, 2 µl of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (SibEnzyme Ltd. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 µl by DEPC water. The mixture was then re-incubated in the thermal Cycler at 37°C for one hour, then at 90°C for 10 min to inactivate the enzyme. For semi-quantitative RT-PCR analysis, specific primers for examined genes (table 1) were designed using Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, GAsa-dong, Geumcheon-gu. Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl of 10 pM of each primer (forward and reverse), and 12.5 µl PCR master mix (Promega Corporation, Madison, WI), the volume was brought up to 25 µl using sterilized, deionized water. PCR was carried out using Bio-Rad T100^{TM} Thermal Cycle machine with the cycle sequence at 94 °C for 5 minutes one cycle, followed by variable cycles (stated in table 1) each of which consists of denaturation at 94 °C for one minute, annealing at the specific temperature corresponding to each primer (table 1) and extension at 72 °C for one minute with additional final extension at 72 °C for 7 minutes. As a reference,

expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined (table 1). PCR products were electrophorized on 1.5% agarose (Bio Basic INC. Konrad Cres, Markham Ontario) gel stained with ethidium bromide in TBE (Tris-Borate-EDTA) buffer. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using Image J software version 1.47 (http://imagej.en.softonic.com/).

Table 1. PCR conditions for rat antioxidants, cytokines and acute phase proteins genes

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	PCR cycles and conditions
GPx (406 bp)	AAGGTGCTGCTCATTGAGAATG	CGTCTGGACCTACCAGGAACTT	40 cycles 57°C 1 min
SOD (410 bp)	AGGATTAACTGAAGGCGAGCAT	TCTACAGTTAGCAGGCCAGCAG	35 cycles, 55°C 1 min
α2 –macroglobulin (325 bp)	GCTCCTGTCTGTTTCCTTAGTT	ATTGGCCTTTCGTGGTTTAG	30 cycles, 56°C 1 min
GAPDH (309 bp)	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA	25 cycles, 52 °C 1 min

Statistical Analysis

Results were shown as means ± standard error of means (SEM). Data were analyzed using analysis of variance (ANOVA) and post-hoc descriptive tests by SPSS software version 11.5 for Windows with $p<0.05$ regarded as statistically significant. Regression analysis was performed using the same software.

Results

Serum renal and hepatic biochemical measurements

Renal and hepatic changes after paracetamol administration were tested. Paracetamol overdose increased serum levels of urea, albumin, GOT and GPT (table 2). Administration of curcumin together with paracetamol inhibited the increase in serum parameters of kidney and liver (table 2). Serum GOT values in paracetamol group were 156±9.4 *Vs.* 83 ± 2 for control group, while GPT values were 136±27.9 for paracetamol group *Vs.* 58.3 ± 6 for control group.

Table 2. Protective effect of curcumin on paracetamol induced changes in serum levels of renal and hepatic parameters and hepatic antioxidant activity in Wistar rats.

	Control	Curcumin	Paracetamol	Curcumin + Paracetamol
Urea (mg/dl)	35.6±3.8	35.3±2.3	42.0 ±1.5*	31.0 ±1.0$^{\#}$
Albumin (g/dl)	3.2±0.1	3.1±0.1	4.8±0.4*	3.5±0.1$^{\#}$
GOT (U/L)	83 ± 2	72.7± 9.6	156±9.4*	90.3±3.7$^{\#}$
GPT(U/L)	58.3± 6	64.7±6.01	136±27.9*	98±4.9$^{\#}$
MDA (nmol/g tissue)	9.78±1.9	10.7±1.0	18.2±0.5*	12.6±0.4$^{\#}$
Catalase (U/g tissue)	33±6.1	37±1.1*	21±0.4*	33.9±2.6$^{\#}$

*Values are means ± standard error (SE) for 5 different rats. Values are statistically significant at *p<0.05 Vs. control and #p<0.05 Vs. paracetamol group.*

Serum lipids measurements

As shown in table 3, paracetamol induced significant increase in cholesterol, TG, LDL and a decrease in HDL levels. Curcumin co-Administration induced normalization in all parameters measured. Moreover. Curcumin coadministration increase HDL levels and normalized the changes induced in atherogenic index and cholesterol ratio.

Table (3). Protective effect of curcumin on paracetamol induced serum changes of lipid profiles in Wistar rats.

	Control	Curcumin	Paracetamol	Curcumin + Paracetamol
Cholesterol	96.3±5.8	97.7±6.1	137.7 ± 8.1*	103.7 ± 3.7#
Triglycerides	55.7±7.9	72±4.2	100.3 ± 1.5*	86.3 ± 6.7#
LDL	66.3±4.1	62.2±7	107 ± 7.8*	65.7 ± 2.2#
VLDL	11.1±1.6	14.5±0.8	18.1 ± 0.2*	19 ± 1
HDL	19.1±0.5	22.1±0.5	12.7 ± 0.6*	19.7 ± 0.5
Cholesterol ratio	5±0.2	4.4±0.2	10.9 ± 0.3*	5.3 ± 0.1
Atherogenic Index	0.5±0.1	0.5±0.0	0.8 ± 0.0*	0.6 ± 0.0

*Values are means ± standard error (SE) for 5 different rats. Values are statistically significant at *p<0.05 Vs. control and #p<0.05 Vs. paracetamol group.*

Hepatic antioxidant activity

The results for the protective effect of curcumin on MDA as oxidative stress marker, and catalase as antioxidant enzyme are illustrated in table 2. The current results revealed that, MDA increased significantly (P<0.05) in paracetamol administered rats compared to control (18.2±0.5 for paracetamol group *Vs.* 9.78 ±1.9 for control). Administration of curcumin to paracetamol group normalized MDA activity (12.6±0.4 for paracetamol plus curcumin group *Vs.* 9.78 ±1.9 for control group). In paracetamol group, the activity of catalase enzyme was decreased significantly compared to control rats (P<0.05) (21±0.4 for paracetamol *Vs.* 33± 6.1 for control). There is a significant increase in catalase activity (U/g/protein) in curcumin administered group (37±1.1). Administration of curcumin plus paracetamol inhibited significantly the decrease in catalase activity and returned to normal levels (33.9±2.6 for curcumin plus paracetamol *Vs.* 33± 6.1 for control).

Molecular findings

Semi-quantitative RT-PCR analysis of hepatic antioxidant enzymes

RT-PCR analysis for antioxidants expression is illustrated in figure 1. Parallel to tissue catalase antioxidant activity (table 2), mRNA expressions of SOD, and GPx were significantly decreased in paracetamol administered rats and increased in curcumin administered rats (Fig. 2). Curcumin administration plus paracetamol reversed the decrease in antioxidants expression reported in paracetamol administered rats.

Semi-quantitative RT-PCR analysis of hepatic alpa-2 macroglobulin expression

To explore the possible involvement of acute phase proteins in curcumin protective effect, we examined the expression of α-2M in liver of treated groups. The expression of α-2M was down-regulated in paracetamol group and returned to control expression in curcumin plus paracetamol administered rats (Fig. 3).

Discussion

This study demonstrated that paracetamol overdose induced hepatotocity in rats and curcumin administration attenuated hepatic toxicity through re-impairment of antioxidants capacity of hepatic cells together with a decrease in the expression of some cytokines that initiate the inflammatory cascade in the body. Previous observations studied in mice and rats (Yousef et al., 2010) focused on the biochemical alterations in levels of liver and kidney parameters. Here, we focused on the molecular regulation of hepatic toxicity and possible attenuation by curcumin. During inflammatory conditions like hepatitis, curcumin shows beneficial effects through its antioxidants activity (Samuhasaneeto et al., 2009).

Cellular antioxidant defenses are classified into primary antioxidant enzymes which include SOD, catalase and GPx, and secondary non-enzymatic antioxidants such as ubiquinol, vitamin E, vitamin C and β carotene. SOD converts superoxide radical to hydrogen peroxide, catalase enzyme catalyzes the decomposition of hydrogen peroxide to water (H2O) and oxygen molecule (O2) where as GPx catalyzes the reduction of hydrogen peroxide and organic hydroperoxide into water and corresponding alcohol at the expense of glutathione (Evans et al., 2002).

Lipid peroxidation represented by MDA as an oxidative stress marker and reduced GST, SOD and catalase as indicators of antioxidant potency for cells were used to assess the degree of hepatic cell stability and integrity. Our results showed that oxidative damage was caused by overdose of paracetamol significantly attenuated by curcumin administration. Therefore, we can postulate that curcumin could protect against free radical mediated oxidative stress by scavenging for free radicals that limits lipid peroxidation involved in membrane damage through attenuation of antioxidants depletion (Del Rio et al., 2005). The protective effect of curcumin can be explained by induction of gene expression for SOD, GPx, and catalase. Curcumin is known to protect cell membrane against oxidative damage. Most of the antioxidants have either a phenolic functional group or a β-diketone group. Curcumin is a super H-atom donor by donating the H-atom from the central methylenic group rather than from the phenolic group in acidic and neutral aqueous and acetonitrile solutions (Priyadarsini et al., 2003). It has been suggested that curcumin was not able to prevent MDA production (Reyes-Gordillo et al., 2007). The most likely explanation is that about 75% of oral consumed curcumin is excreted in the feces and only traces appeared in the urine, suggesting poor absorption of curcumin. However, it is confirmed that curcumin is bio-transformed to dihydrocurcumin, tetrahidrocurcumin, and hexahidrocurcumin; subsequently, these products are converted to glucuronide conjugates, which are more polar and have better absorption than curcumin. Therefore, the pharmacological actions of curcumin are mostly due to its hydrosoluble derivatives (Maheswari et al., 2006).

Alterations in serum levels of hepatic transaminases (GOT and GPT) are used as markers and their increase indicates liver damage. In our study, there is a significant increase in GPT and GOT levels in paracetamol overdose administered rats. Such increase was reduced by the administration of curcumin coinciding with the changes in urea and albumin

levels as their increase was decreased by curcumin administration confirming curcumin role in protecting liver and kidney from paracetamol toxicity.

Acute-phase proteins are proteins that increase in response to inflammation. The variability in protein plasma levels, and following impact on drug binding extent, cause modifications in the mode of drug action, distribution, disposition and elimination. One of the most important acute phase proteins is alpha-2 macroglobulin. Our findings confirmed that the expression of α-2M expression was increased after curcumin administration and decreased in paracetamol overdose administered rats. Co-administration of curcumin with paracetamol normalized α-2M expression. α-2M is a large plasma protein found in the blood. It is produced mainly by the liver and locally by macrophages, fibroblasts, and adrenocortical cells. α-2M acts as an antiprotease and is able to inactivate an enormous variety of proteinases. α-2M functions as an inhibitor of fibrinolysis by inhibiting plasmin and acts as a carrier protein because it binds to numerous growth factors and cytokines such as IL-1β (Lyoumi et al., 1998). It has been shown that α-2M secretion is decreased during acute liver inflammation induced by turpentine oil and the possible cause for the reduction in α-2M expression is presumed to be hepatocyte dysfunction irrespective of cytokines profiles (Kuribayashi et al., 2012).

In summary, the data from biochemical and molecular findings reveal that curcumin is a natural antioxidant and anti-inflammatory polyphenol food supplement that attenuates paracetamol overdose induced hepatic toxicity in Wistar rats.

Figure 1.

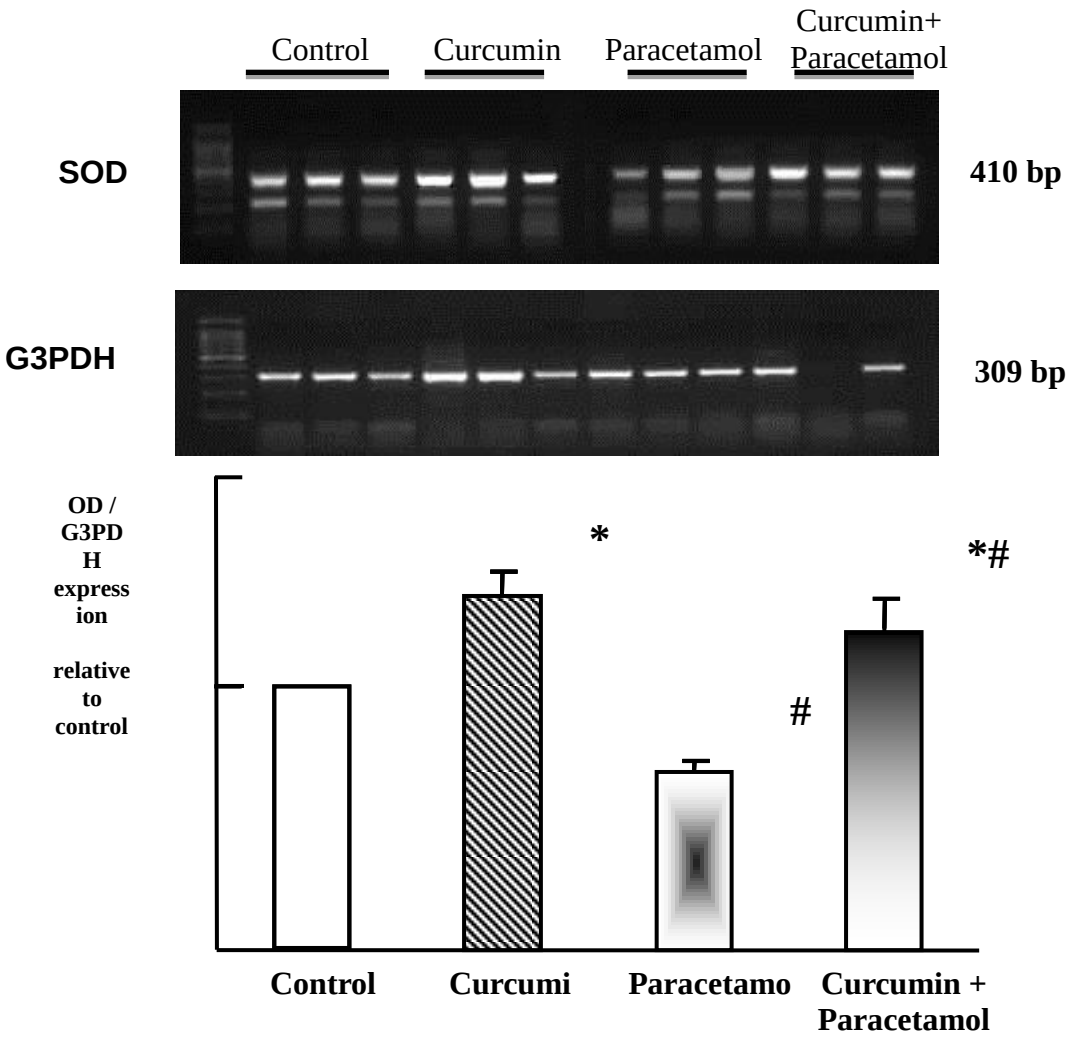

Figure 1. Semi-quantitative RT-PCR analysis of SOD mRNA expressions and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 vs. control group and P#< 0.05 vs. paracetamol administered group.

Figure 2

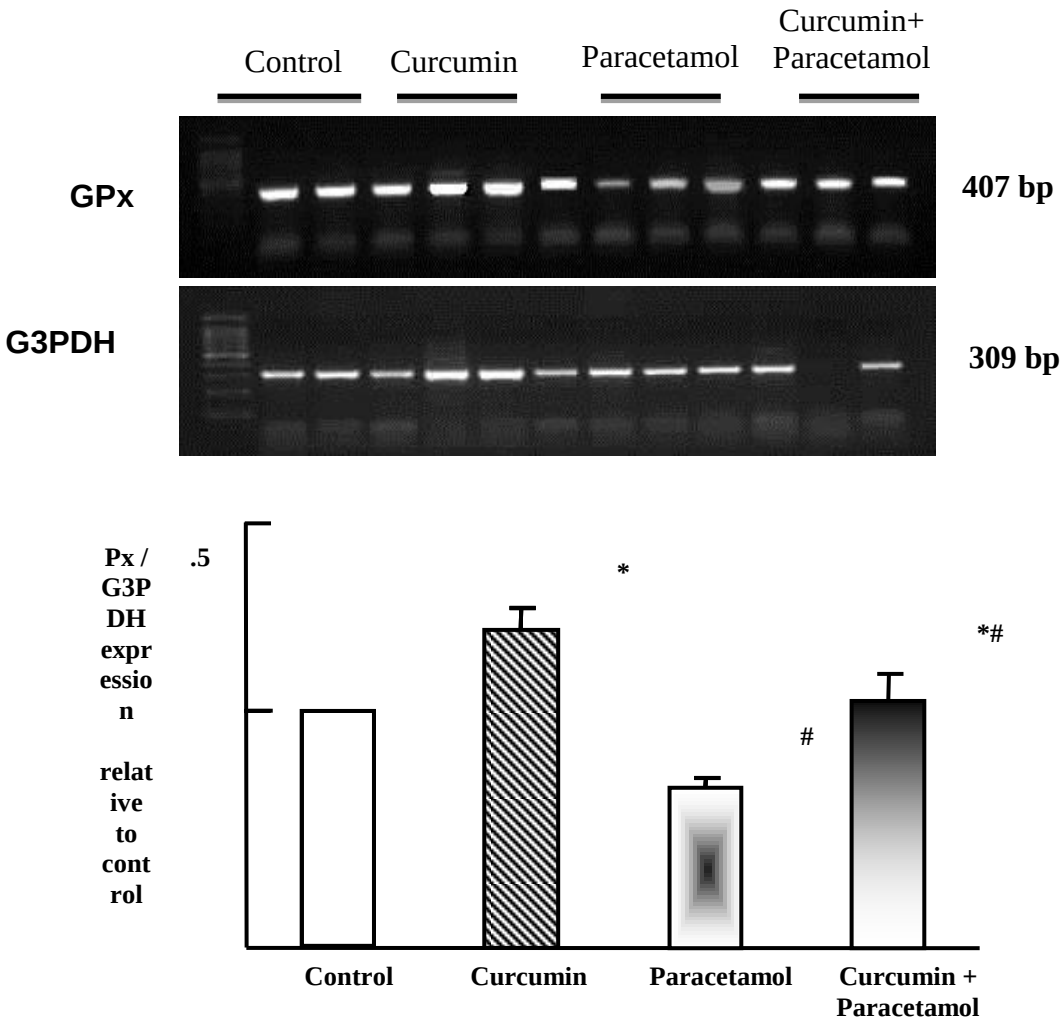

Figure 2. Semi-quantitative RT-PCR analysis of GPx and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods.. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 *vs.* control group and P#< 0.05 *vs.* paracetamol administered group.

Figure 3

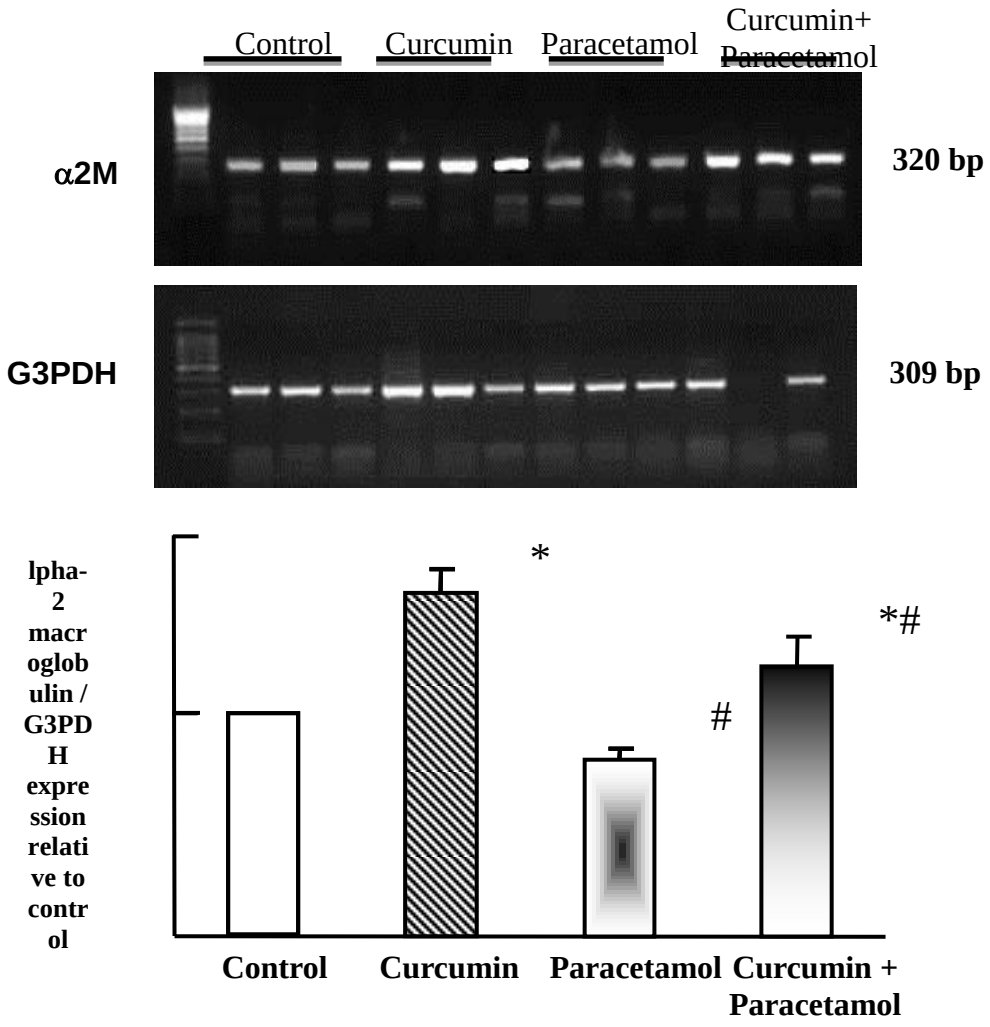

Figure 3. Semi-quantitative RT-PCR analysis of acute phase proteins □-2M mRNA expressions and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 *vs.* control group and P#< 0.05 *vs.* paracetamol administered group.

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IMMUNOPATHOLOGICAL AND ANTIMICROBIAL EFFECT OFBLACK PEPPER, GINGER ANDTHYME EXTRACTS ON EXPERIMENTAL MODEL OF ACUTE HEMATOGENOUS PYELONEPHRITIS IN ALBINO RATS

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Abstract

Recent studies showed prominent antimicrobial activity of plant extracts on some pathogenic microorganisms so we prepare crude aqueous extracts of black pepper, ginger and thyme followed by invitro study by measuring antimicrobial activity of these extracts using agar well diffusion method. Invivo study was carried out on 50 adulthealthy male albino rats which were divided into 5 groups, 10 rats each; Group (1): negative control group (received saline solution intragastric daily).Group (2): Positive control group (injected with mixed bacterial suspension of S. aureus and E.colias a model of pyelonephritis then received saline solution intragastric daily).Group (3): injected with the same dose of mixed bacterial suspension then received 100 mg/kg/day black pepper extract intragastric. Group (4): injected with mixed bacterial suspension then received 500 mg/kg/day ginger extract intragastric.Group (5): injected with mixed bacterial suspension then received 500 mg/kg/day thyme extract intragastric. All the groups were sacrificed after 1 and 4 weeks. Serum and blood samples were collected for measurement of lysozymes activity by using agarose gel cell lysis assay, measurement of nitric oxide production and finally, lymphocyte transformation test in addition to counting of both total and differential leukocytic and erythrocytic numbers and kidney samples were tested histopathologically.Bothinvivo and invitro results confirm the efficacy of these plants extracts as natural antimicrobials and suggests the possibility of employing them in treatment regimen.

Key Words:*black pepper, ginger, thyme, pyelonephritis, extract.*

Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased worldwide. According to World Health Organization (Santos et al., 1995) medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Ellof 1998).

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Almagboul et al., 1985; Artizzu et al., 1995; Ikram and Inamul, 1984; Izzo et al., 1995; Kubo et al., 1993; Shapoval et al., 1994; Sousa et al., 1991). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for

example, the phenolic compounds which are part of the essential oils (Jansen et al., 1987), as well as in tannin (Saxena et al., 1994).

Black pepper (piper nigrum Linn.) is a flowering vine of Piperaceae family and has been a prized spice since ancient times. The volatile oil of pepper has been shown to have antimicrobial activity(Dorman and Deans, 2000).Black pepper has many medicinal prorerties like its use to treat vertigo, asthma, chronic indigestion, colon toxins, obesity, sinusitis, congestion, fever, paralytic, arthriticdisordersand also advised in diarrhea and cholera(Sashidhar, 2002; Ravindran, 2000).

Ginger rhizome (*Zingiberofficinale* Roscoe, *Zingiberaceae*) has long been used in the world as a popular spice as well as a medicinal herb because of its high content of antioxidants and anti-inflammatory properties (Kikuzaki and Nakatani, 1993; Lantz et al., 2007).Ginger rhizome comprises various kinds of chemicals including 6- and 8-series of gingerols and shogaols, among which gingerol is the major ingredient representing a variety of bioactivities including antitumor andantiproliferative promotional (Surh 1999).

Thyme essential oil (Thymus Vulgaris) is utilized as a flavour enhancer in a wide variety of foods, beverages, confectionery products and in perfumery for the scenting of soaps and lotions(Arora and Kaur, 1999). It possesses some antiseptic, bronchiolytic, antispasmodic and antimicrobial properties that make it popular as a medicinal herb and as a preservative for foods(Briozzo et al., 1989; Cosentino et al., 1999).

The present study was carried out to evaluate the potential of these plant extracts as natural antimicrobials and immunomodulatory agents on an experimental model of acute hematogenous pyelonephritis in the rat.

Materials and methods

The present studywas carried out on 50adulthealthy male albino rats. The experiment was carried out in the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were examined to rule out the presence of any disease. The animals were housed in separate cages, supplied with food and water and allowed to live in optimal conditions according to the hospital housing protocol of the Veterinary Hospital,Zagazig University.

I I - Methods:

Preparation of the crude aqueousblack pepper extract:

Black pepper aqueous extract

For extraction, the freshly collected plant parts were thoroughly washed with tap water followed by sterile distilled water. The material was dried in an oven at 50°C for 48 hrs followed by grinding into a fine powder. The powdered material was stored in air tight jars in refrigerator at 4°C, and then 25 g of powdered plant material was dissolved in enough sterilized distilled water to make 100ml of aqueous extract (25% w/v). The mixture was kept undisturbed at room temperature for 24 hrs in a sterile flask covered with aluminum foil to avoid evaporation then subjected to filtration through sterilized Whatman no.1 filter paper. After filtration, the extract was evaporated in water bath until 25 ml extract was left in the container(Pundirand Jain., 2010).

Preparation of Aqueous ginger extract

Fresh ginger root (Zingiberofficinale) wasobtained from local market, washed with distilled water and dried in air, then ground and stored in air tight container under refrigeration. 100g of cleaned, air dried plant material of ginger obtained was blended and soaked in 100ml of sterile distilled water for 24hrs in a sterile glass container. The pulp obtained was shaken vigorously to allow for proper extraction of active ingredients. The crude extract was then filtered using sterile Whatmans No. 1 filter paper. The filtered extract was then stored in the refrigerator at 4°C. (Tagoe and Gbadago, 2010).

Preparation of Aqueous thyme extract

A quantity of 100 g thyme and basil material (stem and leaves), gathered at the maturity phase, were subjected to extraction. The added quantity of water was 200 g, the mixture being stored in hermetically-sealed recipients at the temperature of 30 °C, and shaken firmly at regular time intervals. After 48 hours, the liquid part was separated, being subjected to concentration at the temperature of 80 °C, for 3 hours, respectively at 100 °C for 30 minutes then stored for 6 days in a refrigerator, at the temperature of 3°C, until the in vitro sensitivity testing was carried out. (Tuchila et al., 2008).

Invitro study:

Test organisms

A total of 2 strains were used in the study. (*Staphylococcus aureus, and Escherichia coli O151*, as a model for acute hematogenous pyelonephritis). These strains were freshly prepared before working by overnight culturing on tryptic soya broth (TSB) and tryptic soya broth with 7.5% NaCl tubes. (Oxiod Company).

Antimicrobial activity of black pepper, ginger and thyme extracts against the tested organisms

Antimicrobial activity of black pepper, ginger and thyme extracts was measured using agar well diffusion method according to (Hazir and Keskin, 2001). An inoculums of10^5 CFU/ml of the tested organisms(*S.aureus and E.coli*) wasprepared by taking few colonies of freshly prepared suspension on nutrient agar(Oxoid Company) by sterile loop to 5ml of sterile saline, then spreading them on the surface of Muller hinton agar plates by using sterile cotton swab at 60º angle on the surface of the plates. After that wells were made in these plates by using sterile borer at 4 wells to each plate(8mm in diameter) , the cultured plates were left to dry at room temperature for 15minutes. Different volumes of crude black pepper, ginger and thyme extracts (20, 50, 100µl) were added to wells. After the solution settled down, all plates were cultured at 37°C for 24 hour. Control positive plate were also prepared using Gentamycin antibiotic disc (10µg) and ciprofloxacin. The negative control plates contain sterile saline agar wells instead of the extracts. The results were expressed as the zone of inhibition around wells.

Invivo study:

Invivo studywas carried out on 50 adulthealthy male albino rats which were divided into 5 groups, 10 rats each;

*Group (1):*Negative control group (received saline solution intragastric daily).

Group (2): Positive control group (injected with mixed bacterial suspension containing 1.5 x 10^6 colony-forming units (CFU) of *S. aureus* and 3.0 x 10^6 CFU of *E.coli* in the caudal vein at a dose of 0.5 ml/kg. then received saline solution intragastric daily).

Group (3): injected with mixed bacterial suspension containing 1.5 x 10^6 colony-forming units (CFU) of *S. aureus* and 3.0 x 10^6 CFU of *E.coli*in the caudal vein at a dose of 0.5 ml/kg then received100 mg/kg/dayblack pepper extract intragastric daily (Bang et al., 2009).

Group (4): injected with mixed bacterial suspension containing 1.5 x 10^6 colony-forming units (CFU) of *S. aureus* and 3.0 x 10^6 CFU of *E.coli*in the caudal vein at a dose of 0.5 ml/kg then received500 mg/kg/day ginger extract intragastric(Al-Noory et al 2013).

Group (5): injected with mixed bacterial suspension containing 1.5 x 10^6 colony-forming units (CFU) of *S. aureus* and 3.0 x 10^6 CFU of *E.coli*in the caudal vein at a dose of 0.5 ml/kg then received 500 mg/kg/day thyme extract intragastric daily (Abd El Kader and Mohamed 2012).

All the groups were sacrificed after 1 and 4 weeks. Blood samples were collected for measurement of lysozymes activity, nitric oxide production in blood and lymphocytes proliferation 4 weeks after treatment.

Detection of both total and differential leukocytic and erythrocyticcounts wasdone after 1 and 4 weeks.

Induction of a model of acute hematogenous pyelonephritis in the rat

Mixed bacterial suspension containing 1.5 x 10^6 colony-forming units (CFU) of *S. aureus* and 3.0 x 10^6 CFU of *E.coli* was inoculated in the caudal vein at a dose of 0.5 ml/kg. Control animals received the same amount of saline solution. Pyelonephritis was confirmed by differential leucocytic count and histopathological study of the kidneys (Tancheva et al, 2011).

Histopathological examination:

The collected specimens of kidneys form the sacrificed rats were fixed in 10% buffered neutral formalin solution for at least 24 hrs and then routinely processed. Paraffin sections of 5 micron thickness were prepared, stained withHematoxylin and eosin stain (H&E) according to Bancroft and Gamble, 2008 and then were examined microscopically.

Counting of both total and differential leukocytic and erythrocytic count: Samples were taken into an evacuated tube containing dry K2 EDTA and a full blood count, including an automated differential count, was performed on a Bayer-Technicon H.2 counter.**(**Greer et al.,2013).

Measurement of lysozymes activity by using agarose gel cell lysis assay according to the method described byNaglaa et al, 2008:

The lysoplateswere allowed to be in their plastic bags at room temperature. Wells were filled with 25 µl of serum samples from each group treated with black pepper, ginger and thyme aqueous extracts in addition to negative control group. Each filled plates contained 5 working lysostandard.The plates were carefully and tightly covered and then returned to their plastic bags. The plates were incubated at room temperature on the surface level for 12-18 hours, and then the lysis zone of each sample was measured and compared with the standard according to standard curve.

In vitro Lymphocytes Proliferation test assay:

Lymphocyte proliferation test using MTT (3-(4, 5-di-methyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide)was doneas described by(Rai-Elbalhaa et al., 1985) with modification. Heparinizedrat blood samples were collectedin sterile tubes. lymphocytes separation were doneby layering of blood in Ficol(Al Haj and Al Kanhal, 2010;Ereifej, et al.,

2011) and centrifuged at 400× g at 4˚C for 30 minutes giving blood cells withgranulocyte, interface layer (which contain lymphocytes)and upper plasma layer. The interface layer was carefullyaspirated using sterile glass Pasteur pipette, then put in sterile tubes containing 2 mL RPMI 1640 medium.Cells were washed 3 times with RPMI 1640 medium and centrifugation at 400 × g for 10 min at 4˚C was done. After the lastwash, the sedimented lymphocytes were resuspended in 1mL of RPMI 1640 medium containing 10% fetal calfserum (FCS). RBCs contaminationwas removedby the distilled water lysis method. Lymphocytes wereseeded in triplicate in flat-bottom 96-well micro titerplates (Costar) at 1×10^6 cells per well in 150 µL of culture medium either alone or with various concentrationsof black pepper, ginger and thyme aqueous extracts (10 µl/mL, 20 µl/mL and 30 µl/mL) or 15 µg ofPhytohemagglutinin (PHA) control per mL. Another 100µL of cell suspension was added to three sets of triplicatewells of a RPMI-1640 plus 50 µLPHA in concentration of 15 µg/mL as positive control.Another 100µL of cell suspension was added to three sets of triplicatewells of a RPMI-1640 without Phytohemagglutinin (PHA) as negative control.The plates were incubatedfor 3 days under 5% CO2 at 37˚C. Then 100 µL of supernatant was removed from the wells and 10 µL of MTTsolution was added to all the wells. The plate was incubated further for 4 h at 37˚C. The MTT formed zone wasextracted from the cells using dimethyl-sulphoxide (100µL/well). Then the OD was taken using an ELISA readerat a test wavelength of 570 nm. All experiments wererepeated at least two times.

Measurement of nitric oxide production in blood as described by rajarman et al.,1998:

The test depends on the nitrite, the stable product of nitric oxide oxidation which correlates with the amount of nitric oxide produced in blood; The tissue culture plates of 96 wells were filled with 50 µl of serum samples and 50 µl of colorless Griess reagent (1%sulfanilamide and 0.1% naphthylenediaminedihydrochloride in 2%phosphoric acid) and left in dark place at room temperature for 20 minutes, the purple color was measured using ELISA reader at 570 nm. The results of samples were compared with a standard curve of a known concentration of nitric oxide.

Results

1-Results of sensitivity test:

The obtained results included testing of whole aqueous extracts of three preparations of black pepper, ginger, andthyme extracts against *Staphylococcus aureus* and *Escherichia coli* as recorded in table (1) and figure (I). The obtained results showed a powerful effect of thyme extract on both *E-coli* and *S.aureus* strains with higher effect on *staphylococcus aureus* than *E-coli* as shown in table (1). Ginger extract had antibacterial effect on all tested organisms with varying degree but the highest activity was recorded on *S. aureus* followed by *E-coli* as shown in table (1).Also the obtained results included the effect of black pepper extract on *Staphylococci* and *Escherichia coli* as shown in table (1).

Table 1: Inhibition zones (mm) of natural plant extracts (µl) on tested organisms.

Organism / Extract		*E.coli*	*S.aureus*
Black pepper	20µl	4 mm	5 mm
	50 µl	6 mm	10 mm
	100 µl	8 mm	13 mm
Ginger	20µl	9 mm	8 mm
	50 µl	15 mm	14 mm
	100 µl	18 mm	20 mm
Thyme	20µl	12 mm	20 mm
	50 µl	16 mm	28 mm
	100 µl	20 mm	32 mm

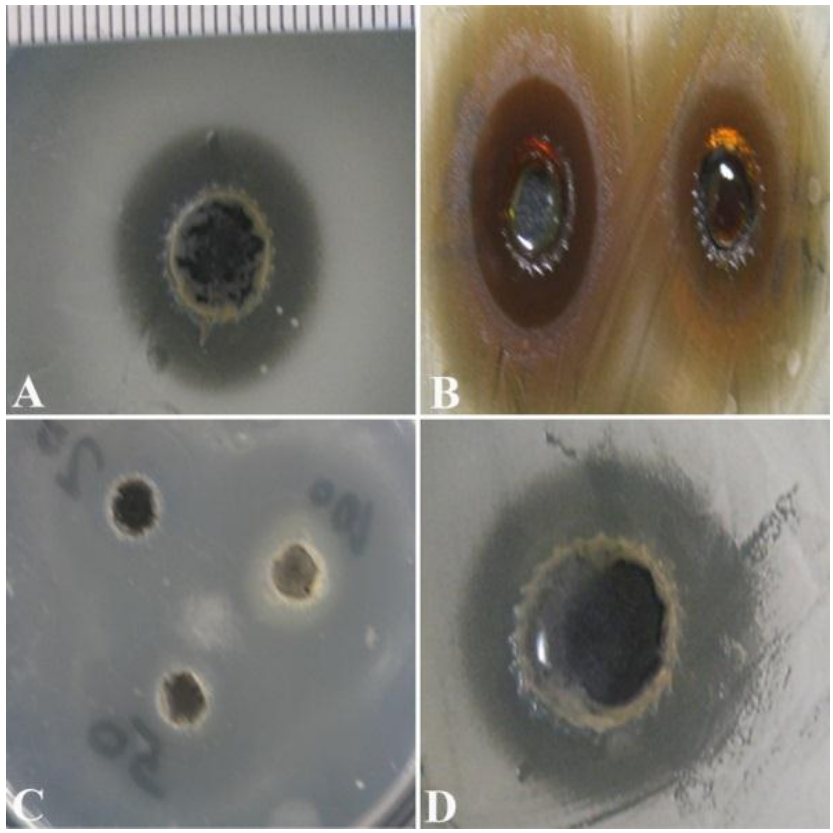

Fig I. Results of sensitivity test. A. &D. Inhibition zone of thyme extract with *E.coli* B. Inhibition zone of ginger extract with *E-coli* strain. C. Inhibition zone of black pepper with *S. aureus*.

2-Results of histopathologic examination:

Infected group

After 1 week the kidney showed congestion of renal blood vessels with perivascular oedema, interstitial leukocytic infiltration and haemorrhage (Fig.II1). Cloudy swelling of renal tubules was seen together with cystic dilatation of the surrounding tubules (Fig.II2). Intra-luminal hyaline casts were also detected with severe degenerative changes of renal tubules by the end of the 3rd week (Fig.II3). Massive leukocytic infiltration was detected among the renal tubules (Fig.II4). Severe haemorrhage was also seen among the renal tubules (Fig.II5).

Black pepper group

Congestion of renal blood vessels was observed. Cloudy swelling of the renal tubules with mild leukocytic infiltration were also detected (Fig.II6).

Ginger group

The kidney showed normal renal structure except for mild cloudy swelling of the renal tubules after 3 weeks from beginning of the treatment (Fig.II7).

Thyme group

Kidney showed normal renal structure with normal glomerular and tubular architecture and absence of apparent pathological changes (Fig.II8). No difference was detected after 1 and 3 weeks.

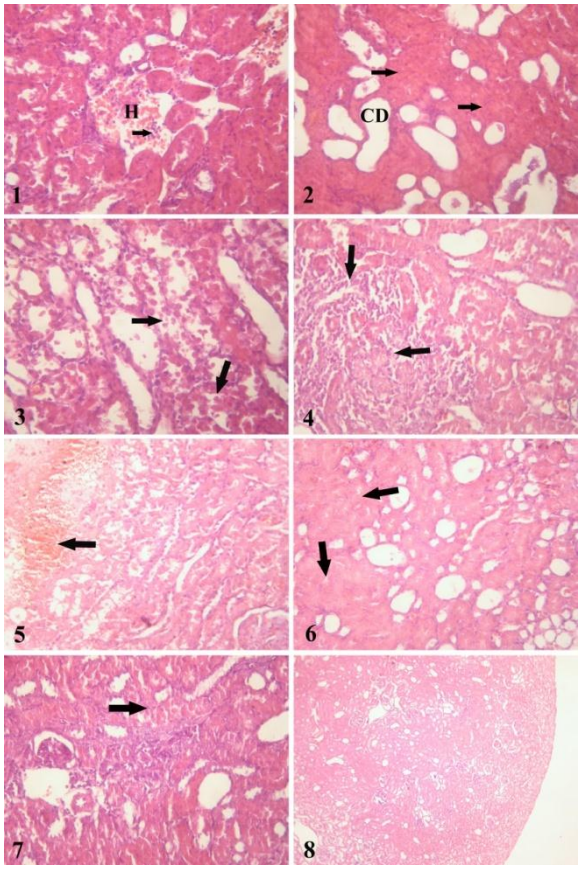

Figure II, (1): Kidney of infected group showing interstitial haemorrhage (H) and mild leucocytic infiltration (arrow) (HE X300).Figure (2): Kidney of infected group showing cloudy swelling of renal tubules(arrows) together with cystic dilatation (CD) of the surrounding tubules(arrows). (HE X300).Figure (3): Kidney of infected group showing intra-luminal hyaline casts with severe degenerative changes of renal tubules (arrows). (HE X300).Figure (4): Kidney of infected group showing massive leukocytic infiltration among the renal tubules. (HE X300).Figure (5): Kidney of infected group showing severe haemorrhage among the renal tubules.(HE X300).Figure (6): Kidney of black peppergroup showing mild cloudy swelling of the renal tubules (arrow). (HE X300). Figure (7): Kidney of ginger group showing mild cloudy swelling of the renal tubules.(HE X300).Figure (8): Kidney of thyme group showing normal renal structure with normal glomerular and tubular architecture and absence of apparent pathological changes. (HE X300).

3-Total and Differential leukocytic count:

1 week post-treatment:

Results of WBCs, lymphocyte, Monocyte, granulocyte and erythrocyte count were summarized in table (2):

Table (2) showing results of WBCs, lymphocyte, Monocyte, granulocyte and erythrocyte counts in infected and treated groups 1 week post treatment.

Group / Papameter	Black pepper	Ginger	Thyme	Infected	control
WBC	10.2 x 10^3/ul	8.8 x 10^3/ul	7 x 10^3/ul	15.1 x 10^3/ul	8.6 x103/mm3
LY	7.9 x 10^3/ul	5 x 10^3/ul	6 x 10^3/ul	9.8 x 10^3/ul	5.12 x 103/mm3
Mo	1 x 10^3/ul	1.3 x 10^3/ul	0.2 x 10^3/ul	1.7 x 10^3/ul	0.03x 103/mm3
Gr	2.5 x 10^3/ul	1.3 x 10^3/ul	0.9 x 10^3/ul	3.6 x 10^3/ul	1.78 x 103/mm3
RBC	7.69 x 10^6/ul	8.30 x 10^6/ul	7.50 x 10^6/ul	8.17 x 10^6/ul	7.75 x 106/mm3

4 weeks post-treatment:

Results of WBCs, lymphocyte, Monocyte, granulocyte and erythrocyte count were summarized in table (3):

Table (3) showing results of WBCs, lymphocyte, Monocyte, granulocyte and erythrocyte counts in infected and treated groups 4 week post treatment.

Group / Papameter	Black pepper	Ginger	Thyme	Infected	control
WBC	6.9 x 10^3/ul	7.3 x 10^3/ul	9.2 x 10^3/ul	15.1 x 10^3/ul	8.2 x103/mm3
LY	5.3 x 10^3/ul	5.7 x 10^3/ul	6 x 10^3/ul	9.8 x 10^3/ul	6.12 x 103/mm3
Mo	0.7 x 10^3/ul	0.8 x 10^3/ul	1.1 x 10^3/ul	1.7 x 10^3/ul	0.02x 103/mm3
Gr	3.1 x 10^3/ul	1.9 x 10^3/ul	0.8 x 10^3/ul	3.6 x 10^3/ul	1.1 x 103/mm3
RBC	8.45 x 10^6/ul	7.47 x 10^6/ul	7.32 x 10^6/ul	8.17 x 10^6/ul	8.10 x 106/mm3

4- Results of serum lysozyme concentration test by µg / ml.

The obtained results revealed that increasing of lysozyme value in all treated groups with ginger extract and thyme after challenged with *S.aureus* and *E.coli* suspension but not with black pepper as shown in table (4).

Table (4) showing lysozyme activity using lysoplate assay of black pepper, ginger and thyme extracts in addition to negative control serum

Sample code	Lysozyme value of negative control group (µg / ml)	Lysozyme value of Black pepper treated group (µg / ml)	Lysozyme value of ginger extract treated group (µg / ml)	Lysozyme value of Thyme extract treated group (µg / ml)
S1	2.2	2.2	3.4	3.2
S2	2.2	2.1	3.5	3.7
S3	2.1	2.2	4.0	4.1
S4	2.23	2.2	3.6	4
S5	2.2	2.1	2.7	3

*S means sample code

5- Results of nitric oxide test

The obtained results revealed that increasing of lysozyme value in all treated groups with ginger extract andthyme after challenged with *S.aureus* and *E.coli* suspension but not with black pepper as shown in table (5).

Table (5) nitric oxide test of serum samples from groups treated with black pepper, ginger and thyme extracts in addition to negative control group.

Sample code	Nitric oxide value of negative control group (µg / ml)	Nitric oxide value of Black pepper treated group (µg / ml)	Nitric oxide value of ginger extract treated group (µg / ml)	Nitric oxide value of Thyme treated group (µg / ml)
S1	10.5	10	38	49.5
S2	13	12	40	48.8
S3	12.7	12	36	45
S4	15.2	15	36	49
S5	10	11	35	45

*S means sample code

6-Immunomodulatoryeffect ofblack pepper, ginger and thyme using lymphocyte transformation test (LTT).

Immunomodulatoryeffect of aqueous extracts of black pepper, ginger and thyme extracts was studied using (LTT)and Phytohemagglutinin (PHA) was used as a control. The obtained results showed that the lymphocyte transformation mean value of PHA was2.20 ± 0.04 (Table 4). While the lymphocyte transformations mean values of ginger extract with PHA at concentrations of 10µl/mL, 20 µl/mL and 30µl/mL were 0.703 ± 0.030, 0.925 ± 0.045and 0.980 ± 0.053respectively, while for thyme extract at the same concentration used in ginger was 2.201 ± 0.030, 3.601 ± 0.045and 3,901 ± 0.053respectively while for black pepper after using the same concentrations was 2.21 ± 0.04,2.261± 0.04 and 2.243± 0.04respectively

Table 6. lymphocyte proliferation test assay of black pepper, gingerandthyme extracts with positive control PHA alone

Parameter	LTT means ± SE
PHA alone	2.20 ± 0.04
Black pepper extract + PHA 10µl/ml 20 µl/ml 30 µl/ml	 2.21 ± 0.04 2.261± 0.04 2.243± 0.04
Ginger extract + PHA 10µl/ml 20 µl/ml 30 µl/ml	 0.703 ± 0.030 0.925 ± 0.045 0.980 ± 0.053
Thyme extract + PHA 10µl/ml 20 µl/ml 30 µl/ml	 2.201 ± 0.030 3.601 ± 0.045 3.901 ± 0.053

Discussion

Black pepper, Ginger and Thyme extracts had some antimicrobial activities and are used in variousfood preparations as flavour enhancers and in herbal medicine (Arora et al., 1999;Cosentino et al., 1999). Mode of action of the extracts could mainly be attributed to its content of the active ingredients which have antimicrobial properties includingthymol, zingerone, shogaolsand gingerols,pipperine, terpenes, eugenol, flavones, glycosides of phenolic monoterpenoids, aliphatic alcohols and volatile oils among other elements(Dorman and Deans, 2000; Hammer et al., 1999). These substances acting alone or in combination may result in a broad spectrum of antimicrobial activity exhibited for both bacteria and fungi.This study revealed the antimicrobial effect of aqueous extracts of black pepper, ginger and thyme on *S.aureus* and *E.coli*. The highest inhibition zone was obtained with thyme on *S.aureus* followed by *E.coli*.Ginger extract had antibacterial effect on *S.aureus,*followed by *E. coli*.Finally, black pepper had the lowest effect on all tested organisms except at concentration of 100ml on *S.aureus* which give zone of 13mm. Our results were agreed with that of Ficker et al., 2003; Grange and Davey, 1990; Zahra et al., 2009. While the obtained

results were nearly similar to that recorded by Hoque et al., 2008, as they used ethanolic extracts instead of aqueous extracts.Thyme and ginger extracts were also the most effective antimicrobial agent against other bacteria as multi drug resistantP.aeruginosa (Wagih and sulaiman, 2009).

Inhibition zones of ginger and thyme extracts were also recorded byChang et al., 2001.Potent antimicrobial activity of aqueous extract of thyme and ginger against *Esherichia coli*isolated from urinary tract infection was recorded by Nahed et al., 2010.In the present study the obtained results showed that thyme extract had the potent antibacterial effect followed by ginger extract while black piper had the lowest level, these results go in a hand with those obtained by Al-Jiffri et al., 2011 who concluded that thyme and ginger extracts were the most effective against *Esherichia coli*isolated from urinary tract infection.Small inhibition zone diameter was recorded by Nanasombat and Lohasupthawee, 2005 by using crude and ethanolic extract of thyme, ginger, black and white pepper against different gram positive and negative bacterial strains. These results somewhat disagreed with our results.The difference in results may be due to plant origin and some ecological factors.

Experimental model of acute hematogenous pyelonephritis in the rat showed congestion of renal blood vessels with perivascular oedema, interstitial leukocytic infiltration and haemorrhage,cloudy swelling of renal tubules together with cystic dilatation of the surrounding tubules. Intra-luminal hyaline casts were also detected with severe degenerative changes of renal tubules by the end of the 4th week. Massive leukocytic infiltration and severe haemorrhagewas detected among the renal tubules. These results was similar to those in the study of Mustonen et al., 1984; Hotchkiss et al., 1999; Diaz de Leon et al., 2006 who showed that tubulointerstitial renal changes were the predominant histopathologic finding. These changes could be attributed to the virulence factors of *E.coli* and *staph aureus*which enable them to enter urinary tract, and cause symptomatic disease (Raksha et al., 2003). Virulence factors of uropathogenic*E.coli* include its ability to adhere to uroepithelial cells and certain specific serotypes O and K antigens which enable it to resist phagocytosis and bactericidal action of normal serum. Other factors known to contribute to the virulence are the production of α hemolysins, colicins, aerobactin, cytotoxic necrotizing factor, and cell surface hydrophobicity (Kausar et al., 2009).

Herrera-Luna et al. (2009) reported that adhesin has been most closely associated with uropathogenic *E.coli* is the P fimbria. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cell membranes.

Staphylococcus aureus is a complex pathogen with numerous classes of virulence factors. Protein secretion principally occurs via the secretory system and is required to render many virulence factors functional. Compounds which selectively block bacterial protein secretion while leaving the host system unaffected may lead to novel antimicrobial therapies. (Bartlett and Hulten, 2010).*S. aureus* expresses many potential virulence factors: (1) surface proteinsthat promote colonization of host tissues; (2) invasins that promote bacterial spread in tissues; (3) surface factors that inhibit phagocytic engulfment; (4) biochemical properties that enhance their survival in phagocytes; (5) immunological disguises (Protein A, coagulase); (6) membrane-damaging toxins that lyse eucaryotic cell membranes (hemolysins, leukotoxin, leukocidin; (7) exotoxins that damage host tissues or otherwise provoke symptoms of disease; and (8) inherent and acquired resistance to antimicrobial agents. (Todar 2008; Plata et al., 2009).Results of blood count showed increased numbers of granulocytes in case of challenge with *S.aureus*and*E.coli* that confirms the infection. Granulocyte count

decreased to the normal in case of using thyme and ginger extract and little effect was detected in case of black pepper extract.

Our study showed increasing values of lysozyme in all tested samples in group treated with thyme and gingerextracts means improved immunity after dietary intake of the aqueous extract of ginger and thyme while that of black pepper extracts showed no change. Those results agreed with that of Heping et al., 2008who proved that immunity improved after dietary intake of thyme and ginger extracts.AlsoManach et al., 1996; Cook and Samman, 1996 reported thatthyme extend the activity of vitamin C,act as antioxidants and may thereforeenhance the immune function.

The results obtained were disagreed with that proved by Abdulkarimi et al., 2011who concluded that supplementation of 0.2, 0.4 and 0.6%thyme extract in drinking water did not improve the immune status in broiler. Thedifference in results may be due to difference in sourceand methods of thyme extract. Our research denotes that serum nitric oxide concentration of the group treated with aqueous extract of thyme and gingerextracts afterchallenging with mixture of *S.aureus*and *E.coli* suspension was elevated in all tested samples but no change had occurred in samples collected from group treated with black pepper extract, this indicating thatnon specific immunity was improved with thyme and ginger. The obtained results were agreed with that of Heping et al., (2008).

Theanti-inflammatoryeffectsofgingerandits extract have been reported insome diseased conditions asrheumatoidarthritis(AltmanandMarcussen, 2001; Wigler et al., 2003). Other researchers proved that ginger extract inhibits the secretion of IL-2 production from mixed culture of lymphocytes in addition to IL1α from activated macrophages Subramanian and Handa(2004).

The present results showed that aqueous extract of thyme increase lymphocyte proliferation test assay in dose dependant manner. The highest transformations was recorded at concentration 100 µl/ml while transformations decreased with ginger extract in all tested samples, finally the black pepper showed no effect. Comparable results were also approved by Al-Kassie, 2009 who stated thataddition of thymeextract in diet of chicks improve all immune parameters including T-cell mediated immunity.

These results agreed with that recorded by hua et al., (2006) reported that immunomodulatory effect of ginger extract at concentration of 0.0001-10ng/ml in vitro significantly inhibited Tlymphocyte proliferation ,decrease the number of total lymphocytes and T helper cells in a concentrationdependant manner but increase the percent of Suppressor in mice.

Conclusion

Both invivo and invitro results showed a strong antimicrobial activity of thyme, ginger and relatively black pepper against experimental model of acute hematogenous pyelonephritis. Also thyme extract modify the immune response and may be used as immunostimulant thus suggest the possibility of employing them in treatment regimen.

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THE IMPACT OF VARIOUS FACTORS ON THE OCCURANCE OF THE RUSTERHOLZ'S ULCER AND THE PROPOSAL TO REMEDY THIS PROBLEM

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Abstract
Rusterholz's ulcer represents extremely painful condition of the animal, induced by inadequate, improper and unprofessional hooves' treatment. The presence of untreated laminitis in cattle population represents an incubator of increased presence of Rusterhoz's ulcers. Increased costs of treatment and reduced production are directly proportional to prevalence of Ruserholz's ulcer. The factors that induce occurrence of Rusterholz's ulcer are: inadequate bearings, unprofessionally done treatment of hooves, weakened horn caused by improper feeding, increased number of the pathogens in the surroundings.
Keywords: *diseases of hooves, Rusterholz's ulcer, cattle production*

Introduction

Problems caused by inadequate bearings, laminitis, as well as by unprofessionally done treatment of hooves may be various (Hadžić et al., 2012.). One of the major represents appearance of Rusterholz's ulcer, which is extremely painful condition of the animal and significantly reduces its productivity. Too short bearings or slope greater than 2% represents excellent prerequisite for the emergence of Rusterholz's ulcer.

Rusterholz's ulcer is caused by the stretching of deep flexor of ligament, through hoof's bone where comes to contusion, therefore the ignition, microcirculation disorder of corium, irregular growth of plantar horn, to the final stage – necrosis of tissue on plantar part of hoof (Kos, 2009.).

One of the problems in intensive cattle production in Republic of Serbia, is old buildings for keeping cattle in tied system of farming, most of them were built decades ago, where bearings were adequate to then formats of animals which were placed in such stables. Due to market development, genetic selection went in the direction of increasing productive characteristics, and as a side effect obtained a larger animal format. This leads to the basic problem of short bearings which remained neglect by cattle possessors, due to expensive repairs. Architecture of bearings on which the ox resides should be adjusted to its physiognomy and zootechnic, this means long enough for specific cattle breeds and types and certain bearing's slope for adequate drain (Somers et al., 2003.). In too short bearings, due to insufficient space for the normal position, an animal stands on the edge of bearing and by that comes to disorder of posture of back legs. Slipping may occur during standing up and by that possibility of injury of front hoof's wall and its crown edge increases, with the mechanical injuries which appears during the manure removal.

Materials and methods

Different experts have different approaches to solving the problem so called Rusterholz's ulcer. Attitudes are unique in terms of the appeared wound on plantar part of hoof shod be as best as it can be unballast, i.e. mechanically free of surrounding tissue which pressures on the problematic spot , as far as possible it's necessary to preserve as much as

possible crown – carrying edge of hoof (Nüske,2007). After these actions, procedures by accepted methods are varies. A group of experts represent an attitude that necrotic tissue should be surgically remove, then the affected area should be treated by termocauter, to prevent excessive bleeding and to prevent penetration of pathogenic agents into the affected area. After that the wound should be fulfill with an acrylic mixture to restore firmness of plantar part of horn and to enable the animal normal support to the affected hoof. Animal's completed recovery is expected in a month or month and a half by this method. According to another group of experts, after has been unballasted, necrotic tissue (in case it's aseptically) it's necessary to spray the antibiotic spray for external use, then isolate by gauze, and to bandage a hoof with special bandage with one-way permeable performance, to drain excess of blood out of wound, and to prevent environmental influence on the wound (to keep it aseptic). Wound healing by this method may be expected in a seven days in most of cases (about 87% of cases) and after the first control and removal of bandages it's noticeable drying of necrotic tissue and re-epithelialization of the surrounding tissue. In a negligible percentage (4%) it is necessary to re-bind a wound, while for the total return of function of the affected hoof is required about 14 days. There are some cases of this disease which requires use of additional assets such as block made of plastic or wood, which are placed with permanent glue on a healthy hoof. The affected hoof is separated from the surface and tread surface by setting these block. The block is removed after one or month and a half (individually) from the setting day, giving by that enough time for re-forming layer of plantar horn thick enough to stand an ox weight. The method of setting the block is present to the both group of experts.

After the intervention has been done, the animal shouldn't show any larger problems with standing or movement.

Results and discussion

Diagnosed occurrence of Rusterholz's ulcer can be seen in Table 1, which monitors work of a group for the functional treatment of hooves during four cycles of treatment or two years, at seven farms. The model that has been noticed is that during the warm month's occurrence of Rusterholz's ulcer increases while during the winter months it slightly reduces. Also, it's noticeable that as the group for the functional treatment of hooves improves and practical advances in work and experience as number of treated animals increases so as way of treatment (number of placed blocks). In Table no 1. is shown number of placed block as the parameter for the number of animals in which the Rusterholz's ulcer was in a critical faze.

Table 1 Number of placed block as the parameter for the number of animals in which the Rusterholz's ulcer was in a critical fazes

Circle	I	II	III	IV
Date	25.01.-05.03.2010	01.08.-03.09.2010	02.02.-10.03.2011	09.08.-21.08.2011
A	**Σ 1681**	**Σ 1708**	**Σ 1585**	**Σ 1706**
	229-13,62%	372-21,77%	229 -14,44%	489-28,66%
	/	42-11,29%	28-12,23%	176-35,99%
Date	30.03-20.05.2010	29.10.-17.11.2010	13.05.-07.06.2011	03.11.-08.12.2011

B	**Σ 1725**	**Σ 1381**	**Σ 1277**	**Σ 1447**
	199-11,53%	321-23,24%	345-27,07%	484-33,44%
	/	29-9,03%	118-34,20%	161-33,26%
Date	13.11.2009-21.01.2010	19.05.-11.06.2010	18.11.-13.12.2010	06.06.-22.06.2011
C	**Σ 1259**	**Σ 1179**	**Σ 1197**	**Σ 1246**
	268-21,28%	168-14,24%	349-29,15%	358-28,73%
	3-1,12%	2-1,19%	118-33,81%	131-36,59%
Date	03.12.2009-16.01.2010	10.06.-05.07.2010	14.12.-31.12.2010	21.06.-14.07.2011
D	**Σ 1273**	**Σ 1124**	**Σ 1157**	**Σ 1137**
	241-18,93%	128-10,48%	202-17,45%	229-20,14%
	/	9-7,03%	39-19,31%	72-31,44%
Date	04.03.-29.03.2010	04.09.-30.09.2010	11.03.-07.04.2011	20.09-17.10.2011
E	**Σ 1531**	**Σ 1322**	**Σ 1321**	**Σ 1287**
	205-13,38%	404-30,55%	264-19,98%	479-37,21%
	/	36-8,91%	41-15,53%	232-40,43%
Date	25.01.-23.04.2010	01.10.-30.10.2010	08.04.-12.05.2011	17.10.-18.11.2010
F	**Σ 1787**	**Σ 1319**	**Σ 1335**	**Σ 1405**
	221-12,36%	420-31,84%	236-17,67%	538-38,29%
	/	36-8,57%	93-39,41%	219-40,71%
Date	21.02-22.02.2010	06.07.-31.07.2010	31.12-01.02.2011	15.07-17.08.2011
G	**Σ 983**	**Σ 1097**	**Σ 1208**	**Σ 1216**
	117-11,90%	281-25,61%	166-13,74%	330-27,13%
	/	26-9,25%	15-9,04%	129-39,09%

Legend :

1. Σ- The total number of treated cows at farm
2. The number of cows with a diagnosed Rusterholz's ulcer - % from the total number of treated cows
3. The number of set block by diagnosed animal - % from the total number of the diagnosed Rusterholz's ulcer

We mustn't exclude a nutrition problem which arises due to inadequate prepared meal as well as management-feeding of dairy cows (Shwab and Shaver, 2005, Goof, 2009). During such fluctuations comes to disbalance in meals (different nutrition technologists have different nutrition methods, of course in purpose to increase productive characteristics). However, frequently changing the meals regime, quality of nutrients and supplements, as the ultimate effect has growth and structural problem of horn (Laminitis) (Hadžić et al. 2013), and by that its function is weaken, causing frequent occurrence different hoof diseases (Cheli and Mortellaro,1974, Hadžić et.al. 2011), hence Rusterholz's ulcer.

Conclusion

Rusterholz's ulcer leads to a significant reduction in production characteristics to affected animal, reduction in milk yield to the 35%, which represents significant loss of profits for owners. Adequate, regular, professional and timely done treatment of hoof can prevent consequences of loss or expensive production. To the therapy of Rusterholz's ulcer is necessary that the treatment conducts professionally trained and experienced professional, in order to the therapy lasted as short as possible and its effect is visible through the production results. Regular hoof treatment and its control should be done twice a year and more often if necessary.

The procedure of suppressing Rusterholz's ulcer in cattle confirms sentence "complete understanding of the problem leads to the solution" and proves that it's absolutely useful in correcting this problem.

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PROTOZOAN INFECTIONS IN DOGS AND THEIR IMPORTANCE IN THE CONTAMINATION OF THE BELGRADE URBAN ENVIRONMENT

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Abstract

Most parasitology studies of urban contamination with dog faeces emphases helminths which dogs are main hosts and who certainly have great epidemiological significance. Much less attention has been paid to a protozoan infection that seen in the feces of dogs that also has a important epidemiological significance of contamination of urban areas. For these reasons, the aim of this paper is to highlight the potential epidemiological significance of protozoal infections in dogs in the urban environment like Belgrade. The survey was conducted in the period 2012-2013 and included examination of feces of dogs taken from the green areas of Belgrade and samples collected in veterinary clinics from dogs that have had intestinal problems. Faecal samples of dogs were collected from 22 green areas and the eco-zone (dog park) in 5 most urbanized city's municipality at old and new Beograd and Zemun, where the biggest dogs concentration, and consequently fecal contamination. Samples of fresh feces were collected with method of randomization and marked in relation to the location in relation to whether the origin of the "eco-zone". A total of 800 samples were examined by the method of the Pataki; McMaster-in, Stoll and Richardson-Kendell and 114 samples were examined using the IDEXX Snap Giardia test. The same method was used in the examination of ownership dogs faeces from veterinary clinics in which 220 samples were examined. In our study during 2012 Giardia intestinalis was found in 15.78% and in 2013 found 32% samples. Also, coprological examination of dogs with clinical symptoms of disease is confirmed giardiasis by a more than 65% of the cases.The presence of Entamoeba histolytica was observed in 7.9 and 9.3% of samples of feces from green areas and 23.7% of feces of dogs with clinical signs of disease.Cryptosporidium spp in the course of our research establishment in 4.7 and 5.3% of samples of feces from green areas and 12.4% of feces of dogs with clinical signs of disease

Keywords: *protozoa, dogs, Giardia intestinalis, Entamoeba histolytica, Cryptosporidium spp*

Introduction

A steady increase in the number of dogs is a serious epidemiological problem of urban areas. Special problems of large cities are non owner dogs that are outside the control of health so that these problems solve more attention in the world. Continuous increase of dog's population, both strays, and pets present permanent epidemiological problem at urban environmental condition worldwide. Those animals permanent contaminated those areas with faeces which present a significant health problem from human (Fok et al.2001, Pavlović et al.1997,2009,2013) Dogs are carrier of numerous parasites species important from human health which eggs were eliminated by faeces and permanent contaminate public and grassy places (Blazius et al.2005, Dubná et al.2007, Pavlović et al.2014 Those parasites, transmitted to human by faecal contamination, especially to children which play at those dirty places.

Most frequent parasites are eggs of zoonotic helminthes *Toxocara canis, Dipylidium caninum, Ancylostomidae spp., Taenia spp., Toxascaris leonina, Trichuris vulpis* i *Strongyloudes stercoralis* and protosoa *Giardia intestinalis, Amoeba spp.* and *Cryptosporidium spp.* (Kulišić et al.1998, Giangaspero,2006, Pavlovic et al .2013). Much less

attention has been paid to a protozoan infection that seen in the feces of dogs that also has a important epidemiological significance of contamination of urban areas. During the last 20 years of constant monitoring of parasitological contamination of public areas and parks of Belgrade, we found that the percentage of the presence of protozoal infections is increasingly present in the faeces of dogs who meet in the streets (Pavlović et al.2007, 2014).

For these reasons, the aim of this paper is to highlight the potential epidemiological significance of protozoal infections in dogs in the urban environment like Belgrade.

Material and methods

The survey was conducted in the period 2012-2013 and included examination of feces of dogs taken from the green areas of Belgrade and samples collected in veterinary clinics from dogs that have had intestinal problems. Faecal samples of dogs were collected from 22 green areas and the eco-zone (dog park) in 5 most urbanized city's municipality at old and new Beograd and Zemun, where the biggest dogs concentration, and consequently fecal contamination.

Samples of fresh feces were collected with method of randomization and marked in relation to the location in relation to whether the origin of the "eco-zone". A total of 800 samples were examined by the method of the Pataki; McMaster-in, Stoll and Richardson-Kendell (Euzeby,1981) and 114 samples were examined using the IDEXX Snap Giardia test (Pavlović et al.2007,Jezdimirović et al.2011). The same method was used in the examination of ownership dogs faeces from veterinary clinics in which 220 samples were examined.

Results and discusion

In our study during 2012 *Giardia intestinalis* was found in 15.78% and in 2013 found 32% samples. Also, coprological examination of dogs with clinical symptoms of disease is confirmed giardiasis by a more than 65% of the cases.

Giardiasis (Lambliasis) is a protozoal disease which affects the small intestine where its dysfunction causes, or asymptomatically flows. Causative agent of the disease is *Giardia intestinalis* a flagellated protozoan parasite. It occurs in vegetative and cystic form. Vegetative form is 10-20 μm in length, showing bilateral symmetry and pear-shaped. There are two symmetric nuclei and reproduce the binary distribution. He lives in the duodenum and may reach in the liver and bile ducts. Form cyst is oval in shape, with thick walls. It has 2-4 nuclei and parasite in the lower part of the small intestine and the large intestine where feces are ejected into the environment. The disease causes the vegetative form as cystic form is used for transmission of infection (Giangaspero 2006)

Giardia infection can occur through ingestion of dormant cysts in contaminated water, food, or by the faecal-oral route (Pavlović et al.2013a) The cyst can survive for weeks to months in cold water especially stagnant water sources. *Giardia* infects humans, but is also one of the most common parasites infecting cats, dogs and birds (Bianciardi et al.2004).

In dogs giardisis usually characterized by diarrhea, sometimes accompanied by vomiting, and in many cases causes a mash feces, or chronic diarrhea that can last, or occasionally in the course of several weeks. The parasite may sometimes be present in the intestines and causing no (or only mild) symptoms (Nikolićetal.2002, 2008). The presence of infected dogs (and people) that their feces contaminate public surfaces acquire the conditions for the spread of giardisis and permanent possibility of infection of dogs, cats and people in urban areas (Brandonisio.2006, Pavlović et al.2013a)

The presence of *Entamoeba histolytica* was observed in 9.3% of samples of feces from green areas and 23.7% of feces of dogs with clinical signs of disease. Amoeba at dogs can live as commissural form that does not cause pathological effects but also under certain conditions and are becoming extremely enteropathogenic (Jordan,1967, Visvesvara,1999) Amoebas have two forms - trophozoite that exists only in the final host and "fresh" feces and cystic form. Cystic form is highly resistant and long-lived in the external environment (water, moist soil, and food), capable of infecting the host after ingestion of cysts, developing stage trophozoite and cause infection in the digestive tract.

Place of parasitism is small and large intestine, but can spread through the bloodstream at other organs. Clinical symptoms can include fulminating dysentery, bloody diarrhea, weight loss, fatigue, weight loss, dizziness, abdominal pain and the presence of amoeba in the feces. Intestinal amoebae damaging the mucosa, causing a characteristic ulcers, and then enter the blood stream. Through blood, amoebas are due to other vital organs of the host, most commonly to the liver, spleen and lungs, and sometimes to the brain when you can cause a clinical picture of epilepsy (Wittnich1976).

Cryptosporidiosis is a zoonotic parasitic illness that causes diarrhea caused by *Cryptosporidium spp.*,protozoan pathogen of the Phylum *Apicomplexa* A number of *Cryptosporidium spp*. infect mammals: *C.parvum, C. hominis* (previously *C. parvum* genotype 1). *C. canis, C. felis, C. meleagridis,* and *C. muris* Parasites were located extracitoplasmatic, at parasitophorus vacuole, primarily in the brush border of the ileum and especially in the dome epithelium covering the Payer's patches. When only a few organisms were detected they consistently could be found in dome epithelium. In the jejunum parasites always were located on the villous epithelium (Anderson,1982,Pavlović et al.2013b)

Cryptosporidiosis is typically an acute short-term infection. Cryptosporidiosis parasites are passed in the stool of infected animals and persons. Cryptosporidiosis is clinically manifestly usually occurring only in young cats and dogs.

Symptoms of acute cryptosporidiosis include lack of appetite, weight loss, and diarrhea which is usually yellow to yellowish-brown in color and of a creamy texture. The rapid loss of nutrients and fluids during diarrhea results in severe dehydration. Since instestinal tract cells are disrupted, absorption of feed nutrients is restricted, and the animal loses more nutrients through the digestive tract than it takes in (Anderson,1982)

During our research oocyst of *Cryptosporidia spp.* establishment in 5.3% of samples of feces from green areas and 12.4% of feces of dogs with clinical signs of disease

Conclusion

Protozoan infections play an important role in the contamination of public and green areas in cities. This is confirmed by our research in the Belgrade area. For these reasons should be given much more attention to the parasitology control of these areas, clean them and take all measures to prevent faecal contamination with dog feces.

In order to solve this environmental problem in Belgrade is approached by adopting Strategies to solve the problem of non-proprietary dogs in the city of Belgrade, the introduction of eco-zones or parks for dogs, baskets with plastic bags to collect feces of dogs from the streets by the owners and regular parasitological control of grassy areas and dogs that are found there (Terzin et al.2012) The results obtained showed that the degree of contamination of the parasitic reduced by 45% as compared to the past studies (Terzin and Pavlović 2012).

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IDENTIFICATION OF STRAINS BELONGING TO QX PATHOTYPE IN AN OUTBREAK OF AVIAN INFECTIOUS BRONCHITIS

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Abstract
In infectious bronchitis virus(IBV) were identified several serotypes (pathotypes) known to infect the respiratory system, the ovaries and oviduct, kidney and glandular stomach. The research was conducted in a flock of 10,500 broiler, Cobb hybrid, reared to the ground. Chickens of these outbreak were vaccinated against IB with a vaccine containing the vaccine strain H120, one day age, with a vaccine that contains the vaccine strain of Ma 5 to 21 days of age. For detection of viral RNA samples were taken from lungs, kidney and proventriculus, that were frozen until they are used.Viral RNA was detected RT-PCR. Were used design primers, for serotype Massachusetts, for serotype QX and for serotype It02, positive controls were vaccine strains: IB 88,Ma 5, H 120 and H 52 and negative samples were strains LS 79 and PCR sterile ultrapure water (Promega). During the research tests used were constructed based on gene sequences, which encodes glycoprotein S1, present in a Gen Bank, for discrimination Massachusetts serotype strains, of classical vaccine strains(H52, H120 și Ma 5), of strains from QX serotype and strains of serotype It 02. Identification of strains of serotype QX has been correlated with the presence of proventriculus lesions induced by these strains and represented by the increase in volume of proventriculus and hemorrhagic ulcers located in the lining of the organ. The results demonstrate the movement strains of serotype QX in broiler flocks and represents the first signal of these strains in Romania.
Keywords: *IB virus, serotype QX, RT-PCR;*

Avian infectious bronchitis (IB) is an infectocontagious diseases caused by avian coronavirus that develops in chickens and adult poultry (1). In infectious bronchitis virus(IBV) were identified several serotypes (pathotypes) known to infect the respiratory system, the ovaries and oviduct, kidney and glandular stomach (1)

Since 1996, in broiler chickens have been reported strains, known to infect proventriculus, being named ”proventricular type” , wich was included in serotypes (pathotypes) QX și Q1 (2,3).

Intense trade with poultry material (eggs, broiler chickens, young chickens), contributed to the spread of these pathotypes, including in Romania, to outbreaks of infectious bronchitis evolving range anatomoclinical.

Materials and methods

The research was conducted in a flock of 10,500 broiler, Cobb hybrid, reared to the ground. Chickens of these outbreak were vaccinated against IB with a vaccine containing the vaccine strain H120, one day age, with a vaccine that contains the vaccine strain of Ma 5 to 21 days of age.

At the age of 28 days, in this outbreak increased morbidity and mortality. To establish the diagnosis were carried epidemiological, clinical and anatomopathological exams, and for confirmation were performed ELISA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

For detection of viral RNA samples were taken from lungs, kidney and proventriculus, that were frozen until they are used.Viral RNA was detected RT-PCR.

With Q/AmpRNA minikit (Qiangen 52906), viral RNA was extracted, and the confirmation was carried out by gel electrophoresis (RT-PCR SYBR GREEN), with

spectophotometric amplifier Mx 3005R Stratagene. Were used design primers, for serotype Massachusetts, for serotype QX and for serotype It02, positive samples were vaccine strains: IB 88,Ma 5, H 120 and H 52 and negative samples were strains LS 79 and PCR sterile ultrapure water (Promega).

Results and Discussion

The results obtained in these researches are shown in fig. 1. Analyzing the results is observed in samples taken, from broiler chickens carcasses, 4 strains were detected, assigned to serotype (pathotype) QX, but were not detected strains belonging to serotype It 02 and to serotype Massachusetts including classical vaccine strains.

IB virus has several serotypes (pathotypes) which included strains with respiratory, genital and proventricular tropism. Specific importance of these serotypes is given that post-infectious immunity or post-vaccination immunity induced by strains of a serotype, not protect against infection with strains of other serotypes.

The avian coronavirus genome RNA is single stranded, unsegmented, of 27 to 30 Kb, with positive polarity. For these reasons, the viral RNA can be moved immediately by the host cells, similar to a messenger RNA, this particularity giving raised infectivity. The viral genome encodes three structural proteins having the role in the pathogenicity and immune response after infection or post vaccination represented by S glycoprotein (spike), glycoprotein M (of membrane) and nucleocapsid protein (N) (1,4).

The glycoprotein S, located in pericapside spikes,has an adhesin role for the attachment of viral particles to the host cells and contains the most important epitopes responsible for the induction of neutralizing antibodies. Due to these epitopes are serological differences between strains, and on this basis the virus strains are classified into several serotypes.

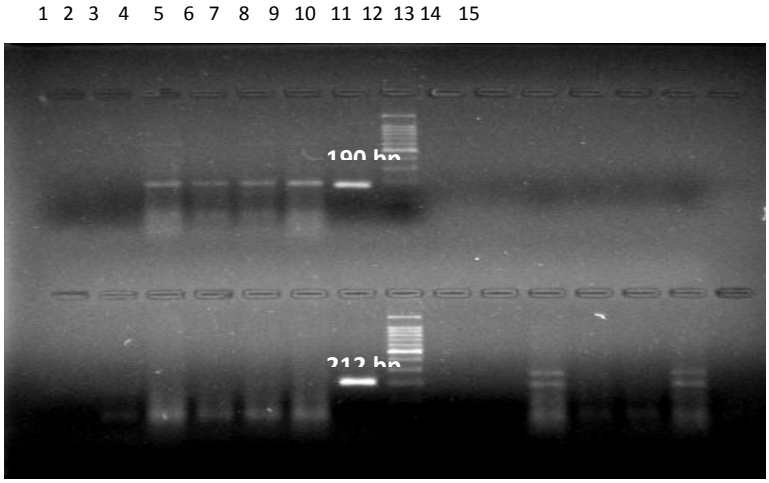
During the research tests used were constructed based on gene sequences, which encodes glycoprotein S1, present in a Gen Bank, for discrimination Massachusetts serotype strains, of classical vaccine strains(H52, H120 și Ma 5), of strains from QX serotype and strains of serotype It 02. In the researches were not detected strains from serotype It 02 and vaccinal strains H52, H120 and Ma5.

Identification of strains of serotype QX has been correlated with the presence of proventriculus lesions induced by these strains and represented by the increase in volume of proventriculus and hemorrhagic ulcers located in the lining of the organ. In that outbreak, sick chickens have evolved following symptoms: depression, eyelid edema, diarrhea, dyspnea and nasal discharge, and mortality was 10%.

The results demonstrate the movement strains of serotype QX in broiler flocks and represents the first signal of these strains in Romania.

These strains have been identified, for the first time, in China from where spread including our country, with the sale of poultry (2,3).

The researches show that it is necessary to study outbreaks of IB, that evolves in broiler flocks in Romania, to identify strains of avian cornavirus, in order to develop strategies to control the disease.



Confirmation by gel electrophoresis for pentru IBV rPCR Sybr Green for type / serotype Qx (CR/FR, 4/91) A, lines 1 – 7, for type / serotype Mass (H120 / H52 / Ma5), B, lines 1 – 7, and for type / serotype It02, B, lines 9 - 14. Lines 1, 9: negative samples, ultrapure water(NFW, Promega); lines 2, 10: negative samples LS79 (NDV, Pasteur). Lines 8: Standard ADN: 100 bp DNA Ladder (Promega).

A: line 3: lung 3554 (positive Qx, amplicon 192-200 bp); line 4: lung 3555 (posive Qx, amplicon 192-200 bp); line 5: lung 3556 (positive Qx, amplicon 192-200 bp); line 6: lung 3557 (positive Qx, amplicon 192-200 bp); line 7: positive sample H120, Merial for Qx (amplicon 190 bp).

B: line 3: lung 3554 (negative Mass); line 4: lung 3555 (negative Mass); line 5: lung 3556 (negative Mass); line 6: lung 3557 (negative Mass); line 7: Positive sample Ma5, Intervet for Mass (amplicon 212 bp).

B: line 11: lung 3554 (negative It02, nonspecific amplicons); line 12: lung 3555 (negative It02); line 13: lung 3556 (negative It02); line 14: lung 3557 (negative It02, nonspecific amplicons).

Conclusions

Using RT-PCR were identified, for the first time in Romania strains belonging to serotype QX, which caused an outbreak of BIA in broilers.

The vaccines used did not protect chickens against strains of serotype QX, due to antigenic differences.

To prevent BIA in broiler flocks should be carried out screening examinations, in order to identify circulating strains to substantiate immunoprophylactic strategies.

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TAQMAN REAL-TIME PCR ASSAY FOR DETECTION OF *ESCHERICHIA COLI O157:H7* IN BEEF

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Abstract
The aim of this study was to detect and quantify E. coli O157:H7 in naturally and artificially contaminated beef samples by a TaqMan PCR assay. The detection of E. coli O157:H7 in fresh beef has performed by EN ISO 16654/2001 standard method. The TaqMan real-time PCR assay (Applied Biosystems), requires specific primers and a TaqMan probe. The target gene was rfbE.All beef samples that we investigated were negative for E. coli O157:H7. The specificity of the TaqMan PCR assay was 100%, and detection limit was 10 ufc/ml in pure cultures and 100 ufc/ml in artificially contaminated samples. These results shows that the prevalence of E. coli O157:H7 is very low, but this pathogen could serve as a potential risk for consumers if proper hygienic and cooking conditions are not maintained, also, rapid and efficient methods, such as real-time PCR are required for food pathogens detection.
Kay words*: detection, E. coli O157:H7, real-time PCR, fresh beef;*

Introduction

Escherichia coli O157 is one of the most dangerous pathogens that cause hemorrhagic colitis and severe hemolytic uremic syndrome (HUS), which may result in death because of the acute or chronic renal failure. The *E. coli O157* is commonly found in ground beef, raw milk, cold sandwich, vegetables and drinking water (Oberst et. al., 2003, Guangfu et. al., 2006). Food poisoning by *E. coli O157* occurred in many countries, including the U.S., Canada, Japan, England or Sweden. *E. coli O157* serotypes were first isolated from the feces of a patient suffering from hemorrhagic colitis in 1986 in China. The infective dose of *E. coli O157:H7* is extremely low. Some reports show that fewer than 10 bacteria would cause disease (Si et. al., 2006).

Traditional culture methods based on biochemical characteristics are labor intensive and typically time consuming, which would be easily overwhelmed during outbreaks. Enzyme immunoassays based on detection of verocytotoxins can provide false-positive results, therefore, due to its sensitivity and efficiency, polymerase chain reaction (PCR) assays, have been integrated to detect this pathogen in food or other biological samples.

Materials and methods

Classical microbiological method

The 40 (27 fresh beef and 13 minced beef) were first processed by classical microbiological methods to obtain the presumptive strains of *E. coli O157:H7.*

Food samples were performed in Food Safety Laboratory (Iasi Veterinary Medicine Faculty), according to the EN ISO 16654/2001. The initial dillutions containing 1 g of sample and 9 ml of mTSB – modified Tryptone Soya Broth (Oxoid LTD., Basingstoke, England) with novobiocin were incubated at 41,5ºC, 18-24 hours. An aliquot of 1 ml was used for selective isolation on SMAC - Sorbitol MacConkey agar (Oxoid LTD.,

Basingstoke, England) and incubated at 37°C, 18-24 hours to observe the typical colonies of *E. coli O157:H7*. The confirmation includes biochemical and serological tests.

Specificity of the TaqMan real-time PCR assay

For specific detection of *E. coli O157:H7* we used three strains that belong to *EHEC O157:H7* serotype *(E. coli O157:H7 EDL, FMVB* and *ATCC 2904i*) and other ten strains included in *fam. Enterobacteriaceae*: five strains of *E. coli biotype 1*, one strain of *Enterobacter spp*., one strain of *Proteus spp*., one strain of *Klebsiella spp*. and two reference strains of *Salmonella*: *S. enteritidis ATCC 13072 and S. tiphymurium ATCC 14028).*

Detection limit of the TaqMan real-time PCR assay

The detection limit of the assay was tested with five serial dilutions of DNA (10^1-10^5 CFU/ml) of from pure enriched culture of *EHEC E. coli O157:H7 EDL*. A standard curve was obtained plotting the number of CFU/ml against the Ct value. The DNA samples were analized in triplicate. For artificially contamination, 1 g of minced beef was inoculated with 9 ml of each dilution of a reference strain of *E. coli O157:H7 ATCC 2904i*.

DNA extraction

The extraction of bacterial DNA was performed with PrepMan Ultra (Applied Biosystems) from enrichment tryptone soya broth. One ml was transferred in the 2-ml microcentrifuge tubes and centrifuged for 3 minutes at 14000 rpm, to pellet bacteria and residual food or other debris, and then the supernatant was removed. The cell pellets were resuspended in 100 µl reagent and placed in a 100ºC heating block for 10 minutes, and cooled at room temperature for 2 minutes. The tubes were centrifuged at 14000 rpm for 3 minutes and the supernatant was transferred in other tubes. We used 2,5 µl of DNA solution for a PCR reaction.

Real-time PCR conditions

Oligonucleotide primers, TaqMan probe (table 1) and real-time PCR conditions were adopted from a previous study (Sharma V.K., 2006), targeting a 129-bp amplicon from the *rfbE* gene of *E. coli O157:H7*.

Reactions and data analysis were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Amplification reaction (25 µl) contains: 2,5 µl DNA sample, 12,5 µl TaqMan Environmental Master Mix 2X, 0,5 µl of each F and R primers (100 µM), 0,25 µl TaqMan probe (50 µM), 8,5 µl RNase/DNase-free water. The PCR conditions were: 3 minutes at 94ºC for enzyme activation and DNA denaturation, followed by 40 cycles: 94ºC 30 s., 55°C 30 s and 72ºC 1 min.

Table 1. Primers and TaqMan probe for real-time PCR (Sharma V.K., 2006)

Microorganism	Target gene	Primers and TaqMan probe structure
E. coli O157:H7	*rfbE gene*	F 5' – TC AA AA GG AA AC TA TA TT CA GA AG TT TG A-3' R 5'–CG AT AT AC CT AA CG CT AA CA AA GC TA A-3'
	TaqMan probe	TET-AA TA AA TT TG CG GA AC AA AA CC AT GT GC AA-TAMRA

Results and discussions

None of the forty fresh beef and minced beef was contaminated with *E. coli O157:H7*. Although *E. coli O157:H7* has been commonly isolated from beef and beef products in many studies, there are only a few number of studies about the prevalence of this pathogen in beef and beef products in Romania. These studies revealed that this serotype is rarely found. Mocuţa et. al., (2001) found one strain which belongs to serotype *O157:H7* from 87 strains of *E. coli* isolated from different food and Drăgan G. (2008) isolates 7 strains of *E. coli O157:H7* in meat and meat products and 6 in cow and sheep milk products. Nica et. al. in 2009, found a prevalence of 0,5% (2 strains belonged to serotype *O157:H7*) in two patients feces. The pathogen is generally present in the intestine of animals, particularly in cattle, without causing disease. *Stx*-producing *E. coli* also have been isolated from the feces of chicken, goats, sheep, pigs, dogs, cats, and sea gulls.

Elder et al., in 2000, studied the association between *E. coli O157:H7* carriage and carcasses contamination during cattle slaughter. In 30 groups studied, the authors found a prevalence of 20% in the cattle feces, but the number of carcasses contaminated with *E. coli O157: H7* was higher, of 43% before evisceration stage.The high prevalence of the carcasses contamination can be explained due to cross-contamination that can occur during the processing stages, most likely during skinning and evisceration, when meat is contaminated with feces.

The specificity of TaqMan PCR assay

The specificity of the primers and TaqMan probe was 100%. The inclusivity and exclusivity were 100%. No false negatives, false positives or cross-amplification were observed during specificity testing. All three reference strains of *E. coli O157:H7* were positive for *rfbE* gene and gave a strong fluorescence signal. For the exclusivity test we used other ten strains of non-*O157:H7*, which were negative for *rfbE* gene and had only minimal background signals. The results are presented in table 2.

The *rfbE* gene, encoding an enzyme necessary for O antigen biosynthesis, is highly conserved in *E. col iO157* serotypes (Sharma., 2006).

Table 2. Specificity of TaqMan real-time PCR for *rfbE* gene

E. coli O157:H7	No. strains	CT Mean values	*non - E. coli O157:H7*	No. strains	Results
E. coli O157:H7 EDL	1	+ 21,9	*E. coli* bovine feces	2	-
E. coli O157:H7 FMV B.	1	+ 16,8	*E. coli* poultry feces	2	-
E. coli O 157:H7 ATCC 2904i	1	+ 19,4	*E. coli* calf feces	1	-
			Enterobacter spp.	1	-
			Proteus spp.	1	-
			Klebsiella spp.	1	-
			Salmonella enteritidis ATCC 13072	1	-
			Salmonella tiphimurium ATCC 14028	1	-

Although, *rfbE*-based RT-PCR assay is suitable for specific detection of *EHEC O157:H7*, it can produce false positive results for those *E. coli* strains that possess *O157*-type O antigen but do not belong to *EHEC O157:H7* serogroup.

*The detection limit of TaqMan real-time PCR as*say

To establish the PCR detection limits, triplicate reaction for each serial dilution were prepared. The standard curve of *E. coli O 157:H7 ATCC 2904i* showed a linear correlation between the values of C_t (threshold cycle) and cell numbers (cfu/ml). The Mean C_t values ranged between 21,5 to 37,5 in pure culture and 17,8 şi 36,01 in artificially contaminated sample. The detection limit of the TaqMan real-time PCR assay was 10 ufc/ml in pure culture and 100 ufc/ml in artificially contaminated meat. The results are showed in table 3.

The use of sensitive, quantitative methods for detection of *E.coli O157:H7* during food processing could be used to determine the stages where contamination occurs and where controls could be introduced to reduce or eliminate this pathogen from retail food products, thereby reducing the risk to the consumer (Sarimehmetoglu B., et. al., 2009).

Table 3. Detection limit of *EHEC E. coli O157:H7* in pure cultures and artificially contaminated samples

EHEC **UFC/ml**	**DNA from pure culture** ***E. coli O157:H7 EDL***			**DNA from artifcially inoculated samples** *E. coli O 157:H7 ATCC 2904i*		
	CT Mean	**SD**	**Q ng/µl**	**CT Mean**	**SD**	**Q ng/µl**
10^5	**21,5± 0,1**		**1,8**	**17,8±0,1**		**1,3**
10^4	**23,8 ±0,1**		**0,18**	**22,3± 0,2**		**0,13**
10^3	**26,2 ±0,1**		**0,01**	**25,6±0,2**		**0,01**
10^2	**29,4 ± 0,4**		**0,001**	**33,2 ±0,3**		**0,001**
10^1	**37,5 ±0,8**		**0,0001**	**36,01±2,2**		**0**

Conclusions

1. None positive sample was find in fresh beef and minced beef. Foods of animal origin, in particular beef and beef products, have been identified as a risk factor for human infection.
2. Real- time PCR is an alternative method that can be used in detection of *E. coli O157:H7* in enriched samples. It is a fast, specific and sensitive method which can be performed in 24 h.

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THE DISTRIBUTION OF RAMBON DUCK POPULATION BASED ON GEOGRAPHIC INFORMATION SYSTEM TECHNOLOGY

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Abstract

This study was conducted to determine the distribution map of rambon duck population based on information technology in Cirebon District, West Java, Indonesia using Lansat (Land Satelitte). The purpose of this study is to get the information that support development planning of rambon duck. The method of this study is a non-experimental method with descriptive data analysis towards overlay form information about poultry population and land vegetation which is supporting area. Support equipments including Satelite Imaginary Landsat ETM-7, ER-Mapper software, and ArcGIS software. Conclusions of this study obtained there is variation population distribution of Rambon Duck which can be divided into several clusters.

Key words: *Rambon Duck, population distribution, information technology, GIS*

Introduction

The increasing of poultry resources in Indonesia has been done with development based on local poultry which needs integrality study in bio-socio-economic system. The most important aspect in this system is apropos local poultry's genetic potential inventory that gives best contributions in one area[2].

Most of local duck in Indonesia has not been classified as original family. The existence as one of the local resource needs to be conserved. Tegal Duck, Alabio Duck, Mojosari Duck, Magelang Duck, and Rambon Duck are some from many type Ducks in Indonesia[3].

Rambon Duck, often called by Raja Cirebon Duck, is Cirebon's duck race. This type of duck is one of the germplasm poultry that play role in fulfilling the needs of animal protein, and as a source of income for farmers [4].

Along with the advance development, dissemination and exchange of technology and information could be perceived by all people quickly and easily, for example Geographic Information System [6]. This system is used for entering, keeping, processing, analyzing, and generating geographically referenced data or geospatial data, which used to support the decision making in planning and managing land use, natural resources, environment, and another public service facilities [5].

Rambon duck are concentrated at north area in West Java such as Cirebon, Indramayu, and Subang. These areas are known as rice-producing areas, and also have port and fishermen. Geographic Information Technology Application is used to describe the distribution of Rambon Duck population. The purpose is to get the availability of food and areal climate information, which hopefully can be one of supporting materials in decision making process for the farm developers [6].

Material and method

1. Research Location

The objects in this research are located in Cirebon, West Java, Indonesia. This area is known as the source of Rambon Duck. It located at 108°40'-108°48' EL and 6°30'-7°00' SL (Figure 1).

Figure 1. Cirebon District, West Java, Indonesia

2. Research Equipment

In the implementation of this study the authors use some support equipments, including:

1. Satellite Imagery Landsat-7 ETM Cirebon area
2. One set of computer
3. ER-Mapper software and ArcGIS.
4. Rambon Duck Population Data in Cirebon

3. Research Method

The method used in this study is non-experimental method with overlay descriptive analysis. This method shows the integration between Cirebon area map and duck population data. The land cover data is verified by secondary data from Annual Activity Report of The Department of Agriculture Cirebon.

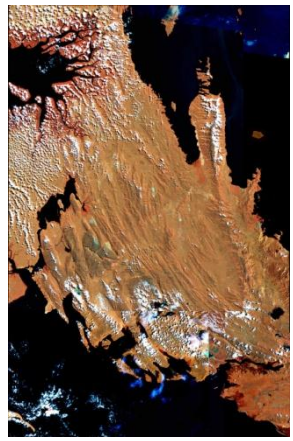

Figure 2. Lansat-7 ETM Satellite's image [6]

Results and discussion

Social Mapping to Support Rambon Duck

Cirebon district is part of West Java Province, bordering with Indramayu District in the north, Majalengka District in the west, Kuningan District in the south and Brebes District in the east. The topography of this area is lownland between 0-10m from sea level in the north and 11-130m from sea level in the south.

Agricultural sector is a leading sector for community in Cirebon District. Agricultural sector contributes more than 30% to the Gross Areal Domestic Income. This sector includes food crops, plantation, animal husbandry and fishery. Food crops include rice, corn, tubers and beans. Rice production data showed that with 10.532 hectares harvest area can produce 71.829 tons rice. In 2010, production reached 544.785 tons [4].

Distribution of Rambon Duck Population

Local duck population in Cirebon District, based on statistic data in 2010, is 1.918.631 ducks, distribution in north beach of Java. Results image from GIS Satellite Data is shown below [6].

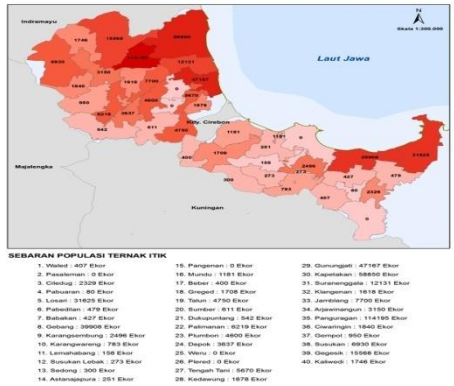

Figure 3. The Distribution of Duck Population

Based on Distribution Map, duck population in this area is varies. Meanwhile there are some areas not having local duck population, such as Pasaleman, Pangenan, Weru and Plered. Generally, these areas are industrial and trade bases, densely populated by people. Most of area which having Rambon Duck is agricultural base areas. These areas have enough irrigation supply, parallel with Rambon Duck as a waterfowl. Water supply came from wastewater irrigation and large river, which is natural existence where ducks lives.

Illustration above explained that Rambon Duck which spreading in Cirebon District have adequate capacity according to waterfowl ecosystem paradigm. However, according to the area, Cirebon has fisherman port in the north which has a great influence for duck population development in Cirebon. These ports located in Gebang and Gunungjati.

Rambon Duck Basis

Relatively, there are 34 locations in Cirebon Districts that have duck population. Some area or locations called duck population basis if there are have duck population above

the average constantly. Constant population shows the presence of poultry, farmers, and stratifications of land use to support continuously.

Results from this research showed that population continuity determined by the existence of organized farmers institutional. The existence of Rambon Duck also determined by the existence of elite groups that produces basic seeds (foundation stock) on community level [1].

There are two elite groups in Cirebon that produce Rambon Duck :

- *Branjangan Putih Muda,* located in Losari, with 10.000 population ducks. This group produces eggs and meats. There are two types of eggs, consumption egg and hatch egg. Hatch egg was chosen from duck and drake that have of production eggs characteristics above the average. Day Old Duck (DOD) with high quality are distributed to Indramayu, Subang, Jakarta, and Central Java such as Brebes, Tegal, Pemalang and Pekalongan.
- *Tigan Mekar,* located in Kroya, Panguragan. The institutional of this group is different from the group in Losari, specifically only produces Rambon Duck. The hatch egg are obtained from farmers around Panguragan and Kapetakan.

The existence of Rambon Duck nursery and the distribution are shown in satellite image (Figure 4).

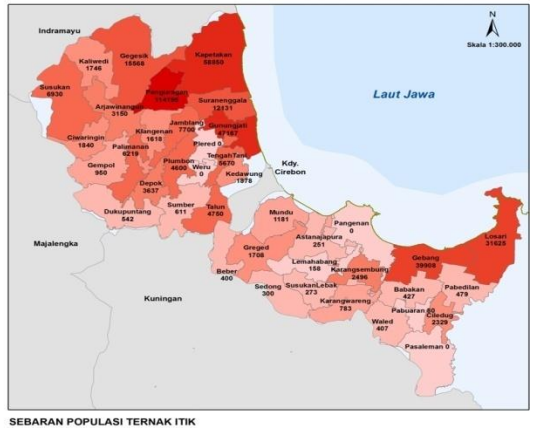

Figure 4. Rambon Duck Population Basis in Cirebon Map

According to satellite image from GIS technology, population basis can divided into clusters.

Cluster 1 : Dark red, shows area with high population more than 31.000 ducks. This cluster includes Losari, Gebang, Panguragan, Gunung Jati dan Kapetakan.

Cluster 2 : Red, shows area with moderate population, between 5.000 – 10.000 ducks. This Cluster includes Gegesik, Susukan, Suranenggala, Jamblang, Plumbon, Palimanan, dan Tengah Tani.

Cluster 3 : Light red, this area hardly called as population basis. This area only have several ducks and far from fish resources [6].

Results from this clustering explain Rambon Duck population basis is:

1. Losari, the population in 2010 is 31.325 ducks. In 2012 increase by 5%, along with increasing needs of duck meats.

2. Gebang, the population in 2010 is 39.908 ducks. Rambon Duck population distributes as primary producer of eggs.
3. Panguragan, basis with higher population, 114.195 ducks.
4. Gunungjati, the population is 47.167 ducks.
5. Kapetakan, the population is 58.850 ducks.

Saturated Area Duck Population in Cirebon

Geographically, Panguragan area is a saturated area. Saturated area, theoretically, can be seen from the balance between population and land support capacities. However, descriptively, can be seen higher and constant number from year to year. Saturation of the population in one area is caused by some factors:

1. The availability of the land is maximal. So that if there is a poultry immigration, stock farmers will eliminate previous population and there will be a place for the next population.
2. The increasing number of farmers from year to year.
3. The availability of duck food resources [7].

Development Area of Duck Population in Cirebon

The area that should be developed as Rambon Duck conservation area is Cluster One area, Cluster Two area which near the fishermen ports, and Cluster Zero area which near sea fish resources.

1. Cluster 1 (One) Area

There are seed resources that should be developed in Losari and Gebang. So it became people responsibility to develop this area. The development can be used of new technology, subsidized production facilities, and increasing of seed price.

2. Cluster 2 (Two) Area

This area is intended for the population which has the best support capacities, such as Gegesik, Suranenggala, Jamblang, and Tengah Tani. This four geographic areas is second in areas that located near the sea, however the transportation to the Kapetakan and Karangampel sea is quiet good, so that should be developed. Jamblang should be developed because the large paddy area and has more access of resources to Gunungjati.

3. Cluster 0 (Zero) Area

In this area, there are some locations that have good support capacities, such as Pangenan, Pabedilan, Astanajapura, and Mundu. So the development in these areas should be done by developing new farmers, and strengthening the farmer institution to diversification duck farm.

Conclusion

Rambon Duck population distribution in Cirebon District using Geographic Information System based on Basic Technology showed there is variation population which can be divided into several cluster.

Rambon Duck Population Distribution Map based on condition of Cirebon district land support capacity conducted through increasing population in cluster 2 (two) area and cluster 0 (zero) area, increasing the seeds price, and increasing the number of new farmers through strengthening the institution and input cultivation technology of Rambon Duck.

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RESPONSE OF SELF SUPPORTING PROGRAM OF CATTLE BREEDER GROUP IN CIAMIS DISTRICT

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Abstract

This study aims to determine the response of a group of farmers to beef self-supporting program. Methods used are qualitative methods to groups of farmers which were selected intentionally / purposive. Each farmers from each group explained holistically and openly about their efforts in improving maintenance effort of cattle. The research was conducted from October 2012 to December 2013 in Ciamis District, West Java, Indonesia. The results showed that the groups' response to beef self-supporting program variated, between positive response, passive/ zero and negative. Groups of farmers in this study sites are divided into two categories, namely the assistance recipients in the form of cattle or money, and those who did not. The groups who receive assistance provide two responses, namely positive and negative responses, whereas those who did not receive help provide passive response / zero and negative. In the first group, positive response demonstrated through success in business, it is characterized by an increase in income and social status. Furthermore, the negative response group was characterized by the absence of an increase in business, even the tendency to stop their business. This is due to the assistance received is distributed to members, or sell it as capital. In the second category, ie the groups who do not receive assistance, also gave two responses, namely passive response / zero and negative. Passive response groups are farmer groups with characterized by the development of their business or be stagnant, because the maintenance of cattle just for that other state or enjoyment of their hobby. The system in this group is not going well. Breeders can maintain an individual basis, selling cattle without the consent of the group, and the breeder also has another job as a farmer. Latter is not a beneficiary farmer groups and gave negatively response. Independent farmers groups, selling the cattle owned then change the business by raising goat or brown sugar business, without any attempt to buy back the cow. The system in this group do not done according to the program, and this group has lower of the cattle population.

Keywords*: Group breeders, positive responses, negative responses, passive responses / zero.*

Introduction

The construction of livestock sub-sector is one part of the agricultural sector in Indonesia. The success of livestock sub-sector in increasing farmers' income and welfare of farmers in the countryside to be a common goal. Livestock in Indonesia has the potential of substantial resources and has investment opportunities in development. Resource big enough to be managed by qualified human resources.

Response to the process preceded is a person's attitude, because attitude is a tendency or willingness of a person to behave, when he faced a certain stimulus.So, talk about the response or no response can not be separated from the discussion of attitudes. Response also defined as behavior or attitude that is both tangible so yet detailed understanding, assessment, or the influence of rejection, like it or not, and utilization at a certain phenomenon (Sarwono, 1998). Response or responses is experienced impressions if stimulus is not there. If the observation process has stopped, and just stay impressions, such events is called a response. The definition is a picture in response to a warning from the experience (Kartono, 1990).

The magnitude of the potential contribution of the agricultural sector in West Java to economic development is inseparable from the position in the structure of the livestock sector

in the economy. Contributions economic growth in the agricultural and livestock sector to the regional apparently showed a trend is increasing over time. One of the districts that serve as the executor of beef self-sufficiency in the province of West Java is the Kudat District. This is because until the year 2010, this district is the largest contributor to the cattle population in West Java with a number of ± 37 397 head of cattle (Jawa Barat dalam Angka, 2011). Kudat District has total area of 740.76 $km^{2,}$ with the area of Kudat District population density is 558.74 people / $Km^{2.}$ (Jawa Barat dalam Angka, 2011).

Table 1.1. Beef Cattle Population in West Java and Kudat District (Target Population and Population Outcomes).

Year	Beef Cattle (heads)					
	West Java	Target Population	Achievement Population	Kudat District	Target Population	Achievement Population
2000				21 370		
2001	189 518			22 199		
2002	205 843			22 532		
2003	223 818			24 781		
2004	232 949			26.440		
2005	234 948			27 794		
2006	254 243			29 789		
2007	272 262			31 837		
2008	295 554			34 292		
2009	309 609	309 609	309 609	36 020	36 020	36 020
2010		319 128	32 7. 750		37. 090	37 129
2011		332 012	422 989 *		38. 574	37 397 *
2012		345 318	44 1,350 (*)		40 117	38 945 (*)
2013		359 819			41 721	
2014		383 548			43 397	

Source: Figures of West Java in 2011; Kudat District in Figures 2000-2012;
* Kementerian Pertanian (2011); (*) Figures While the Animal Husbandry Department of West Java Province.

Program of national self-supporting in meat since year 1995 - 2000 is referred to as meat self-supporting programs on trend expected means at least 90% of the available supply in the country, so that only a certain time can be done import. Year 2000 to 2005, the government issued a policy on beef sufficiency program, in 2005-2010 accelerated beef self-supporting program. Sufficiency beef listed in the Minister of Agriculture Number: 59 / Permentan / HK.060 / 8/2007 on Guidelines for Achieving Self-Sufficiency Acceleration Beef (P2SDS). In this case it is expected that by 2010, beef demand for people already in the country can be met at a minimum of 90% or 281.90 thousand tons (Permentan, 2007). However, the program is not reached and doomed to failure. Further more, the Ministry of Agriculture in the Ministry of Agriculture Strategic Plan years 2010-2014 were compiled in order to meet the mandate of the Act No.25 Year 2004 on National Development Planning System re-launched as a commodity beef self-supporting target by 2014, with the main target production reached 550 thousand tons in 2014.

The purpose of the study was to determine the response of farmers to beef self-supporting program and what factors affect the response. Hoped this research can open-ideas and the ideas for researchers, universities and related institutions, and even groups of cattle ranchers in supporting beef self-supporting program in 2014.

Focus Research

Based on the background and the phenomena that occur, related to meat self-supporting program that had failed to accomplish with this problems, then formulate a research problem, namely how the response shown by the group of farmers to policy implementation beef self-supporting program in Kudat District Province West Java.

Research Questions

Based on the research focus, it can be stated that one of the research questions: How is the group response of farmers against beef self-supporting program (PSDS)?

Research Methods

This study used a qualitative method using four main techniques in the investigation, namely through participant observation, in-depth interviews, documentation, and triangulation (Sugiyono, 2010) to determine and study the life of a group against the cattle ranchers beef self-supporting program. Qualitative research does not use the term 'population' but by Spadley in Sugiyono (2010) called social situation that consists of three elements, namely place, actors, and activity that interact synergistically. The situation can be expressed as the object you want to know what's going on inside the program (Prastowo, 2012).

Source of data collected in this qualitative study is primary data and secondary data. The main data sources used in this research is a statement and action sources, field notes, descriptive data, and other informants in groups/groups of cattle ranchers. Informant ie those observed in the group, that provide data and information in the form of statements, words, or actions, and to know and understand the issues that are being investigated. The determination was performed according to the informant expected goals and objectives, which can be present and can describe the characteristics of the object of research. Key informants is directed to provide the data and correct information. The fact required includes the words, actions informant, information and testimony regarding the response of farmers to the group policy implementation beef self-supporting program (PSDS) in Kudat District of West Java Province.

The researcher as instrument, other instruments is equipped notebooks, tape recorders, cameras and so on, such as the Guidelines for observation (simple observation and non-participant observation), interview (open interviews and interview survey), and the use of documents (documents, photographs, recordings). Researchers in this study are as primary data collectors, but could also be assisted by the other party.

Stage and Procedures Research

The study began with a pre-field steps, ie research plan compose in accordance with the research context. The next phase of field work done, to understand the background starts since entering the research field, gathering the data, each of the activities noted in the daily memo, recorder, whether it be the result of in-depth interviews, focus group discussions, observations, or findings of the documents; then use triangulation techniques from the same source simultaneously and continuously conducted until data saturated.

Data Analysis Research

Analysis data using qualitative research methods approach is carried out continuously from the beginning to the end of the study; with inductive, and look for patterns, themes and

theoretical models (Prastowo, 2012). Furthermore, this study uses inductive-abstractive logic, a logic which starts from "particular to the general." Conceptualization and developed on the basis of a description of events *(incidencei)* obtained when the fieldwork took place. By the reasons between data collection and data analysis take place simultaneously or in progress simultaneously, even the data collection as well placed as an integral component of the data analysis activities (Bungin, 2010). Data analysis in qualitative research carried out before entering the field, as long as at field, and upon completion in field. Furthermore, according to Moleong (2011), declared analysis has commenced since formulate and explain the problem, before plunging into the field, and continues until the writing of research results.

Location and Schedule Research

This research was conducted in Kudat District of West Java province's response to the topic of the research group of farmers to policy implementation of self-supporting program. Study has been carried out from October 2012 to December 2013. Plan for more research activities are listed on the schedule of the study. Research activity is regulated in order to study the research schedule and neatly structured.

Results and Discussion

The results of group of breeders to study the response of beef self-supporting program in Kudat District of West Java Province shows that the policy of beef self-supporting program (PSDS) in 2014 has been implemented up to farmer group level. This self-supporting program was implemented since 2014 in the district of Kudat up to download the farmer groups level, have described that the PSDS program issued by the Ministry of Agriculture through the Directorate General of Livestock and Animal Health at national level, provincial level and district level is going according to the program. Generally speaking, the PSDS program has reached the level of farmer groups, right on target, but it has not been uniformly accepted by groups of farmers in the district of Kudat. Programs and policies that have not been uniformly demonstrated the presence of a group of farmers who receive aid and the presence of a group of farmers who did not receive assistance.

Farmer groups that receive assistance and farmer groups who do not receive help show differences in behavioral responses to the program or PSDS. Group of breeder's program recipients responding PSDS with positive and negative responses, while for the group of farmers who did not receive assistance programs responding to the response PSDS are zero and negative responses. The big difference in the level of groups of farmers receiving assistance, causing the targeted goals of PSDS national self-sufficiency in beef realization in 2014 is still pending or has not been achieved, due to the aid of farmers have not been evenly grouped.

The national level PSDS program can not be realized in the year 2014, but the purpose is specifically for beneficiary farmers group has been realized with an increase in income / welfare in a group of farmers and ranchers group members. Furthermore, in addition to the increase in revenues, beneficiary farmer groups receive recognition from the local community and was followed by an increase in the social status of the farmer group members.

Table 2. Beef Cattle Breeders response group in Kudat District

No.	Breeders Group	Group		Response Group Breeders		
		Beneficiaries	Inappropriate Receiving Help	Positive	Zero Fixed	Negative
1	Aware works	v		v		
2	Minaharja Sari	v		v		
3	I Prosperous	v		v		
4	Jackfruit Pandak	v		v		
5	Rays Saluyu	v		v		
6	Rancage II	v		v		
7	Harmonious	v		v		v
8	Maesa Jaya	v		v		
9	Sangkan Hurip	v		v		
10	Sabilil MUT	v		v		
11	Da'arul Falah	v		v		
12	Sri Rahayu	v		v		v
13	Binawarga		v		v	v
14	Mukti Sugih		v			v
15	Love Nature		v			v
16	Bangkelung 3		v		v	
17	Jagaraksa		v		v	
18	Wargi Bloom		v		v	
19	Bloom		v			v
20	Source Lucky		v			v

Source: Research Findings, 2013

The results showed that the PSDS program up to level group has been successful, whereas the goal of national beef self-supporting program in 2014 can not be realized, yet realized its program of national self-supporting in beef, because of the factors help the farmer group level, not evenly acquired by farmer groups.

The results of the study can be explained and can give an idea of the national beef self-supporting program in 2014 in the district of Kudat. The response of farmers groups to beef self-supporting program, there are three different responses from the resource persons who have been in this research is a positive response, the response zero / fixed and negative responses.

Response Breeders Group Beneficiaries

Based on the results of this research on the response of farmers to the group policy implementation beef self-supporting program in Kudat District of West Java province on the results obtained by farmer groups of beneficiaries in the study. Farmer groups beneficiary

obtained after fixing sources by 2011 and in line with the research process took place in 2013, where there are groups of farmers with a population increase but there is also a decrease in the population of cattle breeders group. A reduction in the population the beneficiary farmer groups, due to the presence of non-technical factors of groups such as ranchers cattle seller, and the died cattle. According to the Figure 1 can be explained that the beef self-supporting program in Kudat District for groups of cattle ranchers beneficiaries and funding, from the government agencies to respond positively. As for the group of cattle ranchers beneficiary of private institutions or aid in outside the government program to respond positively or negatively.

Positive response to farmer groups beneficiaries of government that is the increase in income / welfare groups of farmers and ranchers increase family income groups, as well as an increase in livestock population in several groups of farmers after relief is obtained. There is also an increase of farmers view the status of a recognized group in the community because of the successes.

Negative response from the beneficiary groups to national beef self-supporting program is a group that has sold the cattle and replaced with another business.This type is a group with a group of farmers who received assistance cattle from private institutions or agencies donor in outside of government. Negative response intended to the category is the act of a group of farmers who do not maintain aid was given, but change their selling livestock and other businesses from the seller livestock assistance, in addition to the factor group site unchanged breeder with this type of group effort beef cattle farms.

Response Group Not Receiving Aid breeders

Based on the results of research conducted on groups of cattle ranchers in Kudat District we can see that there are groups of cattle ranchers who do not receive financial aid or animal. This proves that the implementation of beef self-supporting program in 2014, according to program and policy of the Ministry of Agriculture through the Directorate of Animal Husbandry and Animal Health, but the implementation in the group level rancher yet evenly, the groups who do not receive assistance has two different responses that response remains / zero and negative response to beef self-supporting program in 2014.

Respons remain the PSDS, are the groups of farmers who do not get help and stop a group of farmers who, because of a fictional group of farmers without livestock. Groups of cattle ranchers hope fictitious without any help, and because without the help of a group of farmers is stopped / broke. The type of the group with zero response / fixed meaning groups that are formed in order to get help, and if it does not get help groups of farmers has been stopped.

Furthermore, respons fixed and the negative response to the PSDS are the groups of farmers who do not get help, but the population there, and little remains. Farmer groups with fixed response type and a negative response in this study, there was a group of farmers. Understanding the response remains as increasing the population are not happen, increase the income and the ownership is not seen merely pleasure or hobby. Meanwhile, for understanding the negative response is a group of farmers who government programs attempted without thinking and without thinking how much labor is expended in farm business or in the farmer groups.

The negative respons in the PSDS, is a group of farmers who did not receive assistance but still running a business group of cattle ranchers, with a population increase in a group of farmers who reduced, due to trying other business opportunities by selling

cattle. Another case of groups with response categories, namely negative; never thought of how much labor expended in their group effort. Furthermore the groups with this type, expects the help from the government, for the purchase of cattle.This is a group of the lives of a group of farmers who did not receive assistance always have a shortage in the purchase of cattle for subsequent maintenance.

Furthermore, the negative response to the groups that did not receive assistance is in when the group sells cattle to be used as capital for other business. It is describe that other businesses have taken the lower value when compared to the cattle business. Another effort is the effort of goat or sheep farming. Sales of one cattle, when they can buy six goats. This is done by groups of farmers with this category because the group of farmers did not receive assistance. This business is conducted by a group of farmers because livestock can be trusted to continue business as cattle farmers, although not anymore. Another point of this group is selling the cattle belonging to the group and was replaced by coconut plantations, for businesses of brown sugar. This is done on the basis of farmer groups, the brown sugar more quickly businesses, make money every day than the cattle business that requires a minimum of time ranging from 4-6 months in a process of fattening cattle for ready to sale, to get the advantage.

Conclusion

There are two groups of farmers in response to the Kudat District national beef self-supporting program. First, the response beneficiary farmer groups consisting of positive responses and negative responses. Positive response beneficiary farmer groups, can improve the overall sustainability of farmer groups, improving the welfare of the group and its members, while a negative response beneficiary farmer groups occurred due to the group to stop. The group stopped for selling livestock, when the price is expensive and used as capital for other business. Second, the response group consists of beneficiaries is not a passive response / zero and negative responses. Passive response / zero obtained on groups of farmers without livestock (fictitious) and without the help of that group of farmers has been stopped. While the negative response was obtained in the group of independent farmers who keep cattle without thinking PSDS program, livestock population slightly, just for fun / hobby. Subsequently the group has sold cattle ranchers but not able to buy back in exchange for the business because it is expensive.

Based on the response shown by the group of farmers who studied showed that, PSDS have beneficial effects for farmers who receive assistance. The PSDS implementation to improve the welfare of the group. But its provision is not evenly distributed, so that the national beef self-supporting is difficult to obtain.

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THE PERVERSE IMPACT OF GLOBALIZATION ON FOOD SAFETY

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As reported by World Health Organisation, out of the 6838 million human beings living on planet Earth, there is an increasing proportion of overweight and obese people (i.e. 17% overweight , 5% obese), but still there are 15% elsewhere malnourished .

In order to cover the requirements to feed the world's population, there are voices that claim that one option would be to reduce within certain limits the production of meat for human consumption,thus being able to devote to humans part of the cereals and soybeans that are at present consumed by animals.

In any case, it is undeniable that during the last centurysignificant imbalances have been produced in this respect, as it seems there is produce enough to feed the entire population of the planet, but there is a poordistribution of it among the various areas, traditionally classified as:

- developed countries: the so-called 'first world', with often overweight population and a very high meat consumption level;
- developing countries, which are struggling on the path to prosperity and welfare in alimentation;
- under-developed countries, whose inhabitants are mostly placed below minimum subsistence levels.

For us as veterinarians, the zoonoses concern us from a sanitary perspective, interms of diseases transmitted by animals or animal products. At the same time, we are concerned as an economic factor affecting the population.

Some centuries ago, upon European colonization of the Americas,across-exchange of pathogen agents between the two continents began: viruses, bacteria, fungi and parasitic microorganisms that cause diseases in plants, animal and humans. Due to expanding globalizationand international trade,in recent decades the relatedriskshave only increased continuously, on a rather exponential trend.Transmittable diseases know no boundaries or limits, and just as it is true that some of them get to be fully controlled or even temporarily banished, there always appear new ones.

I wonder if this is the new challenge in our profession, in the sense that the diseases we were used to treat in our farms haveno longer a 'local' nature, origin and symptoms, but are now worldwide. And therefore their respective solutions should be worldwide, if any.

As regards the future, a question that arises is what will happen in the future with raw materials and food products mostlyoriginating from developing countries? It is obvious that every day Europe produces less and, on the other hand, there are many countries where exportation of farm produce is the easiest or the only way to achieve foreign exchangerevenues.

In these conditions, it is interesting to analyse comparatively the food safety systems in these two spheres: the one of the providers and the one of the final users.

On the recipient end, the rules of the game are clear and therefore the foods produced in the 'first world'are not expected togenerate any problems in the future from a food safety perspective: the controlsare applied in a comprehensive manner, the legislation is broad and is generally implemented correctly by companies; there exists a perfect traceability in the food chain and there is an adequate application of risk analysis and critical control points (HACCP).

At the supplier's end, inevitably in developing countries problems arise, since over there control of both raw materials and processes of transformation is not exhaustive. Often it is not lack of good intentions on the part of these countries, but simply a lack of resources.

I believe we should ask ourselves what is the real level of control over the food chain in the developing countries, that have become in the past decades in prominent suppliers of the EU in several food sectors?

To answer such a question, we should probably start by analysing what the real level of control is there in countries such as, for instance,the African ones, where sanitary-oriented veterinary schools are almost absent, let alone the fact that in some of them there are no vet universities at all. If existing, the veterinaryuniversities are mostly focused on clinical activities or animal production, leaving completely aside animal health and food safety monitoring.

Apart from the human resources needed to implement decent food safety systems, an appropriate infrastructure is needed to control the products exported. As mere rhetoric,we could ask ourselves how many years will need developing or under-developed countries to have even a minimal such infrastructure? I am thinkingfor example at countries such as Ivory Coast, Ghana, Indonesia, Madagascar , Jamaica , Malaysia , Vietnam and Ethiopia.

But on the other hand, if health standards similar to the EU ones would be effectively applied in these developing countries, then obviously the general increase of costs for the respective industries would most probably make them relatively too expensive, leaving only the wage differential help them compete with their EU competitors.

How can the international legislation help?

Though it is difficult to speak about international legislation as such, a minimum legislative uniformity between countries has been achieved through the recommendations of the Codex Alimentarius. These are used by all countries as a reference to create national legislation, so that the highest possible matches result, in order to promote the world trade.

Of course, each country can choose the degree of application, in a more or less accurate manner,the recommendations of Codex in the process creation of their own national law. As a general comment, it should be noted that there is a lack of armonisation of such legislation in many countries of the third world, and to be honest, evenamong EU countries there still does not exist a legislative uniformity, let alone that the real-life application is even less uniform.

There is a natural tendency to assume that everyone does/thinks/is like us, for instance we would tend to take for granted that the application of our European model of legislation and control is universal, but the reality is that often there is no control at all over the raw materials imported from abroad that enter the EU production food chain.

As regards the practical application by governments of underdeveloped or developing countries, sometimes the economical interests and needs dictate or put a lot of pressure on the equation. On one side, there are countries where a single product covers 80% of the country's exportations and 90-95% of the foreign exchange earnings. And on the other side,in Europe there is much need of such raw materials / products as ingredients for the production, since in Europe such produce is zero, due to climate conditions etc, for example cocoa, pepper or coffee.

Quite confidently we think that food safety administrations in developing or under-developed countries would do what is required when it comes about clearly identified diseases. But if in doubt on a health risk (not crucial, but significant), we can ask ourselves what will be the thinking of the veterinarian services in a supplier country: What will come first? Their national economic interests versus the threat of a food chain in another (EU) country? These very important issues are affecting the movement and trade in both directions, and we must admit that inevitably many factors, including political, are involved when the case is one of apotential risk only, rather than a defined disease threat. .

Generally, the case is that the responsibility for the final product stays with the company responsible for the production of the product, while the health authorities only check the documents and examine the controls in place.But while this is applied successfully in developed countries, it can be very difficult to implement in a country where growth via exports food products are the main source of foreign exchange revenues. The liability of the operator is very relative and the subsequent sanctions by the government of the countries very low.

On the other hand, even upon entry in EU, it is unclear who has the responsibility to verify that the suppliers from abroad really control at source according to the same or similar rules? This is valid for both producers in Eu, as well as for supermarkets, where there are every day more exotic, ecological products, whose checking is unclear.

The current situation is that we are in the hands of others,the same time as others are in our hands. The rules have changed. Nowadays, we can have a very low crop and a final price very low, or reversely, have the highest quantitative crop ever and also the best price. For example: in a particular year, a particularly good harvest in Ukraine, can make Russia to import massively from Ukraine and thus cease to be a major importer of grain from other countries (e.g. Brazil, USA), and even convert it into an exporter to Europe for this year. Another example of changes in a different area, such as meat, that can alter dramatically the cereals market: an epidemic affectingbeef cattle, diminished significantly the demand of beef, increasing the one for chicken and pork, which in its turn, provoked imbalance in demand for specific cereals needed to produce the latter. Apart from the economical effect on prices, there is an effect on the availability of suppliers and raw materials, which affects all the food chain.

It can be deducted that in today's world, there is little control over a lot of factors on this final production.

One hundred fifty years ago, in Europe the harvest and the meat and animal products depended onhow good the agricultural year has been locally, of the type of land, of the rain, of the illnesses of the animals of the farm. Therefore it was independent of factors outside the respective production area, but nowadays the final results are tied to circumstances related to the vegetal crops worldwide field, as well as to the illnesses in the different productive species.

It sometimes appear ridiculous that we have prohibited in our laws for certain ingredients or procedures which are used in the normal course of business in the supply countries, whose products areeventually exported to our markets and reach our tables by being incorporated in the final productsin EU.

As an example, we can mention the use of genetically modified organisms (GMOs) authorized in certain countries, which are focusing primarily on corn and soybeans, that are considered safe there. Countries, over 29 now including China, are in favor of GM's widely used both in food and in the animal production for human consumption. The United States is a famous example, while in Europe there is legislation that rejects it. But nevertheless such GMOs inevitably get to our tables through the mechanism explained above.

The same could be told aboutadditives, preservatives, sweeteners, artificial colours and flavours, which are all obviously needed for the mass production to achieve a level acceptable on the international markets from a price perspective, to be competitive. But since all these additives are used in developing or third world, what kind of assurance could we have in respect of their application?

At presentit appears that the final EU consumer hasfor his use aliments controlled and safe for his health. But in my opinion, if as veterinarians we do not find methods to improve control over foreign sourced products, this illusion will fade more and more in time.

If we are to look for possible solutions, we can look to the history of production in Europe. Thus, the situation in many developing countries could be deemed as similar to what was valid 40-50 years ago in Europa and therefore we can use it to do a parallel between Europe and these countries that are exporting alimentary products to the first world.

A transition similar to whatEurope has lived between the years 1950-2000 could be expected. This will force them on a path similar to the one we have followed, that is to achieve a more intensive production with steps ahead in genetics, in the animal diet and in the intensification of the crops.

Yet in Europe it took 25 years to evolve from of an economy of small production entities to developing a productive sector. And to be honest, we must remember the efforts that were needed to get to a rentable production of eggs or in the swine sector.

Going back to the current situation, we must think on how long it will last?

The big question is if the current situation is expected to keep on a long-term basis, since the competition between developing countries is increasingly fierce.

We must start with the fact that an imported product from outside EU if crosses any border point and is admitted to the EU, then it can be freely transferred and sold throughout EU.Considering the fact that these foods have a limited life span, if the control is limited to the border checkings, inevitably the analysis procedures are rather simple and quick.

These restrictions that currently only apply to the production in EU, suppose an increase of the costs and, therefore, they reduce their competitiveness in front of the pertinent products of third countries, affecting gravely his future. Will EU afford to give for ever subventions to its producers to help them keep the prices down in order to compete with American products or of third countries that, so far, do not have these strict requirements and charges?

Conclusions

It is inevitable that the underdeveloped and developing countries will continue their economical growth and it is natural not to expect otherwise. It should be considered thatthe fastest and easiest way for them to grow their economies is to develop the primary sector (agriculture) and secondary (agrarian transformation and industry).

The future of the production, farmers and of the veterinary profession relates heavily to integrated productive programs that can monitor the full production cycle (if no longer personal control, than at least through procedures), but this no longer can be regardedlocally and/ornationally but to a more global level.

I think that our responsibility as a veterinary is to transmit to the developing countries and of the third world our experience and try to adapt it to the needs of these countries.

At present in many countries, the veterinary profession encompasses both a structure of liberal profession, as well as apublic function. In the past, the veterinary was rather private, and the public had little power, the raport was slightly reversed in past decades. These transformations in our profession have been made in Europe rather rapidly and effectively with the efforts of all, but will these countries be able to follow the same path that we did in Europe? If such solutions will not be implemented, then which will be the ones that we can recommend? To go back to a system of extensive/intensive control at our borders, which does not seem very practical or easy to implement?

I think that we have to attempt to use our experience to help to the developing countries and the same time to improve our food security by increasing the security of the chain from its initialsteps.

Our obligation as a profession is to begin to stimulate the creation of policies of professional growth especially in the countries from which we are making most importations. And leave apart much of the commercial paternalism, together with political pressure,that makes us allow imports without proper vet controls only because they come from countries that are poor.

We have to favour the collaboration between the veterinarians that are involved in the alimentary control by way of visits, meetings and exchanges if we want to grow all together. We could do this at the level of big organisations like the World Veterinary Association, as well as at a lower level: country to country, or at the level of professional associations.

In these aspects (collaborations, formation) not much has been done and yet there appears to be a big demand for it.

If we are go to global, we cannot remain with a regional or national vision.We have to begin to react because this is not a theoretical problem, but a reality due to the increasingly clear trend to importto EU a very important part of our basic products.

Conclusion

Recall that in our society slowly resembles the North American where the basic food becomes terribly cheap . Our grandparents spent 70-80 % of their salary on food, approximately 50% of our parents' salaries, while the younger generations are having trouble in accepting to spend 20-30 % of our salary.

So there is a hypocrisy between what we theoretically declare want to eat and that we buy in the end . The consumer is always looking for products less price in the market according to the current trends will increasingly origin in third world countries or s' are desarrollan .

If you visit any supermarket and take a little time to analyze the origin of what we purchase, we will find that our raw materials and processing of our food is increasingly global meat from Brazil, Denmark milk , cereal USA .. . etc. .

In Romania there is a feeling among consumers that the farmer , the cioban or the industry offer contaminated food and / or harmful to their health. There contonuously exist campaigns repetitive about food contamination problems and in the end there is always the guilt of the food industry . But at the same time, when we go for shopping, we are always looking to buy the cheapest products assuming that food security is always assured indifferent of the product price.

The grain trade is every day more important for the meat production, yet we are increasingly dependent on decisions that are taken away from us, regarding prices, cosechas, enefermedades vegetales etc. que cambian las decisions de importacion hacia EU.

To produce the meat that we do not have sufficient agricultural production itself, so we have to import: soy, wheat, corn. And this is usually done by acquiring from any producer who can offer the best price at the moment

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INVESTIGATION OF BACTERIAL DIARRHEA IN CAMEL-CALVES *(CAMELUS DROMEDARIUS):* CLINICAL AND BACTERIOLOGICAL STUDY

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Abstract

Diarrhea is of complex nature, caused by different infective agents proliferating in the intestinal tract alone or in combination with different microorganisms. A total of sixty five camel-calves (5 ± 1.74 month old, 93 ± 15.45kg) were used to determine hematological and biochemical parameters in control (n=10) and diarrheic camel calves (n=55). In the present study the diarrheic camel-calves revealed the clinical picture of elevated body temperature, polypnea, tachycardia, anorexia, dehydration and pasty to watery feces. Total leukocytic, neutrophils counts, PCV% were markedly increased in diarrheic group when matched with healthy control group. Moreover there was recognizable reduction in the RBCs, lymphocytes counts in diseased calves. Concerning the biochemical analysis, there were significant increase in the levels of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, lactate dehydrogenase, blood urea nitrogen, bilirubin values in diseased camel-calves when compared to healthy ones. Moreover, there was significant reduction in the levels of total proteins, albumen and blood sugar levels in diseased camel-calves when compared to healthy ones. Concerning the bacteriological examination of fecal samples from the diseased calves. It was observed that the most incriminated microorganisms were E.coli (38/55), Salmonella spp (10/55), proteus spp (5/55) and enterococci (2/55). In conclusion, hematological, biochemical parameters and bacteriological examinations play an energetic role in the evaluation of the bacterial diarrhea in camel-calves (Camelus dromedarius). Moreover, E.coli is the most incriminated pathogen in causing camel-calves diarrhea.

Key words: *Hematological, biochemical, bacteriological, camel, calves, diarrhea.*

Introduction

Dromedary camel is one of the tremendously precious domestic animals in Saudi Arabia. Camel is malleable animal that can be used for wool, meat and milk production. The current applications in the dairy camel industry lead to the expansion of camel dairy farms that are accomplished by producing camel milk on the commercial level. Camel milk and meat are considered an imperative donor of proteins for extensive variety of human inhabitants (Breulmann et al., 2007).

Regarding camel illness, camels were previously considered opposing to the majority of the diseases usually affecting farm animals, although many research investigations were pragmatic, camels appeared vulnerable to a great variety of micro-organisms. Numerous issues subsidize to losses in camel-calf, in which camel-calf diarrhea is seemed to be one of the most important incriminated cause of claves mortalities (Agab, 1993).

Diarrhea seems to be of complex nature, caused by various infective agents proliferating in the intestinal tract alone or in combination with different microorganisms (Acres *et al*., 1975). Diarrhea affects about thirty three percentage of the off-spring causing twenty three percentage mortality and accounts for a noticeable drop in calf variability, while many calves die leading to detectable decrease of herd growth rate (Abbas and Musa, 1988). The most incriminated pathogens in the previous research investigations were *Escherichia*

coli, Salmonella spp, cryptosporidium, coronavirus and rotavirus, (Tzipori, 1981). Recently, Clostridium difficle (Hove et al., 1996) was isolated from camel calves with diarrhea.

The scours in calves is a very expensive tricky for many camel breeders. Diarrhea is not a disease but is a sign of disease. Calves suffering from diarrhea will be ill in a very short period. The incriminated pathogens either bacterial (*Escherichia coli, Salmonella spp,*) or viral (coronavirus and rotavirus) that are the causative agents of this diarrhea are not the definite causes of mortality in diseased camel-calves. Excessive losses of water (dehydration state) and electrolytes deficit, and also acid-base disturbances are the fundamental causes of the calf's mortalities (Foster and Smith, 2009).

Camel calf diarrhea in the neonatal period is an economically substantial sickness leading to immense losses in the numbers of camel-calves all over the world (Mohammed et al., 2003). Elevated percentage of camel-calf mortality is measured as one of the chief restrictions to superior productivity in camels' populations in which camel-calf diarrhea is considered the most important cause (Salih et al., 1998). Neonatal mortality in camels was observed to be privileged in camels less than six months old (Khanna et al., 1992). It was stated that a 39.9 of morality percentage in Sudan was due to neonatal camel-calf diarrhea. The majority of the neonatal calves' diarrhea cases are suspected to be caused by bacteria, viruses and protozoa (Ali et al., 2005).

The bacteriological examination of fecal sample collected from camel- calves suffered diarrhea revealed that 69 (66%) cases yielded *E. coli* from a total of 121 cases (Salih et al., 1998). Moreover it was reported by Salwa (2004) that E. coli was isolated from isolated 81 cases out of 100 fecal samples collected from diarrheic camel-calves.

According to Faye et al. (1997) the reason for 68% of the camel calf losses in Niger is diarrhea, while Bada Alambedji et al. (1992) states that the morbidity of diarrhea can reach 80 to 90% with a minimum of 50% mortality. The authors conclude that diarrhea in camel calves is caused by the synergetic effect of predisposing factors, nutritional factors, parasites (such as coccidia), bacteria (such as *Salmonella* and maybe *E. coli*) and virus (e.g. Rota- and Coronavirus). The absence of colostrum intake is described as a contributing factor to camel calf diarrhea (Faye et al., 1997, Kaufmann, 1998, Agab and Abbas, 1999).

The aim of the current study was to investigate the clinical, hematological, biochemical parameters and isolation of bacterial pathogens incriminated in causing diarrhea in camel-calves *(Camelus dromedaries).*

Materials and methods

Animals:

Fifty five camel-calves (5 ± 1.74 month old, 93 ± 15.45kg) were admitted to the veterinary Teaching Hospital, King Faisal University, presenting a clinical signs of enteritis (diarrhea). In addition ten clinically healthy camel-calves were also admitted to the same Hospital for routine examination and are of good body condition and no signs of any clinical problems (control group).

Samples:

Two blood samples will be obtained from each camel-calves. The 1st sample was obtained by jugular vein puncture on heparinized vacuumed tubes and used to cellular examination. The second blood samples obtained in plain vacuumed tube for obtaining clear non hemolysed sera for analysis of total plasma proteins, albumin and glucose, blood urea nitrogen (BUN) and liver enzymes including AST, ALT, LDH, bilirubin.

Fecal samples were collected from each diseased camel-calves to be submitted for bacteriological examinations.

Methodology:

Hematological picture:

Hematological examination carried out using automatic cell counter adapted for camel (VetScan HM5 Hematology system, ABAXIS).

Biochemical parameters

Blood biochemical parameters including total plasma proteins, albumin, glucose, blood urea nitrogen and liver enzymes including AST, ALT, LDH, and bilirubin were measured spectrophotometrically using Beckman CX-7 autoanalyser using commercial kits (Sigma Chemical Co. Ltd., Poole, Dorset, UK).

Bacteriological examination of fecal samples

One loop of fecal sample was taken under complete aseptic conditions from respectively sample, streaked on blood agar and incubated aerobically overnight to obtain discrete colonies. Discrete colonies were sub-cultured on MaCconkey and Xylose lysine deoxycholate agar (XLD), pure cultures were preserved on brain heart infusion broth under -20 C°. The isolated microorganisms were identified using VITEK2, Biomeriux, France.

Statistical analysis

The statistical significance between means was compared using Student's t-test; ($p < 0.05$) was measured significant. All analytical tests were achieved using computer package of the statistical analysis system (SAS, institute, Inc, 2002).

Results

Table 1. The clinical picture of *diarrheic* and healthy camel-claves

Variable	***Control camel-calves***	***Diarrheic Camel-calves***
Heart rate/min	*36.3 ± 3.2*	*47.5 ± 3.3**
Temperature	*37.1 ±0.32*	*40.4 ± 0.42**
Mucous membrane	*Rosy red color*	*congested color(n=40)* *pale (n=15)*
Physical activity	*Excellent*	*Varied from fair to bad*
General appearance	*Bright*	*dull*
Appetite	*Normal*	*Anorexia*
Skin fold test	*Negative*	*Positive (delayed return of skin fold)*
Fecal matter	*Normal*	*Watery to pasty feces*
Sunken eye	*Absent*	*Present in some cases (n=15)*

*Means are significantly different at the level ($P \leq 0.05$)

Table. 2. The Mean hematological values of Diarrheic and healthy camel-claves

Parameters	*Control Camel-calves*	*Diarrheic Camel-calves*
RBC (x 10^6)	*11.15 ± 3.22*	*8.45 ± 1.22**
Hb (gm %)	*8.98 ± 0.88*	*7.78 ± 1.12**
PCV (%)	*46.4 ± 0.08*	*55.2 ± 0.06**
WBC (X 10^3)	*14.20 ± 5.31*	*26.20 ± 2.11**
Neutrophils (%)	*57.0 ± 3.44*	*84.0 ± 2.56**
Lymphocytes (%)	*25.3 ± 3.56*	*19.3 ± 1.26**
Monocytes (%)	*2.4 ± 0.32*	*2.3 ± 0.22*
Basophils (%)	*1.1± 0.12*	*1.0 ± 0.13*
Eosinophils (%)	*2.4 ± 0.21*	*2.6 ± 0.32*

*Means are significantly different at the level ($P \leq 0.05$).

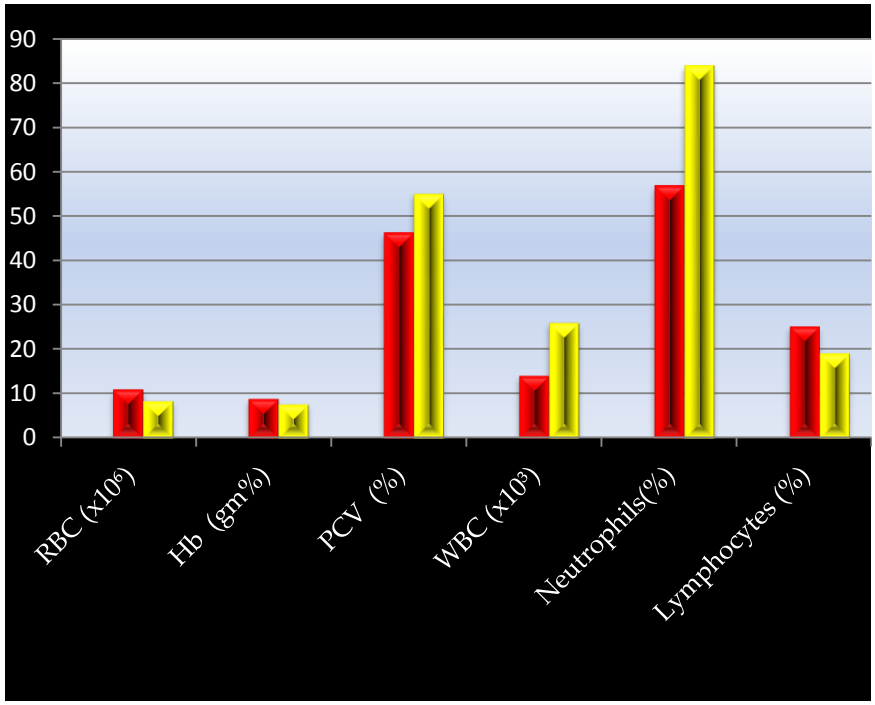

Figure 1. The hematological changes in control and diarrheic camel-calves

Table 3. The mean values of blood biochemical parameters in control and diarrheic Camel-calves

Variables	Control camel-calves	Diarrheic camel-calves
Total Plasma Proteins *(gm %)*	*6.34 ± 0.44*	*4.3 ± 0.31**
Albumen *(gm %)*	*3.74 ± 1.2*	*2.40 ± 0.42**
BUN *(gm %)*	*12.5 ± 0.34*	*22.2 ± 2.4**
Glucose *(mmol/l)*	*37.67 ± 3.1*	*29.5 ± 2.7**
Bilirubin *(mmol/)*	*1.1 ± 0.45*	*1.9 ± 0.5**
AST *(mmol/)*	*139.5 ± 23.4*	*257.21 ± 23.4**
ALT *(mmol/)*	*17.3 ± 4.9*	*153.11 ± 6.5**
LDH *(mmol/)*	*14.02 ± 4.8*	*34.21 ± 3.5**

*Means are significantly different at the level ($P \leq 0.05$).

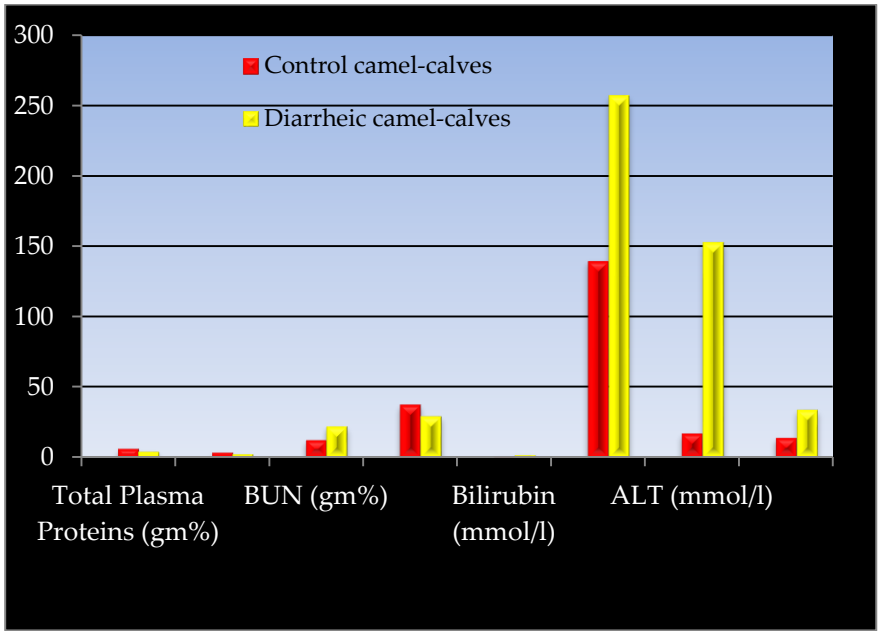

Figure 2. The biochemical changes in control and diarrheic camel-calves

Table 4. The percentage of isolated bacteria from diseased camel-calves

Bacterial isolate	Number of infected camel-calves and the percentage of infection
E.coli	38 (69%)
Salmonella spp	10 (18.18%)
Proteus spp	5 (9.09%)
Enterococcus spp.	2(3.6%)

Discussion

The camel-calf diarrhea is considered one of the most severe obstacles in camel production. The prevalence of calf diarrhea happens all over the year with a few increase in calving seasons.

Diarrhea is of multifaceted nature, caused by a variety of infective agents proliferating in the intestinal tract alone or in combination with different microorganisms. In the present study the diarrheic camel-calves revealed the clinical picture of elevated body temperature, polypnea, tachycardia, congested mucous membrane, anorexia, dehydration and pasty to watery feces (Table 1). The presented results are in covenant with the previously achieved by Abbas et al., (1992), Debroy and Maddox, (2001), Wernery and Kaaden, (2002), Donnenberg, (2002) and Radostits et al. (2007)).

Total leucocytic, neutrophils counts, PCV% were markedly increased in diarrheic group in matching with the same levels in control group (Table 2, Fig.1). Moreover there was a major reduction in the RBCs, lymphocytes counts in diseased calves (Table 2, Fig.1). The increased total leucocytic and neutrophils percentage indicating a bacterial incrimination. Moreover the increased PCV % indicating that affected calves were dehydrated as result of diarrhea. The presented results are in agreement with Venter et al., (1994), Debroy and Maddox, (2001) and Radostits et al. (2007).

Concerning the biochemical analysis, there were an obvious upsurge in the values of ALT, AST, LDH, BUN, bilirubin values in diseased camel-calves when compared to healthy camel-calves (Table 3, Fig.2). Moreover, there were significant reduction in the levels of total proteins, albumen and glucose values in diseased camel-calves when compared to healthy ones (Table 3, Fig.2). The presented results are in agreement with Venter et al., (1994), Wernery and Kaaden, (2002) and Radostits et al. (2007)

The detected elevation in the values of liver enzymes profile in diarrheic camel-calves may be allied with conceivable liver injury persuaded by Spartan irritation of intestinal mucosa. The presented results are in concern with that stated by Venter et al., (1994) and Radostits et al. (2007).

Agab and Abbas (1999) reported that camel-calves diarrhea is an imperative cause of calf's losses, weight loss and subsidizes to retard growth rate of the calves herd. Calves diarrhea previously reported in 21.9% of the examined camel-calves, with a peak percentage in the early summer. The authors refer this peak percentage to the calving seasons of the camels. To the best of their knowledge the reason of diarrhea is not sufficiently recognized, but *Salmonella* infections and poor management strategy are reported. In Morocco the

mortality rate in camel-calves up to six months of age approaches 20.2% resulting in a major hazard for the progress of camel breeding. (Bengoumi et al., 2000).

Concerning the bacteriological examination of fecal samples from the diseased calves in the presented study, it was observed that the most incriminated microorganisms were *E.coli* (9/15), *Salmonella spp* (3/15), *proteus spp* (2/15) and enterococci (1/15). The investigation revealed that The E. coli was the main cause of camel-calves diarrhea. The present findings are in covenant with the data achieved by Salih et al., (1998), Salwa (2004. It was reported by Fouda and Al Mujalii (2007) that *Proteus spp*. and *E. coli* were the greatest incriminated bacteria causing camel-calves diarrhea and *Staphylococcus aureus* was the main causative agent of respiratory troubles in diseased camel-calves. On the other side there was a clinical study showed that the *Clostridium perfringens* was incriminated in 27 cases, *E. coli* in 9 and both *E. coli* and *Clostridium* in 7 samples from 71 fecal samples (total number of samples) collected from calves facing diarrhea (Zakia, 2004). Additionally Abubaker et al. (2006) isolated 52 (27.3%) pathogenic *E. coli* from 190 diarrheic samples collected from camel-calves in the Kingdom of Saudi Arabia.

Abass and Omer (2005) state that diarrhea is a problem in "young camel calves". They describe three clinical syndromes that can be observed in different age groups. The first one occurring in neonates is mainly caused by rotavirus and *E. coli*, the second syndrome occurs in calves between two weeks to two months of age with *Salmonella*, *Clostridium*, *Campylobacter* and possibly coccidia being the major cause of diarrhoea. The third syndrome is mainly found in older suckling calves up to one year of age and caused by the recurrence of an episode of acute or subacute gastroenteritis in this age group with *Salmonella* spp being most common. The authors stress on the importance of the investigation of risk factors, economic impact and control procedures for the diarrhea complex in camel calves.

Furthermore, *E. coli* and *Salmonella* were stated as the main pathogen causing enteritis in camel-calves up to three months of age. Conversely, parasites such as *Strongyle* and coccidia as well as the nutritional state of the dam play a vital role, too. The authors conclude that several factors participate in the etiology of the diarrhea in camel calves Dia et al. (2000).

Conclusion

From this study it could be concluded that hematological and biochemical parameters could be used in diagnosis of diarrhea in camel-caves. The total and differential leucocytic counts could be used to identify the causative infectious agents. Moreover the PCV% will give a good picture about the hydration state of the affected camel-calves. The most incriminated microorganism was *E.coli* (38/55). Further investigations should be carried on the methods of immunization and control of such pathogen in camel-calves populations.

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INVESTIGATION OF BACTERIAL MASTITIS IN GOATS WITH PRECISE ORIENTATION TO NEGATIVE ENERGY BALANCE RELATIONSHIP

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Abstract

Forty-five goats were used to detect selected hematological and biochemical parameters in healthy and mastitis affected goats. Goats were divided into two groups represented as clinically healthy (control N=15) and mastitic groups (N=30). The bacteriological examination of milk samples from diseased goats showed various types of bacterial incrimination. The isolated pathogens were Staphylococcus aureus (15/30), Escherichia coli (10/30), Streptococcus agalactiae (5/30). There were a significant elevation in the values of β-hydroxy butyric acid, triglyceride, non-esterified free fatty acids, low density lipoprotein-cholesterol, serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase and a significant reduction in the values of blood glucose, cholesterol, and high density lipoprotein-cholesterol in mastitic goats compared to healthy goats. Conclusively, Staphylococcus aureus, and Escherichia coli and Streptococcus agalactiae play an important task as causative agents for mastitis in goats under investigation. The present study shed the light on the possible pathophysiological role of Lipid profile in mastitis in goats. Lastly, Ketosis may be considered as contributing factor in the occurrence of secondary mastitis in goats.

***Key words**: Goat, mastitis, HDL-C, LDL-C, β-hydroxybutyrate, NEFA.*

Introduction

Mastitis is inflammation of the tissue of the mammary gland. Such irritation is mainly common as a result of pathogen incrimination (intra-mammary infection) however may be owing to damage (injury) and to lesser extent as a result of allergy and neoplasm (Leite Browning, 2008). The inflammation may be noticed without presence of intra-mammary pathogen. In such clinical condition the udder was injured, it is improving from a pathogen that has overcome, the inflammation is not due to incriminated microorganisms in udder mastitis, or that the culturing process and sampling protocols was defective (Bergonier et al., 2003).

Mastitis is an irritation of the tissue of mammary gland that leads to physical and chemical replies in milk compositions created through goats. Udder injury is very common problem in both meat and dairy producing animals reared under semi-intensive and intensive management programs. Centered on the sternness of the udder injury, the disorder may lead to considerable decrease in revenues for goat producers (Leite Browning, 2008).

Mastitis is connected with pitiable hygienic conditions and instigated by the injury of mammary gland tissue by nursing, bites of flies, mechanical traumas, or any injury to the udder skin. Different causative agents as bacterial, viral or fungal pathogens and their correlated end products (toxins) are obviously liked with goat udder injury (mastitis). Stressful circumstances (state of increased stress) like tremendous atmospheric climate, muddy & wet housing circumstances, the immunity is negotiated and has a very difficult

situation and long time for combating the attack of infective pathogens (Moroni et. al 2004 &2005 and Merck, 2008).

The unusual anatomical portrait of the mammary gland (udder) or teat contour may be considered as an additional contributing factor. Contamination or infection of the udder ensues when infectious pathogens attain mammary gland tissue through different pathways. The infectious pathogens comes into the mammary gland through the milk canal, and then challenge the mammary cells, and then multiplies. Many pathogens yield undesirable end products as toxins. Such immoral toxins react with mammary gland and consequently the gland becomes tender and irritated. The udder infection in Goats may occurs shortly after birth, while infection may occur through milking season and following also dry stages (Leite Browning, 2008).

Sub-clinical ketosis is a significant production disease in early period of lactation in dairy cows. The cause is correlated to the power of elevated levels of milk production, the frequency of this condition is disposed to still imperative. Masked ketosis is extremely linked with many peri-parturient disorders, including subclinical and clinical mastitis. Many emergent evidences that the mechanisms of udder immune defense against clinical mastitis are inhibited in times of negative energy balance and ketosis. (Ken et al., 2000).

Mastitis is a significant disorder in sheep and goats that influences the meat and milk production and good scientific procedures necessity to be considered to compete such clinical problem (Gebrewahid et al., 2012).

The Clinical picture of udder injury is manifested by detectable variations in the consistency of the udder or mammary tissues, changes in the physical character of the milk, which may include clots, pus secretions and be also stained milk could be detected. In meat producing sheep, it may not be probable to state the period of the inflammation as the mammary gland or udder may only be evaluated at parturition or at the end of suckling or if the animal is clinically examined for a certain disease condition (Radostits et al., 2007).

The acute form of udder injury comes suddenly with a protuberant fever over 105° F and tachycardia. A mastitic goat may walk leisurely, and anorexia. Classically, the mammary tissue is too rigid, enlarged, with reddish skin color, hot and so responsive to touch (pain sensation). The infected milk becomes watery consistency and stained with yellow coloration. A mastitic goat's milk may also contains flakes and milk-clots. The major cases are in the risk of loss of production or even death. The untreated cases developed to chronic picture. The udder tissue may have solid swellings as a consequence of bacterial clusters (Merck 2008). The goal of the present investigation was to explore the different causative agents of Caprine mastitis. Moreover to throw the light on the diagnostic importance of blood picture and lipid profile in cases of caprine mastitis. Moreover, to investigate the relationship between ketosis and mastitis in sheep.

Materials and Methods

Animals

Thirty goats (3.8±0.4 years old), native breeds, were referred to the Veterinary Hospital, college of veterinary medicine, presenting acute clinical signs of mastitis (figure 1). Treatment of affected goats was initially attempted using amoxicillin (10 mg/kg, once a day, subcutaneously) and enrofloxacin (10 mg/kg, 24 h, subcutaneously), along with flunixin meglumin (1.1 mg/kg, 24 h, intravenously). In addition, other 15 healthy goats of similar age

and post-partum period were used as a control group. The diseased goats were examined clinically.

Sampling

Two separate blood samples were harvested from both clinically healthy goats and diseased goats with mastitis. One sample was taken in an Eppendorf tube, which was assorted with ethylenediaminetetraacetic acid (EDTA) as anticoagulant for hematological investigations. The other blood sample was taken in plain tubes. Samples were centrifuged for 10 min and the clear non-hemolysed sera was isolated carefully and stored in Eppendorf tubes at −20 C until estimation of selected serum biochemical markers. Milk samples were aseptically collected from diseased goats and acquiesced to microbiological cultivation on 5% defibrinated sheep-blood agar (0.05 g/ml), MacConkey agar, and Sabouraud agar, and incubated under aerobiosis, at 37 °C for 72 h. The same material was submitted to 5% sheep blood agar, and incubated under anaerobic conditions, at 37 °C for 120 h. The isolated microorganisms were identified using VITEK2, Biomeriux, France.

Hematological picture

Hematological examination carried out using automatic cell counter adapted for camel (VetScan HM5 Hematology system, ABAXIS).

Determination of certain biochemical parameters:

The values of blood sugar, cholesterol, triglyceride, blood urea nitrogen, low density lipoprotein-cholesterol, high density lipoprotein- and, cholesterol, , as well as serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase were determined in serum samples on a Beckman CX-7 autoanalyser using commercial kits (Sigma Chemical Co. Ltd., Poole, Dorset, UK). Serum concentration of β-hydroxybutyrate was measured by a kinetic enzymatic method using a commercially available kit (Ranbut D-3-hydroxybutyrate, Randox, UK), following the protocols mentioned by Bani Ismail et al. (2008). Serum concentration of non-esterified free fatty acid was conducted using commercially accessible kits supplied by Randox laboratories, UK.

Statistical methods

The obtained data were studied by using Student's t-test ($P \leq 0.05$) was considered significant. Entirely all tests were completed using computer package of the statistical analysis system (SAS, institute, Inc, 2002). The regression analysis and regression figures were carried out using Minitab 16 software

Results

The mastitic goats displayed fever and anorexia. The mammary gland was swollen or enlarged (figure 1). Concerning the bacteriological investigation of mastitic milk samples, there were two types of bacterial infection. The most incriminated isolated bacteria were *Staphylococcus aureus* (N = 10/15) and *Escherichia coli* (N = 5/15). Regarding the biochemical analysis of mastitic goats, Table 1 declared that there was a detectable increase in the values of beta-hydroxy butyric acid , non-esterified fatty acids, triglyceride, LDL-C, serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase (1.60 ± 0.13 mmol/L, (1.20 ± 0.12) mmol/L, (76.25 ± 1.4) mg/dl, (30.43 ± 0.30) mg/dl, (88.7 ± 2.41) U/L, and (41.22 ± 1.45) U/L, respectively), with an observable decrease in the values of blood sugar, cholesterol, and HDL-C levels (26.20±0.33 mg/dl, 56.32±1.44 mg/dl, and 33.22 ± 0.84 mg/dl, respectively) in goats with clinical mastitis compared to healthy control ones.

The regression analysis of the β-hydroxybutyrate and NEFA showed a significant relationship between both markers in mastitic goats (R^2= 97.57) (Figure 2). Also figure 3 and 4 showed the significant differences between β-hydroxybutyrate and NEFA in control and mastitic goats.

Table 1. Hematological findings (±S.E.) of control and Goat with mastitis

Variable	Healthy goat (n=15)	Goat with mastitis (n=30)
Hb (gm/dl)	12.32 ± 1.34	12.21 ± 1.62
PCV (%)	31.52 ± 1.23	31.53 ± 1.73
RBC (x 10^6/mm^3)	10.54 ± 0.62	10.32 ± 0.42
TLC (x 10^3 / mm^3)	10.62 ± 0.62	15.33 ± 0.53 *
Neutrophil (%)	35.03 ± 1.33	77.23 ± 3.33 *
Lymphocyte (%)	59.62 ± 1.23	26.32 ± 1.32*
Monocyte (%)	5.32 ± 0.43	5.53 ± 0.32
Eosinophil (%)	3.22 ± 0.32	3.11 ± 0.32

*Means are significantly different at the level ($p \leq 0.05$).

Table 2. Selected biochemical parameters in clinically healthy goats and those with mastitis

Variable	Healthy goat (n=15)	Goat with mastitis (n=30)
β-hydroxybutyrate *(mmol/L)*	0.91 ± 0.13	1.60±0.13*
NEFA (*mmol/L*)	0.51 ± 0.12	1.20±0.12*
Glucose *(mg/dl)*	60.4 ± 1.32	26.20±0.33*
Triglyceride *(mg/dl)*	56.8 ± 0.24	76.25 ± 1.40*
Cholesterol *(mg/dl)*	77.2 ± 1.58	56.32±1.44*
LDL-c *(mg/dl)*	26.57 ± 0.24	30.43 ± 0.30*
HDL-c *(mg/dl)*	50.6 ± 0.62	33.22 ± 0.84*
AST *(IU/L)*	45.9 ± 0.73	88.7 ± 2.41*
ALT *(IU/L)*	18.14 ± 0.43	41.22 ± 1.45*

*Means are significantly different at the level ($P \leq 0.05$).

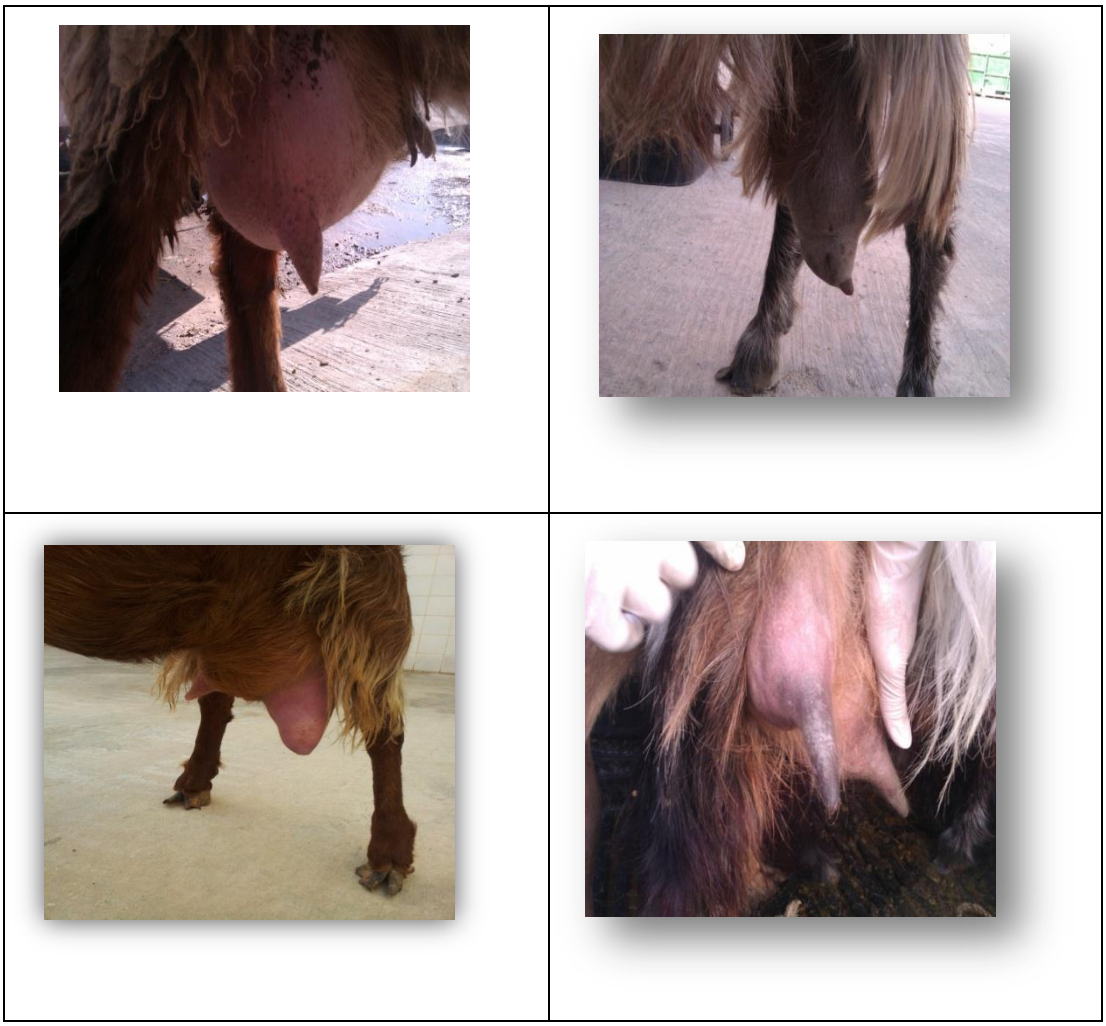


Figure 1. Goats with different clinical ictures of mastitis

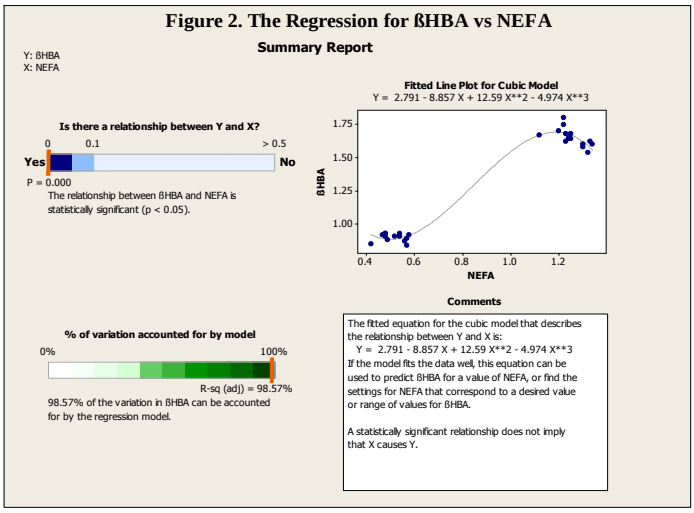


Figure 2. The Regression for ßHBA vs NEFA

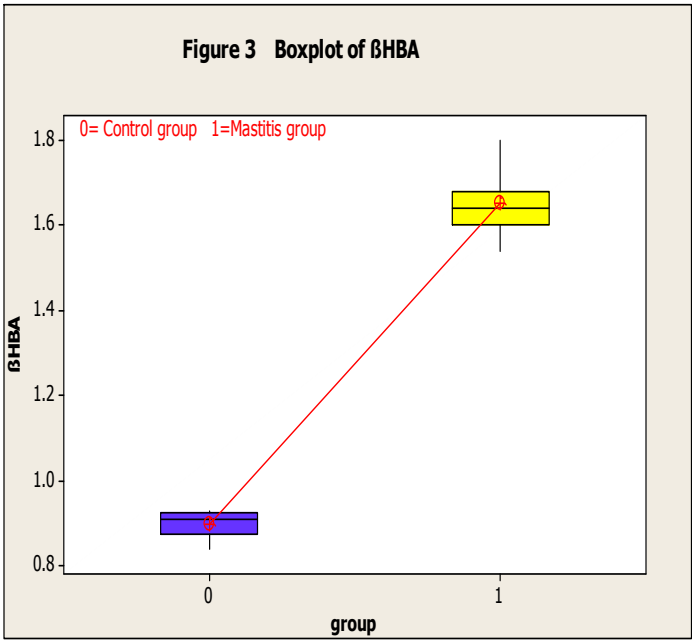
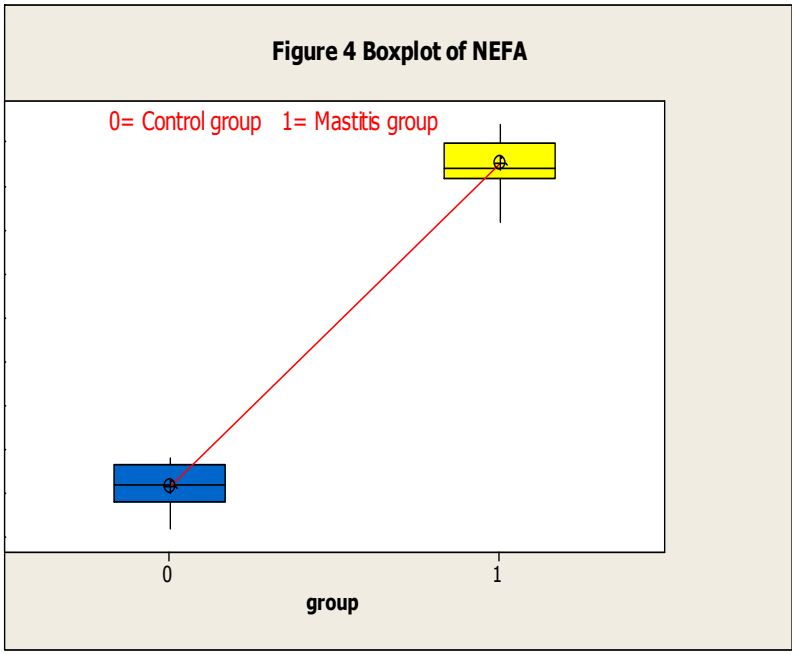


Figure 3 Boxplot of ßHBA



Discussion

The cows in the state of negative energy balance declare an impairment of mammary gland immune defense mechanisms. Ketosis is theorized as one of the greatest imperative aspects leading to decreased udder immune defenses. Potential clarifications for such possessions through each of the mechanisms of defense were present. First of all, the capability for engulfing foreign pathogen (phagocytosis by macrophages and polymorphonuclear cells) may be diminished in the state of negative energy balance (Ken et al., 2000)

Clinical mastitis is considered one of the most imperative disorders of farm animals, caused by numerous etiological pathogens. Transmission of the infectious agents primarily happen by ascending the teats canal, usually concerning pathological agents from animals and environmental source and from the milking method (Anderson *et al.*, 2005). The hematological findings of the current study declared pronounced elevations in the values of TLC & neutrophils % with lymphopenia in diseased goats in matching with control goats. The obtained data are in agreement with the data stated by Radostits et al., (2007). The higher values of TLC and neutrophils % may be attributed to bacterial incrimination (Radostits et al., 2007). The presented study showed a detectable decline in blood sugar values in goats with clinical mastitis in comparison with healthy goats. Additionally, there was detectable elevation in the values of β hydroxybutyrate and NEFA in comparison with healthy goats. The diminished values of blood sugar and increased values of β-hydroxybutyrate and NEFA could be attributed to the loss of appetite in mastitic goats. Amazingly, these laboratory data may present a special correlation between ketosis and clinical mastitis in lactating goats. These results of reduced glucose levels are constant with previously detected data by Çetin *et*

al. (2005) and Hayrettin et al., (2005) and El-Deeb, (2013) in ewes with mastitis and differ with that obtained by Fuguay *et al*. (1975) in cows with coliform mastitis.

Numerous epidemiological investigations have declared that hypoglycemic state (clinical ketosis) is linked with amplified hazard of obvious (clinical) mastitis (Emery et al., 1964, Grohn et al., 1989, Correa et al., 1993 and Oltenacu, and Ekesbo 1994). Additional research studies of negative energy balance (ketosis) in dairy cattle have been investigated for relations between masked (subclinical) ketosis and measures of mammary gland immune status (Duffield et al., 1998).

Additionally, there was a noteworthy increase in the values of total lipids in mastitic goats compared to healthy ones. It has been stated previously that the levels of serum total lipid concentrations elevated in ketotic condition (Marteniuk and Herdt, 1988; Kaneko *et al*., 1997 and El-Deeb, 2012), which was established in this investigation. Serum cholesterol values diminished obviously in liver incompetency (Kaneko *et al*., 1997). The current laboratory outcomes showed that, the values of cholesterol in the diseased goats were lower than that in healthy control goats. In addition, it was observed that the serum values of HDL-C in the mastitic goats were also considerably diminished, while LDL-C was considerably elevated compared to the control healthy goats. The serum levels of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities amplified in mononucleosis and liver & kidney injury (Kaneko *et al*., 1997). In the present investigation, serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities differed considerably between mastitic and healthy control goats. Elevated levels of serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase in diseased animals are linked with compromised hepatic function. The pathway by which inflammation cause udder injury or inflammation to mammary tissues during clinical mastitis are still not completely assumed. It is well recognized that demagogic replies, in which vascular penetrability augments and leukocyte migration happens, comprise oxidative stress radicals or reactive oxygen species, like $H_2O_2\cdot$, $OH\cdot$ and $O_2\cdot$, (Cuzzocrea *et al*., 1998; Poch *et al*., 1999). From the present investigation it could be concluded that hematological picture and lipid profile could be used as diagnostic markers of Caprine mastitis. The most incriminated bacteria were *Staphylococcus aureus* (N=10/15) and *Escherichia coli* (N=5/15).The present study declared a distinctive correlation between mastitis and ketosis in goats. As the ketotic state of the goats could be considered as an imperative factor encouraging the occurrence of clinical mastitis.

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EVALUATION THE INCIDENCE OF INFESTATION WITH BABESIA SPP. IN DOGS

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Abstract
Ticks are important vectors of transmission of viruses, bacteria and protozoa in some animal species and humans. The transmission of babesiosis in dogs reported an increased incidence in recent years, manifested throughout the year. The aim of our study tracked the incidence of infestation dynamics of Babesia spp. In dogs, by performing a descriptive epidemiological study, based on recorded cases of the disease in veterinary clinics with in our faculty in the period January 2011 - September 2014, a total of 392 dogs. Confirmation of diagnosis by laboratory testing was performed in peripheral blood samples but central. We calculated cumulative incidence of cases by age, the annual incidence of babesiosis and seasonal dynamics of infestation values. Results showed that the number of infected animals increased from year to year, with a maximum of infestation in the months from March to May and September to November, most at the dogs dogs aged up to 3 years.
Keywords*: tick, infestation, babesiosis, protozoa, hemosporidioza*

Introduction

Babesiosis is a hemosporidiosis (piroplasmosis) produced by protozoa of the subclass Piroplasmia, order Piroplasmida, disease that mainly affects lymphoid blood counts or both mammals and birds. The spread of parasites is subject to their definitive hosts dynamics, and especially Ixodidae ticks.

In case of babesiosis, Babesia parasite of the genus, family Babesiidae, is fixed in parasitized erythrocytes body in a variable number of 1to 4 parasites, wearing different forms and aspects, depending on the species (pear, bigeminy, ring) (Matijatko *et al*., 2012; Sikorski *et al*., 2010). Life cycle of the parasite has two phases, depending on the host parasite at a time: in ticks that are definitive hosts, sexed phases occur, and in the body of mammals, considered intermediate hosts occur asexual stages (Boozer *et al*. 2003; Irwin, 2010).

Babesiosis is a parasitic disease that affects very serious different species of mammals including humans, is considered serious zoonosis, caused by a unicellular parasite that is transmitted through ticks and reservoir hosts babesii definitive in nature.

Transcutaneous animal contamination is done during feeding infected ticks that with saliva, inoculated parasites. Inoculated parasites pass on the erythrocyte surface plasma then within them where they feed, multiply and destroy them then passing to other RBCs. Pathogenesis babesiosis is extremely complex - serious illness affecting the entire body (Di Cicco *et al*., 2012). In dogs, acute form begins with listlessness, fever, anorexia, jaundice, anemia, respiratory distress and possible nerve (type epileptic seizures, pseudoparaplegie), photophobia with possible retinal hemorrhages. Death can occur 5-10 days after illness. In the chronic form that persists for several weeks, anemia and weight loss is recorded sharp.

Canine infection with Babesia spp. is common throughout the world. Infection is more common in areas where tick infestation pressure is high and when routine acaricide use is not practiced.

Material and metod

Studies were conducted on casuistry veterinary clinics of the Faculty of Veterinary Medicine, January 2011 - September 2014. From a total of 745 cases with suspected babesiosis were considered in 392 dogs showing different specific signs of babesiosis: loss of appetite, apathy, anemic mucous, fever (between 39 to 41.2^0 C), refusing to move and adopt decubitus, jaundice, dark urine, and some dogs hypothermia and was confirmed positivity infestation. The diagnosis was made by corroborating medical history, clinical signs correlated with ultrasound, hematological, biochemical, and with parasitological examination of blood through blood smear of peripheral or central.

Data analysis

All collected data were entered into spreadsheets and analysed with statistical software; a Statistical Package for Social Sciences (SPSS, version 15). Data were summarised as descriptive statistics (mean and percentages) and displayed as tables. The proportions obtained in the study were compared using chi-square test. The confidence level for the analysis was set at 95% with significance level assessed at $p<0.05$.

Results

Reported to type diseases diagnosed in dogs considered in veterinary clinics FMV Iasi found to babesiosis represents over 60% of all diseases.

The diagnosis for each of the 392 patients was confirmed by microscopic examination of a peripheral blood smear, which were highlighted modified erythrocytes (destroyed) or intracellular parasites with characteristic appearance. In cases in which microscopic examination of peripheral blood smears were negative or inconclusive (in cases of infestation in its early stages), and blood examinations were performed centrally thus confirming positivity infestation. Values haematological parameters in blood collected before the start of drug treatment, showed significant decreases in red blood cells (values between 3.85 - 4.5 mil. / Mm3), hemoglobin (6.5 g/100 ml - 8.3 g/100ml) hemtocrit (20.3% - 28.8%).

Clinical symptoms were highlighted in dogs infected at a rate of over 60% represented by a general feeling strongly affected mucous manifested by jaundice, severe hemolytic anemia, convulsions, vomiting, hemoglobinuria, respiratory and cardiac, hepatic and renal impairment. Some patients had fever, while others were hypothermic.

Our findings revealed a significant increase in infestation Babesia spp. in dogs in recent years (Table 1), with an upward trend in incidence dynamics.

Table 1. Dynamics of the annual incidence of infestation with Babesia spp.

Year	2011	2012	2013	2014
Total number of cases		392		
Nr. cases/year	56	71	98	167
%	14,28	18,11	25,00	42,60

$p>0.05$ $\chi_2 = 0.1265$

Regarding the seasonal dynamics of infestation Babesia spp. has been a sporadic incident in the cold season, 2 cases were registered in December 2013 and one case in January 2014. The maximum incidence incidence was found in the spring months - March, April, May and autumn months - September, October and November (Fig. 1).

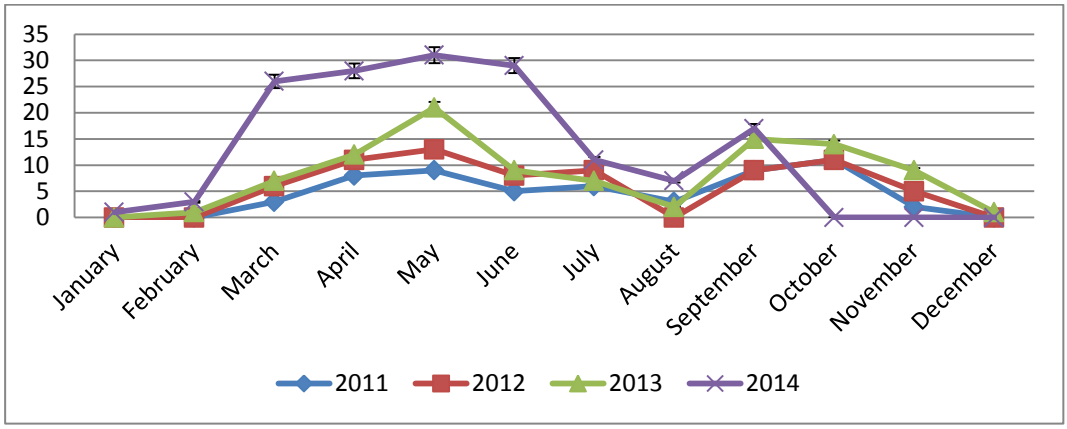

Fig. 1. Monthly dynamics of infestation Babesia spp. in dogs

In terms of patient age were infected dogs of different ages between 7 months - 15 years, but most reciptivitatea found in young dogs aged up to 3 years and a good state of maintenance (table 2). In the races could not establish any correlation between the degree of infestation and racial sensitivity. Also there could be no correlation in terms of the dynamics of which were from patients and infestation, they are from different areas of the city, or even in other cities.

Table 2. Prevalence of Babesia spp. in different age group

Age group	Numer cases infected	%
Yang /0.7 – 3 years	212	54.10
Adult / 4 – 9 years	124	31.62
Old / 10 – 15 ani	56	14.28
Total	392	100%

$p>0.05$ $\chi_2 = 0.0465$

Discussion

Babesiosis, a parasitic disease with evolution theory, seasonal, demonstrated in recent years, however, the existence of illness throughout the year, varying only the frequency of occurrence from sporadic to very frequent during winter when temperatures are positive. Disease is distributed across the globe, which affects humans and animals alike. This disease has now become a subject of intense played both by the disease in dogs and products through potential zoonotic nature.

The season for the start of tick activity is of major interest, especially for veterinarians and pet owners, to determine the best practice for countermeasures against tick infestation. Ticks are strongly influenced by temperature and host seeking behaviour increases with temperature. Higher temperatures have been described to induce a higher level of aggression, leading to the infestation of host species other than the primary ones (Schoeman, 2009).

Increases in the abundance and distribution of ticks and tick borne disease (TBD) within Europe have been reported extensively over the last 10–20 years (Matijatko *et al.*, 2012). In temperate climates, there is a seasonal increase in incidence during the spring and summer months (Ayoob *et al.*, 2010; Gray *et al*., 2009; Jongejan *et. al*., 2004), when the tick vectors are more active and abundant, and a decrease in the fall and winter. In tropical and subtropical climates, the incidence of disease is unchanged throughout the year (Jongejan *et. al*., 2004; Uilenberg, 2006). Changes in climate, habitat management, economic patterns and changes in the abundance of hosts, may all have influenced this change to varying extents.

Babesia spp. infection was observed to be higher in young dogs when compared with either the adult or adolescent hosts. This might be due to the fact that young dogs are agile and once allowed a little chance they move about indiscriminately where they come in contact with the tick vector of Babesia canis and thus high infection recorded. Another possible reason could be the habit formed by the young animals of playing on the grasses around where they pick up a waiting tick that was ready to attach itself to a scavenging host as observed during the study. This finding conforms to the works of Di Cicco *et al.*, 2012, Welc-Faleciak *et al*., (2009) and Hornok *et al.* (2009) who reported high babesia infection in young dogs than the adult hosts.

Increasing abundances of tick populations in urban and peri-urban environments, such as parks, are of particular concern. In these sites, suitable habitat, wildlife hosts, tick populations, people and their pets may be brought into close proximity and hence may provide foci for tick infestation and, ultimately, disease transmission.

Outbreaks of infection in this disease are infected animals or those undiagnosed, who have latent infection and Ixodidae ticks that are actually considered this parasite reservoir. Contamination - tick transition from animal parasites - ticks feeding occurs when infected saliva inoculated transcutaneous.

The parasite initially penetrate red blood cells multiply, secrete various metabolic toxins, causing lysis of red blood cells, so that while anemia occurs. Other effects of such massive destruction can be translated and subsequently by spleno- or hepatomegaly (Shah *et al*., 2011). As a defense reaction of the body can integistra a slight increase in blood pressure accompanied by vasodilation and tachycardia. In more severe cases can register perivascular edema and severe damage in the brain, nervous manifestations, liver and kidney dysfunction - jaundice, albuminuria, or necrosis and degeneration in the affected organs, etc. (Sikorski *et al*., 2010).

To conclude, given the major risks posed this disease both in humans and animals and also increased incidence of infestations of dogs in recent years, we insist on the means of prevention of this disease. The first step in preventing external Babesiosis is regular deworming of the animal, and appropriate products recommended by your veterinarian.

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MOLTING IN TARANTULAS – BASIC CONDITION FOR THEIR DEVELOPMENT IN CAPTIVITY

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Abstract
Growth in tarantulas is based on the periodical shedding of the exoskeleton, process that is realized through molting. The aim of this paper is to follow the degree and rhythm of development in tarantulas from Acanthoscurria geniculata species – the Brazilian white knee tarantula, in captivity, based on the rhythm of molting and to observe the influence of ambient conditions on this essential process. We analyzed the factors that influence the molting process beginning with the youth period, the behavioral and clinical manifestations, the pathological changes, the duration of the process as well as the seasonal particularities. Knowing these factors, that have an influence on this process, allows it to be carried in the best conditions, without affecting the quality of life and the development of tarantulas.
Keywords: *tarantulas, molting, Acanthoscurria geniculata*

Introduction

Being invertebrates, tarantulas possess an exoskeleton that covers the exterior of their body. Molting in tarantulas refers to the periodic replacement of it, in order to allow growth. This happens with the formation of a new exoskeleton underneath the old one. During molting the old exuvium is replaced with a new, bigger one. This is a process that is influenced by several factors: microclimate, accommodation particularities, food, age, and species (Manning, 1998).

Materials and methods

This study was done on one tarantula from the *Acanthoscurria geniculata* species, during 3 years (from 2011 to october 2014). The exoskeletons were collected right after the molting process was completed and were mesured and weighed. The tarantula was also measured before and after molting. Changes observed regarding the exoskeleton were noted. The methods we used were represented by inspection, observation, monitoring and observation of ambient conditions using a thermometer, a hygrometer and a photographic camera.

Results and disscusion

A few signs (Tabel 1) need to be observed to determine whether the tarantula is in its pre-molting period. Some of them can be observed in the post-molting period as well.

Tabel 1. Body changes observed in the pre and post molting phases

Exoskeleton changes		
	Pre-molting	Post-molting
Chelicerae color	black	pale pink
Hairs color	brown	salmon pink
Number of hairs	rare	thick
Exoskeleton strength	rigid	flexible

These signs can appear singularly or all at once: the tarantula refuses food (Tabel 2), seems lethargic, has difficulties in climbing on the terrarium walls, the skin on the abdomen darkens (easy to spot in *Acanthoscurria geniculata* that, when throwing irritating hairs gets to have empty spots on the abdomen, thus permitting us to see the skin) (Rankin, 1994).

Tabel 2. Eating behavior in the post-molting period

Days after molting	Molting day – eating behavior				
	01.01.2013	18.03.2013	14.07.2013	10.12.2013	05.07.2014
day 1	-	-	-	-	-
day 2	-	-	-	-	-
day 3	-	-	-	-	-
day 4	-	-	-	-	-
day 5	-	-	-	-	-
day 6	-	-	-	-	-
day 7	+	-	-	-	-
day 8	+	-	-	-	-
day 9	+	+	+	-	-
day 10	+	+	+	+	-

The molting process has variable duration, from less than an hour to more than 12 hour (Tabel 3). It is important not to disturb the spider during this time.

Tabel 3. Duration of molting in accordance to age

Molting date	Duration (hours)	Period
01.01.2013	2h 45’	day
18.03.2013	5h 20’	night
14.07.2013	6h 30’	night
10.12.2013	10h 15’	night
05.07.2014	7h	night

When a tarantula is ready to molt, normally it will built itself a web rug (Pic. 1) and lay on it with the ventral part up (Pic. 2). In this stage a liquid layer separates the new and the old exoskeletons but it’s soon absorbed by the new one and replaced with an air layer to prevent adherence between the two.


Pic. 1, 2 – Tarantula building a web rug and laying on it with the ventral part up

After a period the cephalothoraxes and the abdomen will open and the tarantula will start to shed the old exoskeleton. Initially, a crack forms on the dorsal part of the shell that will continue horizontally on the abdomen, allowing the old exoskeleton to open like a lid (Pic. 3).


Pic. 3, 4 – The cephalothoraxes and the abdomen open with a horizontal crack and the tarantula starts to flex its legs to eliminate the old exoskeleton

Light movement can be seen, while the animal flexes its legs (Pic. 4). The cycle flex - rest - flex will continue until the legs are completely out of the exuvium (Pic. 5, 6). After full detachment (Pic. 7), the exuvium is left aside and the tarantula lays on its back resting and regaining strength. Even now it can sometimes flex its legs. After a while it will return to its normal position (Pic. 8). For the next few days it will continue to distend in the new exoskeleton. A newly molted tarantula has a rubber appearance (Pic. 9). In the following days it will continue to flex its legs and strengthen the new exoskeleton.
(www.spidy.goliathus.com, www.tarantulaforum.com)


Pic. 5, 6 – Eliminating the old exoskeleton using slight movements of the legs

Molting also occurs in the fangs, the new ones result in being white and elastic. Until they are not strong and hard the spider won't be able to eat. This period can last from a few days up to a few weeks, age dependent. Handling the tarantula is not recommended during this period because it is very vulnerable, weaken and stressed (Escoubas, 2004).


Pic. 7, 8 – The tarantula has eliminated the old exoskeleton and turns to its normal position to rest

An adult tarantula molts once every one or two years. The juveniles, because of the rapid growth rate, can even molt monthly. For them this process is more rapid and easier, allowing feeding even from the second day after molting has completed.


Pic. 9, 10 – The appearance of the tarantula and the old exoskeleton right after molting

A very interesting property of the molting process is regeneration. A tarantula that has lost a leg, after molting, it can grow it back, although it may be smaller and may not work properly (Ballard, 2003).

Tabel 4. Particularities of molting in *Acanthoscurria geniculata*

	Exoskeleton 1	**Exoskeleton 2**	**Exoskeleton 3**	**Exoskeleton 4**	**Exoskeleton 5**
Date	01.01.2013	18.03.2013	14.07.2013	10.12.2013	05.07.2014
Season	winter	spring	summer	winter	summer
Size	6 cm	6.7 cm	8.2 cm	10 cm	14.5 cm
Color	light colored	light colored	dark colored	dark colored	dark colored
Weight	0.05 g	0.11 g	0.24 g	0.37 g	0.68 g
Size of tarantula before molting	6.2 cm	7 cm	9.1 cm	11cm	13.5 cm
Size of tarantula after molting	6.5 cm	7.5 cm	9.8 cm	13 cm	14.5 cm
Pic. 11, 12, 13, 14, 15 Exoskeletons of *Acanthoscurria geniculata*					

Measuring the tarantula before and after molting and also the old exoskeleton (Pic. 16) allows us to appreciate how much it has grown and if the development rate is normal.

It is very important to provide optimum temperature and humidity during molting. It is preferred an increase in humidity, up to 80%, in this stage. Failing in respecting these conditions leads to severe molting problems, mostly lethal (Ballard, 2003).

Pic. 16 – Exoskeletons in several moltings in *Acanthoscurria geniculata*

In the pre-molting period the tarantula will search for a place to built its web rug (Foelix, 2012). That is why it is absolutely necessary to ensure the right dimensions for the terrarium and to remove any decorations that may occupy space.

Acanthoscurria geniculata will refuse to feed a few days before molting, which is why food that has not been eaten has to be removed or it will alter the hygiene conditions inside the terrarium. Is it not recommended to administer small mammals before molting because, given their high concentration in calcium, they can produce mineral dystrophies: calcification of the soft tissues at the exoskeleton as well as its chitinization. Still, we can give the tarantula cockroaches and worms to provide the nutrients that it needs until the new exoskeleton is fully strengthened (Schultz, 2009).

The duration of the molting process varies with the age of the tarantula and consequently, with its size: the biggest, the older (Tabel 4). Young tarantulas will molt more frequently, several times a year. In males, the adult molting means reaching sexual maturity. After this, normally, they will not molt any more, unlike the females (Ballard, 2003).

Conclusions

1. Molting is a very important process that allows growth in tarantulas.
2. Providing the optimum accommodation needs is very important for the natural carry out of the molting process.
3. Careful observation of the tarantula allows us to spot some specific changes (both natural and pathological), gradually, during the entire process.

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OBSERVATIONS REGARDING THE ACCOMMODATION AND FEEDING OF LEISURE REPTILES

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Abstract
The aim of this study is to illustrate the possibilities of accommodation and feeding for some wild, exotic animals, newly appeared among the preferences of breeders, respectively the common green iguana (Iguana iguana) and the corn snake (Elaphe guttata). The special needs that these animals demand arise from the environmental conditions of their original climate, very different from the one of our country regarding temperature, humidity, circadian rhythm, solar radiation level. Each species demands a certain pattern of feeding and caring that every future breeder has to know and to respect in order to ensure animal welfare and optimal development in captivity. Acquiring such an exotic pet can be tempting but has to be made knowing what it imposes in order to prevent premature health problems due to negligence or lack of knowledge, zoonotic aspects or lack of interest from the owner once he realizes the true needs or behavioral particularities.
Keywords:*iguana, corn snake, accomodation*

Introduction

Class *Reptilia*, Order *Squamata*, Suborder *Sauria (Lacertilia)*, Family *Iguanidae*, Species *Iguana Iguana*.

The common iguana or green iguana has its original habitat in the wet, semiarid, tropical woods from Central and South America as well as some islands from the West Indies Archipelagos. They are both arboreal and terrestrial. Males are more aggressive than females and they fight for the best place to get a sunbath or the biggest territory. They can easily reach 2m in length and 8-9 kg in weight.

Class *Reptilia*, Suborder *Lepidosauria*, Order *Squamata*, Suborder *Serpentes*, Family *Colubridae*, Subfamily *Colubrinae*, Genus *Pantherophis*, Species *Pantherophis guttatus (Elaphe guttata)* (Holland, 2002)

The corn snake is easy to keep in captivity. It's not an aggressive species and does not require special accommodation conditions so it can be a prime choice for a beginner in herpetology.

Materials and methods

This study followed the accommodation and feeding possibilities of one adult male green iguana (Pic.1), aged 6, and an adult male corn snake, aged 3. For both we tried to ensure the minimum accommodation conditions that these species require. The methods we used were represented by inspection, observation, monitoring, observation of ambient conditions using a thermometer, a hygrometer and a photographic camera.

Results and disscusion

The Green Iguana

The terrarium can be made from glass, plastic, wood and other materials. When making the design and construction of a terrarium we have to consider the fact that a young iguana, 20-30 cm long, will grow in to a 1.5-2 m long adult. Glass and plastic terrariums are only suitable for youngsters. For an adult the habitat should be built depending on the

dimensions, shape and particularities of the room where it will be placed. At any age of the iguana the terrarium should have two iguanas in length, one iguana in width and two iguanas in heights. The main conditions for a terrarium are: large and spacious, high, properly heated, lightened and ventilated, with climbing areas that may help thermoregulation, with a water recipient for drinking and keeping humidity. The vivarium should provide visibility but should never be set by a window because sunlight can overheat it causing the death of the animal inside. Also radiators, air conditioning or radiators should be at a safe distance too (Hatfield, 2004).

Pic. 1 – Male green iguana

The materials used for constructing the terrarium are not to be rough, reflective or glossy in order to avoid injuries to the animal. For bedding, wrapping paper can be used but also wood shavings and cellulose fiber. Substrates that can be ingested with food are to be avoided (Manning, 1998).

Pic. 2 – Male green iguana bathing in the sunlight

As light sources, one can use natural sunlight (the best and more natural solution – Pic.2), supplemented with UV-B lamps if filtered by glass. Also, usual light bulbs, fluorescent tubes, black light tubes etc. can be used but always placed at a safe distance from the animal (40-50 cm). The optimum temperature is set somewhere between 24^{o} and 31^{o} C with a hotter period during the day (35^{o} C). The humidity should be around 80% and can be provided with a water recipient or a nebulizer. For heating one can use heating lamps or thermal rocks, plaques etc. keeping in mind that these can cause burns if left to the direct access of the reptile.

The day – night light cycle has to be approximately 12 h – 12 h. For the iguana the day should begin around 6 am and feeding should also take place in the morning so that it may use the noon heat and light for metabolizing food (Hatfield, 2004; Girling, 2003).

We chose to accommodate our iguana in multiple spaces such as a room of 4 m per 5 m, a simple cage box of 1 m per ½ m and ½ m and a larger cage 2 m high, 1.5 m long and 1 m in width. From our observations we can say that free living in a room can offer the best conditions for the expression of the natural behavior and also for keeping in good health the iguana. Besides the space that it provides for moving around the room also provided natural sun light and light/dark cycle. Warm baths were given once every two weeks to help the iguana keep clean and to ease the shedding process.

Iguanas are herbivorous but for short periods can be omnivorous, depending on season or availability of resources. They eat mostly leafs (salad, spinach, dandelion, parsley, grass, hibiscus), flowers (rose petals), fruits (apples, pears, grapes, apricots) and vegetables (beans, peas, tomatoes, potatoes, peppers). Carrots can also be used but with some limitations because of their high content in calcium oxalate.

Pic. 3 – Mixed salad for the iguana

The food should be finely chopped and mixed into a salad in order to make sure that the iguana can eat and get all the nutrients that it needs. Adults should be fed daily and youngsters one-twice a day. Vitamin and mineral supplements can be added to the food (Hatfield, 2004; Manning, 1998).

The Corn Snake

For the corn snake there are two main directions for housing: the western way means very simple materials and almost no decorations, the European way means trying to recreate the natural habitat for the snake. The Americans prefer plastic boxes (shoe, blanket boxes) with an absorbent bottom of folded newspaper, paper towels or aspen shavings, a water bowl and a simple hide box (Pic. 5).

The Europeans chose to recreate a nature corner, with plants and branches (Pic. 6) and sometimes even other animals (insects, amphibians) in it (Bartlett, 2006).

The materials that can be used to make a terrarium for a corn snake are the same as for the iguana: glass (Pic. 4), plastic, wood. The adult corn snake reaches about 1 m in length and can climb very well on branches even though it is not classified as an arboreal snake. In view of these particularities the cage can be both long and high and can have natural or artificial branches (Mattison, 2006).

Pic. 4 Glass terrarium for a corn snake

Pic. 5 Hideout made from a ceramic pot

Ventilation is important so, whatever the material used for construction, a series of holes need to be drilled or melted preferably in the lower and upper parts of the terrarium and sometimes even on the top.

Inside the vivarium the ideal would be to have a cold corner with 18-24^{o}C and a warm corner of 24-28^{o}C. This can be achieved with heating pads, heating tapes, hot rocks and ceramic heating units with light bulbs. Light can be provided with different types of lamps, fluorescent or normal. Corn snakes do not need additional UV-B because they get their Ca and vitamin D3 from the prey they eat. While the amount of UV supplied may be negligible, the color-temperature and full-spectrum bulbs may be beneficial to captive snakes (Bartlett, 2006; Girling, 2003).

Pic. 6 – Branches and other decorations may be used but not in excess

Cage humidity can be important and the lack of can cause shedding problems. One can use a simple water bowl (Pic. 7) that, besides serving for dinking, can help regulating the humidity inside the terrarium. Also a water dish large enough for the snake to soak in from time to time could be a nice treat for him and could help while shedding (Bartlett, 2006).

Pic. 7 – Simple setting inside the terrarium of a corn snake

Feeding can be done once a week or one every two weeks in the summer and more rarely in the cold season (once a month). Mice and baby rats can be used depending on the snake size, age and feeding habits. Sometimes we can chose pre-killed food, like frozen mice, but we need to consider that defrosting has to be done slowly, preferably in warm water, to preserve the properties of the food (Manning, 1998).

Conclusions

1. Choosing a green iguana as a pet has to be done with prudence and much consideration with regard to the fact that it will need a lot of space and special habitat conditions all its life.
2. If the special needs that the iguana requires as a species are not satisfied both health and behavior problems can manifest.
3. The corn snake is a reptile that can be kept in captivity even in terrariums with only minimal conditions, being a docile species and relatively easy to handle in the absence of other prepredators..
4. Both these species can be suitable companions for someone that wishes to recreate a wild and exotic corner inside their house while respecting the special environmental needs that they require.

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OBSERVATIONS REGARDING THE ACCOMMODATION NEEDS FOR LEISURE TARANTULAS

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Abstract
In this paper we present the influence of some ambient factors (terrarium – as a preferred space for accommodation, bedding substrate, temperature, humidity, air currents etc.) that condition the keeping of tarantulas in captivity. The artificial microhabitat for the three species taken into account for this study (Acanthoscurria geniculata – the Brazilian white knee tarantula, Pelinobius muticus – the King baboon and Chilobrachys sp. "Blue" – the Vietnam blue tarantula) recreated in an original fashion the environmental conditions of their native habitat, respectively for tarantulas that live in subterranean tunnels or on the surface of the substrate. Knowing these ambient particularities that each species demands allows the normal growth and development of tarantulas in captivity
Keywords*: tarantula, habitat, terrestrial*

Introduction

When considering exotic pets, invertebrates may be considered by breeders that want a quiet, easy to keep and yet fascinating animal. Although they require special needs they are not time consuming animals and besides the initial investment they don't need much more afterwards. Tarantulas are one of the safest and easiest invertebrates to keep. There are over 900 species to choose from, some are venomous and aggressive but some can be handled without problems (Manning, 1998).

Materials and methods

We chose for this study three tarantula species: *Acanthoscurria geniculata* – the Brazilian white knee tarantula, *Pelinobius muticus* – the King baboon and *Chilobrachys sp. "Blue"* – the Vietnam blue tarantula. All three are terrestrial species but the first one lives on the surface of the substrate while the other two inside galleries that they dig up themselves.

Results and disscusion

1. Terrarium

Acanthoscurria geniculata is a terrestrial species and so the base floor surface of the terrarium is more important than its height. It needs a hideout or a thicker layer of substrate so they can build one even though they don't spend too much time inside it. The terrarium needs to have around 50 cm in length and 30 cm in width. For young tarantulas the terrarium can be a plastic box of 20 cm per 10 cm with a detachable lid for feeding and cleaning. Also, the lid needs to have large air spaces to ensure the wellbeing and the minimum habitat conditions for tarantulas (Pic. 1) (Rankin, 1994).

Pic. 1 – Plastic terrarium for a young tarantula (*Acanthoscurria geniculata*)

For large tarantulas the terrarium can be made from glass (Pic. 2), polystyrene, OSB, plaster etc. still respecting the same conditions mentioned above. Regarding decorations, *Acanthoscurria geniculata* does not need any and even more, they can cause injuries. Still, most breeders use decorations to improve the aesthetics of the terrarium (Schultz, 2009).

Pic. 2 – Glass terrarium for an adult *Acanthoscurria geniculata*

For *Pelinobius muticus* (The King Baboon) the terrarium (Pic. 3) may have 20 cm in length and 10 cm in width for the young ones (the body measures up to 6 cm) and for the adults, 40 cm per 40 cm (the body length reaches 22 cm). In both situations height is important because it needs a thicker layer of substrate, around 30-50cm (www.spidy.goliathus.com, www.tarantulaforum.com).

Pic. 3 – Tall terrarium for a young *Pelinobius muticus*

Regarding *Chilobrachys sp. blue* (The Vietnam Blue tarantula) , because they have a rapid growth, the terrarium should have 25 cm in length and 10 cm in width for the young ones (Pic. 4) and 30 cm both in length and width for the adult ones. The height of the terrarium is also important, about 30-40 cm are necessary. Decorations are not recommended because they will be buried in the substrate and may cause injuries to the tarantula (www.spidy.goliathus.com, www.tarantulaforum.com).

Pic. 4 – Vertical spread of the substrate in the terrarium of a *Chilobrachys sp. blue*

2. Substrate

For *Acanthoscurria geniculata* the base of the terrarium should be covered with peat moss, vermiculite, potting soil without additives, coconut fiber etc (Pic. 5, 6). No matter the substrate type, three characteristics are very important: humidity, neutrality and hygiene. The thickness has to be around 3-4 cm to sustain optimum humidity. It is preferable to keep it moist on a daily basis but not always recommended because of different fungi and mites that may develop in it. Also, high humidity in the substrate can cause the death of the tarantula. The alternative is a water bowl (Pic. 11) to avoid dehydration. The bowl has to be in proportion with the size of the tarantula so that we can eliminate the risk of it drowning (Rankin, 1994; Scultz, 2009).


Pic. 5, 6 – Aspects inside the terrarium of *Acanthoscurria geniculata* with coconut fiber as substrate

Being a nocturnal tarantula, *Pelinobius muticus* lives most of the time in its galleries dug in the substrate with web lining (Pic. 7, 8). The tunnels have more than one exit and all but one are carefully covered with web. The tarantula can stay hidden in these galleries for long periods of time, coming out just for food and water. That is why a thicker layer of substrate is necessary, from 6 cm for the young ones to 20 cm for the adult ones. Also, a periodical change in the pattern of galleries will be noticed (Rankin, 1994; Scultz, 2009).

The preferred substrate is coconut fiber because it allows easy digging. To keep the humidity within the accepted limits a water bowl is more recommended than watering the substrate.


Pic. 7, 8 –Coconut fiber is preferable for Pelinobius muticus because it helps in forming its galleries

Coconut fiber is preferable for *Chilobrachys sp. blue* too, in a thick layer up to 20 cm for adults. Being a species that lives almost permanently hidden in galleries (Pic. 9, 10), its maximum activity will be noticed during night time. Unlike *P. muticus*, the web lining inside the galleries is more abundant and the diameter of the tunnels are smaller compared to those of the tarantula (for a 6 cm tarantula the diameter of the galleries will be around 2 cm) (Escoubas, 2004; Foelix, 2012).


Pic. 9, 10 –Coconut fiber or peat moss eases the digging for *Chilobrachys sp. blue*

3. Humidity

Can be measured with a hygrometer (Pic. 12) and has to be around 60-70% for *Acanthoscurria geniculata* and *Pelinobius muticus*, a little bit higher for *Chilobrachys sp. blue*: 70-80%. Humidity has to be maintained within those boundaries for several reasons: low humidity can cause serious problems during molting, low hydraulic pressure can cause motion problems and dehydration can even cause the death of the tarantula. One thing that needs to be avoided is the accumulation of water in the humid part of the substrate or the formation of swamps. The best solution is to create a dry corner in the terrarium so that the tarantula may chose between several degrees of humidity according to its daily metabolic needs (Ballard, 2003).

Pic. 11 – *Acanthoscurria geniculata* drinking from its water bowl

4. Temperature

The body temperature of the tarantula, the metabolism and other biological functions are linked to the ambient temperature.

The optimum temperature can be set between 25-28^{o} C on day time for *Acanthoscurria geniculata* and must not drop below 18^{o} during night time. In order to provide these conditions we can use as a heat source a lamp, heat pads, heat wires etc. Ensuring the right temperature is in direct relation with the growth rate and reproduction. In captivity, tarantulas from this species feel comfortable at room temperature.

Raising temperature over the normal limits can have visible effects on the tarantula meaning the contraction of the cardiac tube with the increase of the number of contractions per minute, an agitation state and an increase in the dynamism of the animal. On the other side, lowering the temperature can induce hypobiosis, with the decrease of the number of heart contractions and respiratory movements as well as motility (Ballard, 2003).

Pic. 12 – Thermometer and hygrometer for constant surveillance of internal microclimate conditions

5. Lightning

Regarding the light-dark cycle, a natural light source is sufficient when avoiding the strong summer day light. Artificial light can cause low humidity and sudden heating of the air can cause burns on the abdomen. To observe the night activity of the tarantula, terrariums can have a red light source, in view of the fact that tarantulas do not see this color (Manning, 1998).

Tabel 1. Accomodation conditions for three tarantula species

Species	Temperature	Humidity	Terrarium length	Terrarium width	Terrarium height	Substrate
Acanthoscurria geniculata	25-28 ^{o}C	60-70%	50 cm	30 cm	30 cm	peat moss, vermiculite, potting soil without additives, coconut fiber
Pelinobius muticus	24-30 ^{o}C	60-70%	40 cm	40 cm	50 cm	
Chilobrachys sp. blue	21-32 ^{o}C	70-80%	30 cm	30 cm	40 cm	

The basic habitat needs are almost the same for the three tarantula species that we studied. The particularities (Tabel 1) originate from the different behavior and lifestyle but also from the natural conditions that their original climates offer.

Conclusions

1. Tarantulas are animals that can be kept in captivity when ensuring a few special conditions both respecting and avoiding their defense possibilities.
2. References regarding keeping tarantulas in captivity are not numerous. They can be kept by a passionate breeder that can provide for them original accommodation conditions, similar to those from their wild, original habitat.
3. In order to ensure proper growth and development a few particularities need to be respected, depending on the tarantula species.

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IN VITRO SCREENING OF API-PHYTOTHERAPEUTIC PRODUCT TO CONTROL NOSEMA SPP. INFECTION

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Abstract

Welfare status of bee colonies in Romania, as well as in other European countries, is affected by specific parasites and pathogens[2, 3, 4, 7, 8, 9, 10]. Nosema spp. are known to be the most infectious among European honeybee cultivators [1, 2, 6, 7] . In order to obtain a natural preparation with an antiparasitic effect as an alternative therapy to control Nosema spp. spores development' there have been tested 10 hydroalcoholic extracts from different plants and also propolise extract. The experiments were performed on naturally infected honey collected from Romanian apiaries diagnosed with nosemosis with an estimated of value of 28 spores/field The infection of the honey samples was determined by OIE 2008 method. The samples were incubated with different plant extracts for eache extract being performed five replicates. The obtained results showed that Origanum vulgare and Rosmarinus officinalis extracts (with a concentration of 0.70 % g/g volatile oils) emphasized the highest antiparasitic action against Nosema spp. After three consecutive treatments, the number of spores was significantly reduced to 4 spores/field. In vitro testing showed antiparasitic activity after 3 administrations of the work variant V_7 (T_1, T_2, T_3) in honey naturally infected with Nosema spp. spores. It was observed the reduction in the number of spores at max. 1 spore / field, compared to the control which remained constant at the initial value of 7 spores / field, which justifies the use of the work variants for testing under field conditions. They have been designed several work variants depending on the hydroalcoholic concentration and the content in volatile oils of the extracts (10 variants including in the formulation also a bee raw material). In the Laboratory of Bees Pathology of the ICDA Bucharest, for each version of the product, organoleptic, physico-chemical and microbiological determinations were performed. compiling product sheets.

These results recommend api-phytotherapeutic product to control Nosema spp. development, as an alternative to veterinary therapy currently in use.

Keywords: *api-phytotherapeutic product, in vitro screening, Nosema apis /ceranae.*

Background: Welfare status of bee colonies in Romania, as well as in other European countries, is affected by specific parasites and pathogens. *Nosema apis* and *Nosema ceranae* are known to be the most infectious among European honey bee cultivators [1, 2, 3, 7, 8, 9, 10].

The aim: The obtaining of a natural product with an antiparasitic effect as an alternative therapy to control *Nosema sp.* Spores development by testing 10 hydroalcoholic extracts from different plants and also the conventional preparation used by owners.

Materials and methods

The experiments were performed on naturally infected honey collected from Romanian apiaries diagnosed with nosemosis, with an estimated of value of 28 spores/field. The infection of the honey samples was determined by OIE 2008 method. The samples were incubated with different plant extracts, and a commercially available phytotherapeutic preparation, for each extract being performed five replicates. There were investigated samples from 32 apiaries, from 11 counties of Romania.

Results and discussion

The obtained results showed that preparation no. 7,(with a concentration of 0.7% g/g volatile oils), emphasized the highest antiparasitic action against *Nosema sp.*, the number of spores was significantly reduced, to 1 spore/field as compared with control, that presented 7 spores/field.

Table no. 1 – The volatile oil concentration of 10 selected preparates

Hydroalcoholic preparation	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
Volatile oils concentration g/g	0.15	0.16	0.31	0.50	0.22	**0.47**	**0.70**	0.19	0.16	0.44

Table no. 2 – Comparative eficiency of V 7 formula after three consecutive „in vitro" treatments

Tested lotes	**E 1**	**E 2**	**E 3**	**E 4**	**E 5**	**E 6**	**E 7**	**E 8**	**E 9**	**E 10**	**Ctrl. treat.**	**Cont rol**	**V 7**
T_0													
Honey infected (g)	30	30	30	30	30	30	30	30	30	30	30	30	30
Number of spores/field	28	28	28	28	28	28	28	28	28	28	28	28	28
Type Na/Nc	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑	a-c↑
Treatment (ml). **I**	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3
T1 (after 1 **day**)													
Nr. of spores /field	0-2	0-4	1-6	2-10	1-8	0-4	2-8	3-16	3-14	0-1	0-4	0-5	0-1
Type Na/Nc	C	a↑-c	C	a-c	A	a-c↑	a-c↑	a-c↑	a-c	a-c	a-c↑	a-c↑	c
T2 (after **2 days**) Nr. of spores /field	2-8	0-4	1-6	0-3	0-3	0-2	0-1	0-2	0-3	0-1	0-3	0-5	0-1
Type Na/Nc	a-c↑	a-c	a-c	a-c↑	a-c↑	a-c	C	C	C	C	a-c↑	a-c↑	a
T3 (after 3 days)													
Nr. of spores /field	0-6	0-3	0-2	0-3	0-3	0-1	0-1	0-2	0-2	0-1	0-3	0-4	0-1
Type Na/Nc	a-c↑	a-c↑	a-c↑	a-c↑	A	A	C	C	a-c	a-c	a↑-c	a↑-c	A
Treatment (ml). **II**	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6
T4 (after **4 days**)													
Nr. of spores /field	0-4	0-3	0-3	0-2	0-4	0-3	0-2	0-2	0-5	0-2	0-2	2-7	0-2
Type Na/Nc	C	a-c	a-c↑	a-c	a-c↑	a-c↑	↑a-c	A	A	a-c	a-c	a-c↑	a
T5 (after **7 days**)													
Nr. of spores /field	0-2	0-17	0-6	0-4	Treat ment	0-4	0-2	0-2	0-2	0-2	0-4	0-7	0-2
Type Na/Nc	C	a-c	a-c↑	a-c	a-c↑	a-c↑	↑a-c	A	A	a-c	a-c	a-c↑	A
Treatment (ml). **III**	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9	0,9
T6 (after 14 days)													
Nr. of spores /field	0-5	0-2	0-7	0-3	0-2	0-2	0-4	0-5	0-4	0-2	0-3	0-7	0-1

The dose was determined after the field research conducted on bee families, taking into account the concentrations of volatile oils compared Witness P product, and depending on the behavior of bees at work managing variants obtained.

Administrations were carried out at T0 and T1 respectively (after 7 days) and T2 (after 14 days) doses of 0,3 ml, 0,6 ml and 0,9 ml and the results summarized in tables 1 and 2 highlights the evolution of the degree of infestation of honey in bottles or Petri dishes, following administration of product variants (3 doses / 7 days interval).

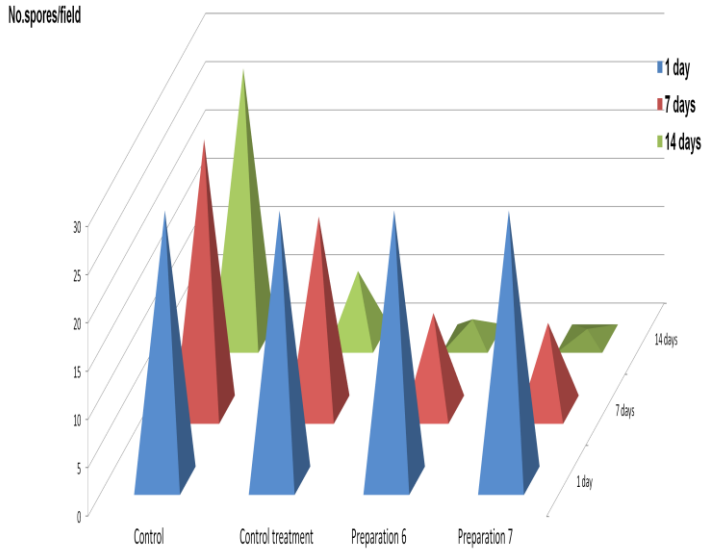

Fig. No. 1 – "In vitro" efficiency after 3 administrations at 7 days period of Preparation V7

After testing "in vitro" activity was found after 3 administrations anti parasitic effective working variant V 7 (Preparation 7) in honey infected with spores of Nosema spp. Reducing the number of spores at maximum 1 spore / field compared to the control which remained at 7 spores / field and Control treatment (within 3 spores per field) justifies the use of V 7 working version for testing under field conditions.

Conclusion

These results recommend the api-phytotherapeutic preparation number 7 as treatment of honey to control *Nosema sp.* development, as an alternative to veterinary therapy currently in use and also for testing in field conditions.

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STUDY REGARDING THE INFLUENCE OF ACCOMMODATION CONDITION ON THE WELFARE OF SOME AQUARIUM FISH (*CARASSIUS AURATUS AURATUS, CORYDORAS ALBINO, ANCISTRUS DOLICHOPTERUS* AND *ANCISTRUS DOLICHOPTERUS ALBINO*)

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Abstract

This study was performed from 2012 to 2014 and it's aim was to observe the influence of accommodation condition (size of the tank, temperature, filtration, oxygenation, water changing period, feeding, etc.) on the welfare of aquarium fish (6 Carassius auratus auratus, 2 Corydoras albino, 1 Ancistrus dolichopterus and 1 Ancistrus dolichopterus albino). We observed that in order to ensure the optimum caring conditions for aquarium fish from the mentioned species, the most important role resides in the filtration system and the use of different materials (charcoal, sponge, synthetic cotton- mechanically cleaned on a 48 h basis), changing 60% of the total water volume once every 14 days as well as providing granulated feed containing proteins of vegetal and animal origin, vitamins, minerals, color intensifiers and lecithin.

Keywords: *Ornamental fish; Carassius auratus auratus; accomodation condition; nutrient requirement; welfare*

Introduction

Ornamental fishes are attractive and colorful species of fishes with peaceful nature and they are the most popular pets in present day world (Singh, A.K. and Ahmed, S.H. 2005). Ornamental fish keeping is emerging as one of the most popular hobbies across the world next to photography (Kurup, B.M, 2003; Das et.al.2005; Singh, A.K. and Ahmed, S.H. 2005). *Carassius auratus auratus* which originated in China were a dull brown. They had been selectively bred by the Chinese over the centuries to develop the different colour, scale and body shape morphs as seen today. The Common Goldfish, Shubunkins, Comets, Fantails, Veiltails, Moors, Pearl Scale, Orandas, Ranchu, Lionheads, Pom Pom, Bubble Eye and Celestials are only a few of the breeds of this single species available. Those "Panda" coloured individuals (orange/black or white/black) are in the process of changing colour and may turn completely orange or completely white. It is a natural occurrence that is not very well understood, but is assumed to be related to hormonal changes as the fish matures. Depending on breed, goldfish can attain a length of between 10 - 20cm with a lifespan of 20 years.

This study was performed from 2012-2014 and aimed at monitoring the influence of maintenance conditions (aquarium size, temperature, filtration, oxygenation and frequency change of water, food, etc.) on the welfare of some aquarium fish (*6 Carassius auratus auratus, 2 Corydoras albino, 1 Ancistrus dolichopterus and 1 Ancistrus dolichopterus albino).*

The criteria for assessing "the degree of comfort" of ornamental fish in this study were represented by: the manifestation of swim behavior (dorsal fins and tail position, the general position of the body, moving at different levels in the aquarium, etc.). Also the manifestation of feeding behavior (reactivity when feeding and foraging granulated in

aquarium), intra and interspecific social behavior, manifestation of the playful behavior and the changes of body color (pigmentation clarity and brightness).

Materials and methods

For this study were assessed 6 *Carassius auratus auratus* specimens, two specimens of albino Corydoras, one specimen of Bushymouth catfish and one albino Bushymouth catfish. The handcrafted glass aquarium equipped with useful water volume of 96 liters equipped with Internal Filter Resun Magi 1000 (max aquariums. 80-250 liters, power: 19W, flow 852 l / h, mechanical and biological filtration, adjustable flow and spray bar), Perlon-synthetic cotton for water filters, air pump, aquarium heater with thermostat, *Vallisneria giant* aquarium plants, natural lighting, water thermometer and hand pump for water removed from the aquarium. Food was provided only by using commercial diet designed for ornamental aquarium fish (pelleted commercial feed). Also for monitoring the behavioral manifestations were used photo and video cameras.

Working method: The study was conducted by monitoring water temperature, recording the periodicity of aquarium water change, observing the filtration efficiency before and after using synthetic wool, and the commercial diet effect on general state of fish.

Results and discussion

Following the study, it was observed that in order to ensure optimal growth conditions of the aquarium fish species, to ensure the water temperature has an important role, also filtering system using different materials (coal, sponge and synthetic cotton - mechanically cleaned 48 hours), change of 60% in volume of water at 14 days, as well as the use of the granulated food based nutrition of plant and animal derived protein, vitamins, minerals, enhancers, color, and lecithin.

Criteria for assessing the "comfort level" of ornamental fish in this study were represented by: manifestation of swim behavior (dorsal fins and tail position, the general position of the body, moving at different levels in the aquarium, etc.); manifestation of feeding behavior (reactivity when feeding and foraging granulated in aquarium); intra- and interspecific social behavior; manifestation of playful behavior; changes in color (pigmentation clarity and brilliance).

The water temperature

The water temperature was monitored for 24 months, it was recorded changes depending on the season, with an average of between 20-33ºC (Fig.1). To raise the water temperature in winter was used heater with thermostat.

Regarding the expression patterns of behavior analyzed it was observed the swimming behavior modification, the ornamental fish swimming at the bottom of the aquarium and a reduction of the playful behavior when water temperature was below 22ºC.

When water temperature was over 25ºC, the behavioral patterns occurred in all individuals, regardless of species, the fish prefer to swim at all levels of the aquarium.

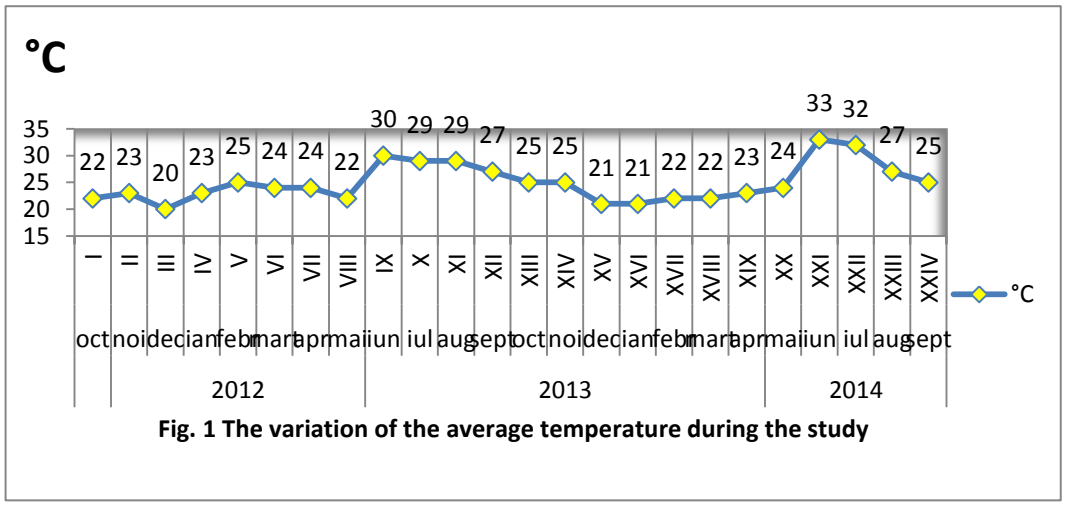

Fig. 1 The variation of the average temperature during the study

The filtering system and the changing of water

Filtration system was represented by a filter equipped with sponge box, with coals and synthetic cotton. It was observed that the best results were obtained by filtering water-using simultaneous of three types of materials, with the change to 48 hours of synthetic cotton and mechanical cleaning of the filter to remove accumulated deposits on its component parts. Regarding the effect on the degree of clarity of the water, it was observed that the best results were obtained by the changing of 60% water volume with a periodicity of 14 days. (Fig. 2).

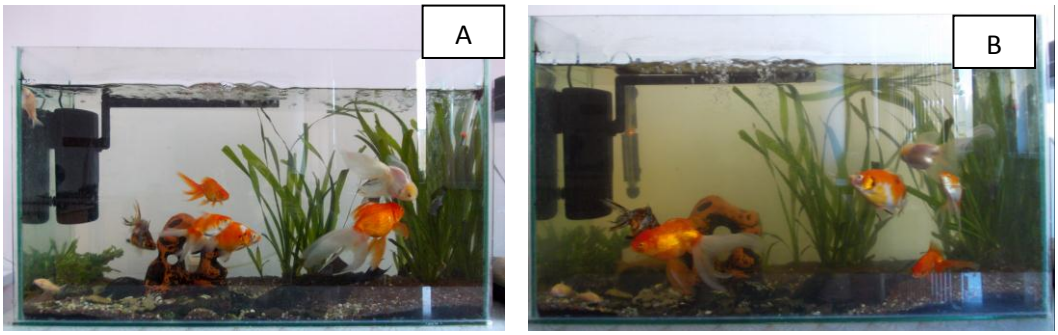

Fig. 2 A- The aquarium after 48 hours of water changing and the use of synthetic cotton filter
B- The aquarium after 48 hours of water changing without using of synthetic cotton filter

The fish feeding

Food was provided only by using commercial diet designed for ornamental aquarium fish (pelleted commercial feed) with fish and fish products, vegetable protein extracts, grains, vegetable products, vegetables, yeast, shellfish and crustaceans, fats, minerals, algae. Also, the main constituents are represented by: crude protein 46%, fat 7%, cellulose 2%, humidity

8%, vitamins and provitamins (Vit. A 30035 UI/Kg, Vit. D3 1860 UI/Kg), trace element (Mn 68 mg/Kg, Zn 40 mg/Kg, Fe 26 mg/Kg, Co 0,5 mg/Kg) and lecithin.

Effect of feeding with commercial diet mentioned was observed primarily on gloss and pigmentation of the fish body (Fig.3).

Fig. 3 Potentiation of pigmentation in *Carassius auratus auratus*

Food is the most important and vital factor for the growth and survival of the living beings on the face of the earth (Islam, M.N., 2004) and feeding is the dominant activity of the entire life cycle of fish. The success on good scientific planning and management of fish species largely depends on the knowledge of their biological aspects, in which food and feeding habits include a valuable portion (Joadder, M.A.R., 2006; Gupta S and Banerjee S. 2009).

Fish eat to satisfy their energy requirement, and protein and energy in the diet should be balanced (Macartney A, 1996). Although fish use energy efficiency as an energy source, excessive dietary intake may restrict protein consumption and subsequent growth (Kruger at al. 2001), started that it would appear that a diet with at least 45% protein at a 6% lipid level is needed for the best specific growth rate and conversion of growing (6-8 weeks of age) swordtails. Fish meal might be substituted by soyabean meal up to37% of the diet, replacing 33% of the fishmeal protein, to normal growth in juvenile tin foil barb (Earle, K.E, 1995).

Dietary lipids are important sources of energy and fatty acids that are essential for normal growth and survival of fish (Sales, J and Janssens G.P.J. 2003) Although fish have a low energy demand, and is thus susceptible to deposition of excessive lipid (Earle, K.E 1995), lipids do have a role as carriers for fat-soluble vitamins and sterols, are important in the structure of biological membranes at both the cellular and subcellular levels, are components of hormones and precursors for synthesis of various functional metabolites such as prostoglandins, and are also important in the flavour and textural properties of the feed consumed by fish. Fish in general require fatty acids of longer chain length and a higher degree of unsaturation than mammals.

No dietary requirement for carbohydrates has been demonstrated in fish. However, carbohydrates present a cheap energy source, that would “spare” the catabolism of other components such as protein and lipids to energy. Minerals are inorganic elements required by fish for tissue formation and various functions in metabolism and regulation. Ornamental fish can absorb some watersoluble minerals from the water, complicating studies in determining

dietary mineral requirements. Of all the minerals required by fish, phosphorus is one of the most important because it is essential in growth, bone mineralisation and lipid and carbohydrate metabolism, and is needed in the diet due to low content in natural water (Sales, J and Janssens G.P.J. 2003).

Fish use oxygenated carotenoids, one of the most important groups of natural pigments, for pigmentation of skin and flesh. Carotenoids commonly occurring in freshwater include beta-carotene, lutein, taraxanthin, astaxanthin, tunaxanthin, alpha-, beta-doradexanthins, and zeaxanthin. As fish cannot synthesise these pigments, they rely on dietary supply of carotenoids to achieve their natural skin pigmentation, one of the most important quality criteria informing the market value of ornamental high value species such as Koi carp (*Cyprinus carpio*) and goldfish (Paripatananont et al., 1999; Lovell, R.T. 2000; Gouveia et al., 2003). Red colouration is imparted in goldfish and common carp by astaxanthin, a carotenoid that is readily metabolised from the yellow pigment zeaxanthin. Goldfish metabolised very little beta-carotene and no lutein to astaxanthin. Under certain well-defined culture conditions (nitrogen depletion, high salinity and light intensity) the algae *Chlorella vulgaris, Haematococcus pluvialis* and *Arthrospira maxima* (*Spirulina*) will accumulate secondary carotenoids and might be used to replace costly synthetic colourings in ornamental fish feed (Gouveia et al., 2003).

One of the main problems is the diversity of species kept in home and public aquaria and how to provide them with adequate diets (Pannevis and Earle, 1995; Macartney, 1996). With the exception of a small number of tropical freshwater carnivorous fish species (Cichlidae), and goldfish and koi carp, ornamental fish are seldom kept in a single-species environment (Macartney, 1996). It is impractical to feed very specific diets to individuals in an aquarium environment (Pannevis, 1993; Pannevis and Earle, 1995). The diet must be suitable for all tank inhabitants, which may include herbivores, omnivores, and carnivores. Not only will these fishes have different nutrient requirements, but also the digestibility of various components of the diet will differ depending on the natural diet and intestinal morphology. Furthermore, the physical characteristics of the diet and the feeding regime must satisfy the different lifestyles and feeding habits, such as surface, middle, and bottom feeders, and diurnal variations in feeding among these groups. Physical characteristics of the diet also play an important role when species of various weights are fed on the same diet. Food particles need to be small enough for the smaller species to ingest, but large enough to be identified and eaten by the larger species (Macartney, 1996).

Conclusion

1. This study was performed from 2012 to 2014 and it's aim was to observe the influence of accommodation condition (size of the tank, temperature, filtration, oxygenation, water changing period, feeding, etc.) on the welfare of aquarium fish (6 *Carassius auratus auratus*, 2 *Corydoras albino*, 1 *Ancistrus dolichopterus* and 1 *Ancistrus dolichopterus albino*).
2. The water temperature positively influenced the fish studied, values being maintained between 20-33ºC, depending on the season, especially depending on the ambient outdoor aquarium.
3. The most effective method of filtering has been obtained by the simultaneous use of coal, and sponge, synthetic cotton, and by changing a volume of 60% water every 14 days.

4. The clarity of pigmentation and brightness on *Carassius auratus auratus* in was obtained and maintained by the administration of balanced commercial diet in protein, vitamin and mineral, with a high content of essential amino acids and color enhancers.

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DERMATOPHILOSIS IN A SIMMENTAL CALF

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Abstract

The aim of this case report was to evaluate cytologic, histopathologic and culture results from skin scraps and biopsy samples in dermatophylosis in 4 month old male Simmental calf in Erzurum, one of the coldest regions of Turkey. In clinic-macroscopic examination, grayish to brown scabs; 0,1-0,5 cm in diameter, were observed on head, neck, dorsal and lumbal region. These lesions spread through the wall of the abdomen and chest. Typical coccoid spores in filamentous shaped were observed with normal and necrotic keratinocytes in smear slides stained with Giemsa. Similarly, gram positively stained spores in one or more filamentous chain shaped were observed in bacteriologic culture. Marked hyperkeratosis and clusters of coccoid-shaped bacteria under hyperkeratotic area and inflammatory infiltrations were detected in histopathological sections prepared from biopsy material. Gram positive coccoids were detected to spread sometimes in the form of long-chain, sometimes as single or double row in gram staining of colonies obtained from blood and chocolate agar. In the treatment, long-acting oxytetracycline (20 mg/kg dose, IM, two times with a week intervals) was administered.

Key words: *Simmental Calf, Culture, Cytopathology, Dermatophilosis, Histopathology.*

Introduction

Dermatophilosis occurred by *Dermatophilus congolensis* which is pleomorphic gram-positive actinomycete, is one form of acute or chronic exudative dermatitis characterized by superficial crusting. The disease is much more common in hot and rainy areas of tropical regions of Australia, Africa and South America (Radostits ark. 2006; Ginn ve ark. 2007; OIE, 2008). Dermatophilosis is a zoonotic disease and reported in cattle (Berhanu ve ark. 1999; Decostere ve ark. 2002; Dalis ve ark. 2009; Dalis et al. 2010; Admassu ve Alemu 2011), horse, sheep and goat (Pal, 1995; Sekin et al. 2002; Awad et al. 2008), cat (Pal, 1995; Kaya et al. 2000), and human (Burd et al. 2007). The most important predisposing factors affecting the pathogenesis of Dermatophilosis is remaining the skin wet for a long time and disruption of the integrity of by mechanical effects. Long-time humidity and wetness of the stratum corneum cells of skin separates from each other and this situation is suitable for vector tick activity.

Under the proper moisture and temperature conditions, natural defense mechanism of the epidermis is disrupted and coccoid spores called zoospores thrive filamentary. Branched filament extending through the epidermis generally can't cross the basal membrane. Bacterial invasion of the epithelium and depending on the response of dermal hyperkeratosis, crusting occurs. Lesions of cattle Dermatophilosis begins along the ridge and spreads towards the chest and abdominal wall. In ticks concentrated areas, lesions are more common on armpits, pubis and scrotum (Radostits ark. 2006; Ginn ve ark. 2007). Dermatophilosis is rare disease in Turkey and often occurs in hot and plentiful rainfall areas.

The aim of this case report was to evaluate cytologic, histopathologic and culture results from skin scraps and biopsy samples in dermatophylosis in 4 month old male Simmental calf in Erzurum, one of the coldest regions of Turkey.

Material and Methods

Case material was 4 month aged and male Simmental calf referred to the Large Animal Clinic, Faculty of Veterinary Medicine, University of Ataturk suffering from skin problem. After the general clinical examination, skin scraps 0.2 and 0.5 cm in diameter were removed under aseptic conditions for cytology, histopathology and bacteriologic culture. Fresh smears were fixed in 70% methanol and stained with Giemsa stain. For histopathology, 10% formalin fixed tissue sample was routinely processed and paraffin sections in 5 μ were stained with Hematoxylin-eosin (HE). All slides were examined and photographed under the light microscopy (Olympus BX52 with DP72 camera attachment).

Microbiologic specimen was cultivated to blood agar, chocolate agar, and Sabouraud agar. Specimen was incubated in blood and chocolate agar at 25 ^{0}C, 35 ^{0}C and 45 ^{0}C until adequate growth in 5% CO_2 oven (1-3 days). Gram staining and biochemical tests such as glucose, lactose, sucrose, mannitol, nitrate, indole, catalase, oxidase, urease, hydrolysis of starch, and gelatin hydrolysis were performed from growing colonies.

No fungal growth was observed in the culture at the end of the four weeks period.

Results

Extensive crusting was observed on the head, around the eyes, neck, back and extends to the chest and abdomen (figure 1a). When these structures are examined carefully, 0.1-05 cm in diameter, red to pale-gray lesions were adherent to lower portion and can be separable only with bleeding. In the examination of skin scraps, poor exfoliation was seen except keratinized structures. Better exfoliation was observed in smears prepared from surface of biopsy material. Coccoid spores typically proliferate in filamentous type among normal and necrotic keratinized epithelium (figure 1b). Epithelial cells were large and polygonal shaped and they have dark stained picnotic nuclei. Clustering tendency of epithelium was partially impaired in this area.

In the examination of histopathologic sections prepared from biopsy material, severe hyperkeratosis, and dense clusters of coccoid-shaped bacteria under the keratinized epithelium (figure 1d). Extensive necrosis and cellular exudation neutrophil leukocyte exudation were detected.

Rapid colony growth was observed in chocolate and blood agar in 5% CO_2 medium at 35 ^{0}C. There was no colony growth at 25 ^{0}C and 42 ^{0}C. Egg-yellow coloured solid colonies were determined adherent to the agar surface, partially made of beta-hemolysis. Glucose, catalase, oxidase, urease, hydrolysis of gelatin and starch hydrolysis were positive and lactose, sucrose, mannitol, nitrate and indole were negative in biochemical tests. In gram staining of colonies, gram positively coccoids were detected to spread in the form of long-chain and as single or double row. Besides, irregular, long and fine shaped, branched filamentous structures were observed (figure 1c). According to clinic-macroscopic, cytology, histopathology and microbiologic results, the disease was described as Dermatophylosis and the calf was treated with Oxytetracycline (primamycin LA 50 ml. enjectable solution, Pfizer), 20 mg/kg dose IM, 2 times with a week intervals.

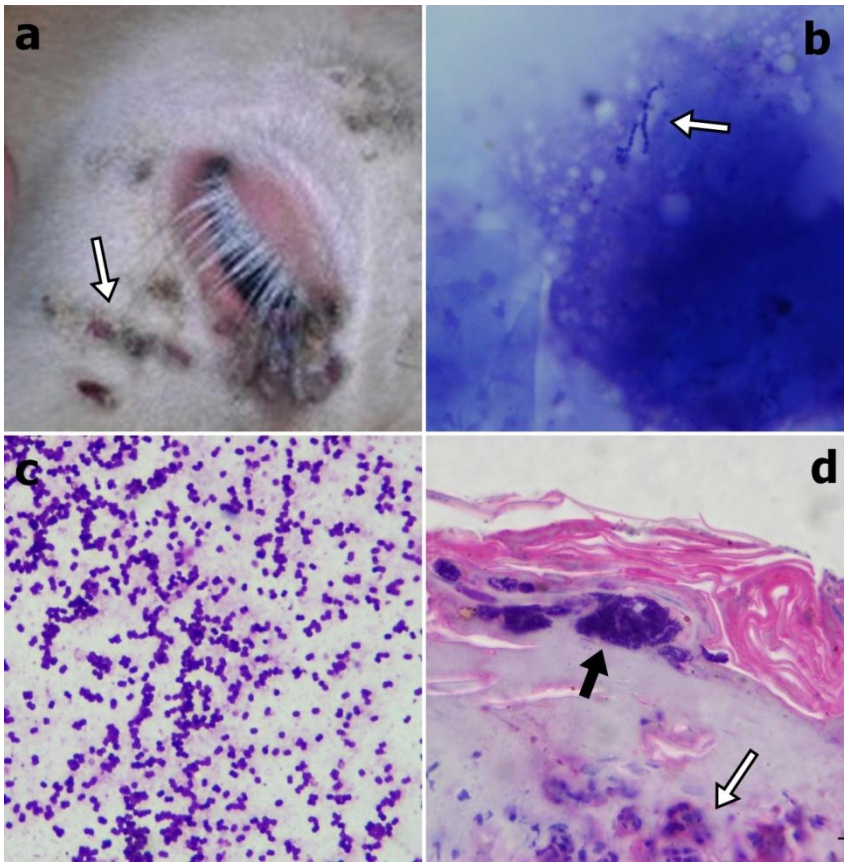

Figure 1. Red to pale-gray colored crusting around the eyes (arrow in a). Coccoid spores in filamentous type among normal and necrotic keratinized epithelium (arrow in b) and epithelial cells with picnotic nuclei, Giemsa's stain. Irregular, long and fine shaped, branched filamentous structures (c), Gram's stain. Severe hyperkeratosis and densely clusters of coccoid-shaped bacteria under the keratinized epithelium (black arrow in d) and inflammatory cells (white arrow in d), H-E.

Discussion

Dermatophylosis is generally seen in hot and rainy seasons in some African countries such as Nigeria (Dalis et al. 2010), Kenya (Wabacha et al. 2007), Ethiopia (Berhanu and Woldemeskel, 1999; Admassu and Alemu, 2011) Egypt (Awad et al. 2008) and the other countries, Australia, South America (Radostits et al. 2006) and India (Pal, 1995). At the same time, the disease was rarely reported in Iran (Shoorijeh et al. 2008), Belgium (Decostere et al., 2002), Turkey (Kaya et al, 2000; Sekin et al. 2002). Some diseased flocks in Africa, the prevalence was reported that reached 100% depending on the season (Radostits et al, 2006). Isolation of *Dermatophilus congolensis* was indicated as 5.1-79.1% in some African countries (Dalis et al. 2010; Wabacha et al. 2007; Berhanu ve Woldemeskel, 1999; Awad et al. 2008). This rate was reported in India as 5.45% (Pal, 1995). In the studies conducted in Turkey, Or et al. (1999) did not isolated *Dermatophilus congolensis* from cattle and sheep in Black Sea and Marmara regions which they prefer to show rainy season. In subsequent years, the disease was reported in a cat (Kaya et al. 2000) and sheep and goat flock in Diyarbakir

province (Sekin et al. 2002). In the present study, Dermatophilosis is detected in the Simmental calf in Erzurum, the city in the northeast Turkey. Clinic-macroscopic symptoms and histopathology were found compatible the current literature (Decostere et al. 2003; Radostits et al. 2006; Ginn et al. 2007; OIE, 2008; Shoorijec et al. 2008; Nath et al. 2010; Dalis et al. 2010). For the diagnosis of the disease, Giemsa staing smears prepared from the infected area and the occurrence of the typical formation of the agent is important. Histopathology, bacteriological culture, serology, immunofluorescence and molecular methods are recommended together with cytology (Skalka and Pospisil, 1994; Ginn et al. 2007; OIE, 2008; Shaibu et al. 2010).

In our study, the diagnosis of Dermatophilosis was strengthened that had been seen typical morphology of the agent on the smears and histological sections. Similarly, bacteriologic culture results were found compatible with the literature (Skalka and Pospisil, 1994).

20000 IU/kg/day procaine penicillin for 3-5 days or single dose of 20 mg/kg oxytetracycline LA, have been reported as effective for the treatment of Dermatophilosis (Radostits et al, 2006). Hamid and Musa (2009) determined gentamicin, penicillin-streptomycin and long-acting oxytetracycline, the most effective antibiotics respectively, in the natural and experimental Dermatophilosis in calves. In the treatment, long-acting oxytetracycline was administered as two times with a week intervals. As the animal has not been brought for control within the recommended time, healing process could not be observed. However, skin lesions and general condition was acknowledged to have improved significantly in the phone calls made by animal owner.

In conclusion, the case of Dermatophilosis in the Simmental calf in Erzurum, TURKEY was diagnosed with gross examination, cytology, histopathogy and microbiologic culture. Oxytetracycline treatment was found effective for the treatment of the disease.

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CORRELATION BETWEEN ESTRADIOL LEVELS AND REPRODUCTIVE SYSTEM DEVELOPMENT IN QUAILS *(COTURNIX COTURNIX JAPONICA)*

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Abstract

The present study followed the correlation between plasmatic estradiol and morphological changes of reproductive system in Japanese quail from 1 day of life until 44 days. Estradiol plays an important role in oocyte and oviduct growth and development. 46 quails were taken into study, from which the ovary, oviduct and blood samples were collected. Histological exam was carried out and plasmatic estradiol was determined for each specimen. A proportional raise of estradiol values was observed from first day until day 44 of life. Also the raise of estradiol was higher starting with day 31. Morphologically the raise of the oocyte and development of glandular structure were observed since day 31. In conclusion a correlation between the level of plasmatic estradiol and morphological observations for quail reproductive system was established.

Key words: *estradiol, morphology, quail, reproductive.*

Introduction

The quail is an avian model for reproductive biology because of the short period of incubation, which may be a determining factor in laying age, described in the literature as being about 6-7 weeks after hatching. These details imply the rapid development of young quails. The development of reproductive system implies differentiation of ovary and oviduct structures. As is reported in literature, estradiol is correlated with reproductive system development. The aim of this study was to correlate the estradiol level with the stages of ovary and oviduct development.

Material and method

To study the development of the ovary and oviduct post-hatching, 46 quails aged between one and 44 days were taken to consideration. From these quails ovaries, oviducts, and blood samples were collected in order to determine the dosage of estradiol. The samples were fixed in bouin fixative for 24 h, embedded in paraffin and cutted at 5-7μm. The sections were stained HE for morphological details.

Results and discussion

Anatomically the ovary is not completely detached by mesonephros. Histologically the ovary presented clear differentiation of cortical and medullar areas. In cortex numerous oocytes were present. Medulla was constituted by numerous blood vessels and connective tissue. In this stage rare primary follicles were identified. The oocyte nucleus is well evidentiated with clear cytoplasm. At the surface, the ovary was covered by a simple germinative cubic epithelium. At 15 days after hatching, the development of ovarian follicles was represented by a raise of oocyte dimensions and a numerical raise of follicular cells. So previteline follicles in primary developmental phase can be observed. With the ovarian growth, the delimitation between cortical and medullar is lost. So at 24 days the cortical comprises almost entirely the ovary surface, numerous previteline follicles being observed. At 31 days tardive previteline follicles are identified, characterized by an oocyte suspended by two cubic cells layers of granulosa. The techa layer is divise in external and internal techa.

Between the follicles numerous interstitial cells could be observed. At 44 days, the quail ovary presents all follicles types and interstitial cells. The number of interstitial cells represents an evident factor of sexual maturity because they are responsible of steroid hormones secretion (Rodler, 2009) which influence the ovulation (fig.1).

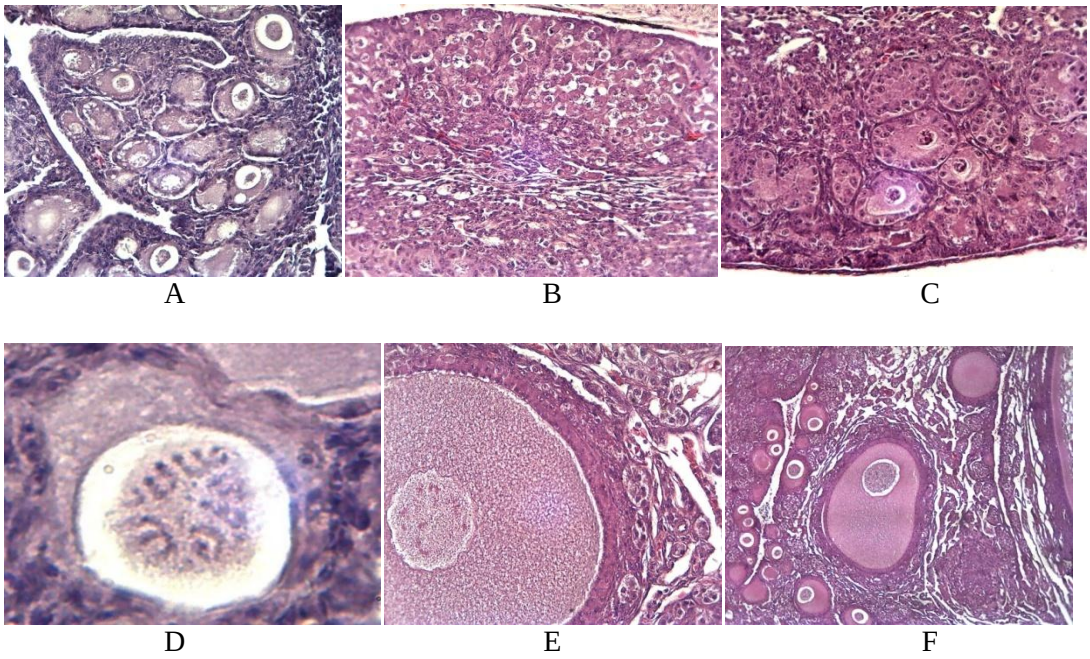

Fig.1. Morphological detail of young quail ovary. First day: A.x100, B.x400 presence of oocyte and primordial, primary follicles; 15 days diverse stages of follicles evolution x100 C; 24 days, presence of chromosom in oocyte nucleu x400 D; 31 days, structur of previtelin follicle, x1000 E; 44 days, follicles ovarian and interstial cells x100 F, HE stain

The development of the oviduct was followed with the ovary development. At 1 day after hatching, the oviduct is composed by pseudostratified ciliated epithelium. In the epithelium, there is no predominant ciliated or secretory cells. The first folds appear at 15 days, but inside the mucosa there is no glandular structure. Morphologically the oviduct is structured by mucosa submucosa, musculosa and serosa layers. The differentiation of oviduct on anatomical segments is evident at 31 days. Histological inside the epithelium there are numerous ciliated cells which can be distinguished by a big nucleus situated centrally and numerous cills evidentiated at the apical pole. The folds present epithelium invaginations which represent a sign in tubular gland formation (fig.2). The tubular gland structure reaches the lamina propria of mucosa which is composed by connective tissue and numerous blood vessels. The tubular gland formation is related to inactive ovaries in quail, data confirmed I literature by Madekuroza 2002 in ostrich.

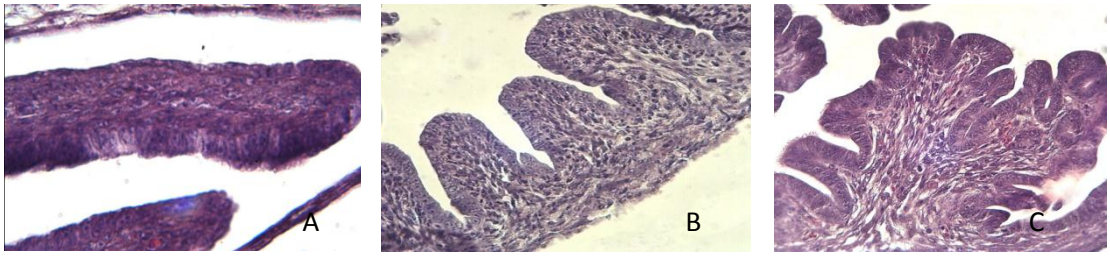

Fig.2. A. Epithelium pseudostratificat ciliat, 1 day, x 1000.
B. Folds formation in quail imatur oviduct, 15 days, x400. C. Tubular glands formation in magnum, 31 days, x400.HE stain

At 44 days the oviduct formation is complete, and all the segments are anatomically differentiated. The infundibulum present a mucosa formed by pseudostratified ciliated epithelium in which secretory cells are evident. The muscular tissue is differentiated in intern and extern layers, between them numerous arterioles and venules being evident.

The magnum is the biggest segment of the oviduct. In the epithelium the alternation of ciliated and basal cells appears. The tubular glands are completely developed and their lumen is well evidentiated. In the muscular area is well evidentiated the longitudinal layer. The isthmus presents smaller folds comparative with the magnum and the tubular glands are more dense under the epithelium. Medial the folds present numerous connective fibers.
The musculosa is thin. The folds in uterus have a leaf aspect.

Morphologically the muscular layer is well evidentiated in uterus and vagina. In vagina rare secretory cells are evident. The pass between vagina to cloaca is sudden. Histologically, cloaca is composed by pavimentous stratified keratinized epithelium (fig.3).

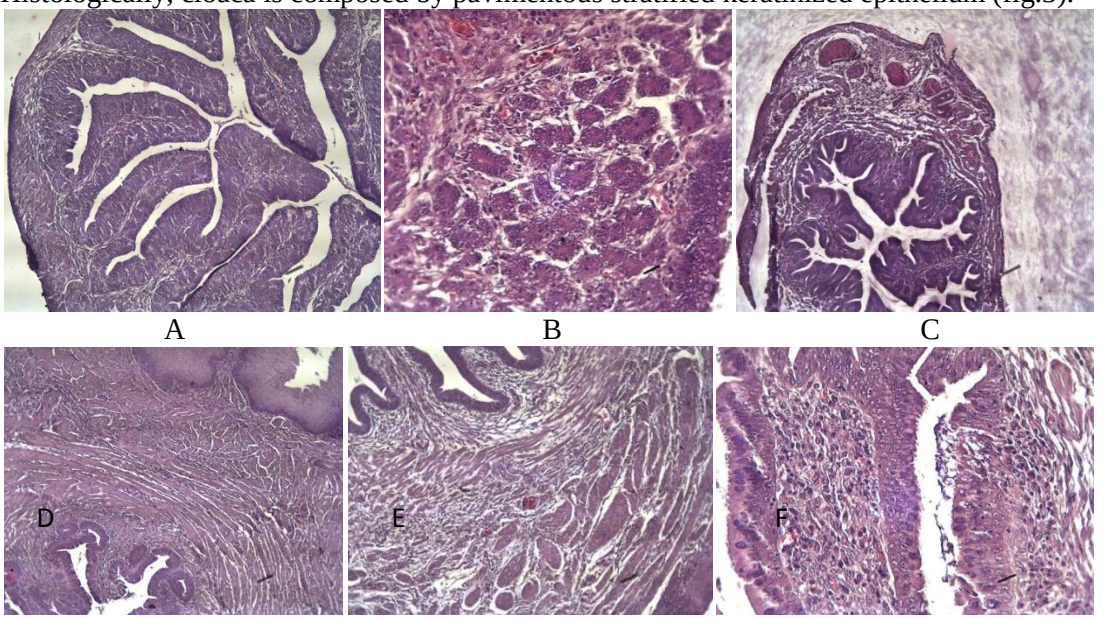

Fig.3. Quail oviduct at 44 days post hatching. Morphological details. A. Infundibulum, X100; B. magnum, x 400; C. isthmus, x 100; D. uterus, x 400; E. vagina, x 100; F.vagina-cloaca, x100.HE. stain

In quail the raise plasmatic estradiol value vas in concordance with follicular development, results confirmed also by Williams in 2004. From 1 day until 31 days the plasmatic estradiol raises with 15 pg/µ, while from 31 to 44 with 19,0 pg/µ. The results confirme the histological exam and the plasmatic estradiol raises starting with 31 days, when the tubular glands developments starts. At 31 days, macroscopically the ovary presents small white follicles smaller than 1 mm, while at 44 days these follicles are bigger than 1 mm, with a yellow color. The oviduct segments could be seen anatomically. These results could indicate the reproductive precocity of Japanese quail (fig.4).

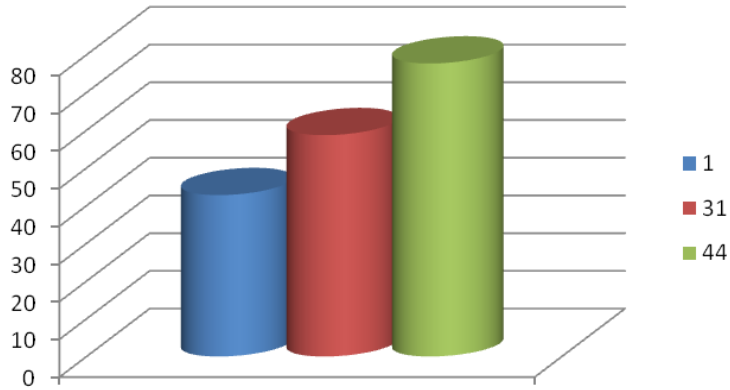

Fig.4. Growth ratio of plasmatic estradiol.

It is known that the FSH in birds has a role in estrogen regulation by developing ovarian follicles. Along the reproductive phase, estrogen are secreted by follicular cells of the ovary, being implicated in egg yolk synthesis (Wallace, 1985; Walzem et al., 1999) and the development of the oviduct (Yu et al., 1971). Estradiol is one of the factors that determines the reproductive tissue growth, and can be considered a growth hormone.

In quail, the level of plasmatic estradiol is dependent of the follicular development stage, being in concordance with granulosa functionality, which implies the oviduct development. In the oviduct, estrogens influence the tubular gland development.

Conclusions

- Folliculogenesis in quail occurs during the embryonic stage and at hatching the ovary presents numerous primordial follicles.
- The oviduct in day-old quail chicks is barely visible macroscopically, while histologically we distinguished only pseudostratified ciliated epithelium.
- The development of follicles occurs by increase in oocyte size and also in number of layers of granulosa cells.
- The folds of oviduct mucosa appear around the age of two weeks. The oviductal segments differentiate anatomically at the age of 31 days.
- Around 4-5 weeks after hatching, the formation of the tubular glands begins with the invagination of the epithelium.
- Estradiol levels in plasma increases starting day 31 after hatching.
- Ovary development occurs more rapidly compared to oviductal development and may influence its development by hormone control.

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THE CONSEQUENCES OF HYPOVITAMINOSIS A IN CAPTIVE TURTLE – CASE REPORT

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Abstract
Hypovitaminosis A is a condition frequently diagnosed in young captive turtle. Vitamin A deficiency is due to depletion of vitelus (which is the greatest source of vitamin A), within a few days after hatching, as well as the administration of food poor in vitamin A and the lack of an appropriate microclimate. The main clinical signs that might be observed are represented by oedema of eyelids, exophthalmia and conjunctivitis mucopurulent discharge, which dries up and sticks the eyelids. If the lack of vitamin A intake prolonged for a longer period, symptoms as anorexia, mucopurulent discharge and lethargy may occur, followed by the death in 2-3 months. This paper describes the occurrence of hypovitaminosis A in captive turtle and its remediation by administration of specific treatment, with the change of nutrition and microclimate conditions.In the case of turtles, like as other living beings, the feeding is an absolute necessity, without which the body would not be able to maintain, develop and perpetuate. In addition to the specific biological particularities, for the turtle diets must be considered also the needs of the body determined by season, age, environmental variations and by their metabolism.
Key words: *hypovitaminosis A, turtle, oedema of eyelids.*

Introduction

The turtles have occurred before the Age of Dinosaurs, in Permian period, reaching a great development in the Cretaceous and Tertiary Periods, and then they decreased constantly, thus from approximately 150 genera, as they were in the prehistoric period, currently are existing only 50 genera, which are similar from biological point of view, although they present several particularities, (Breisch, A. R., J. W. Ozard, 2001).

Turtles are belonging to the most primitive reptiles and are taxonomically assigned to Reptiles Class, subclass **Anapsida** and Suborder **Chelonia**.

The turtles rose in captivity, in order to develop their specific quality values they need conditions as close to those of their natural environment. Their growth in captivity requires the setting of terrariums and aquariums, which ensure the necessary temperature variations and a proper diet, in accordance with their specific regime in the natural environment. The turtle food varies according to the habitat that they occupy.

Vitamin A, axerophthol or vitamin antixerophthalmic is found in nature mainly in green plants, particularly in leguminous plants under the form of provitamin A or alpha, beta, gamma and delta-carotene. Great amounts of vitamin A can also be found in feed of animal origin such as meat, fat, liver, eggs and milk. The liver and the fat marine fish represent the richest sources of vitamin A, (Dixon, J. R., 2000).

Vitamin A is involved in the formation, maturation and metabolism of epithelial cells and is indispensable in the functioning of the retina, adrenal glands and nervous systems.

Hypovitaminosis A is commonly diagnosed in younger turtles of the Trachemys, Chrysemys and Pseudemys genera that are bred in captivity and fed with an inadequate diet deprived of vitamin A, (Elkan E, Zwart P., 1997).

The paper aims to present a case of hypovitaminosis A encountered in a turtle bred in captivity and its specific therapy.

Materials and Methods

A turtle belonging to the *Trachemys scripta* species, about 4 -5 years old was submitted for a clinical examination to the Infectious Diseases and Preventive Medicine Clinic from the Faculty of Veterinary Medicine, Iasi.

From the anamnesis obtained from its owner, it was found that the animal was not eating for two weeks, was lethargic, moving very little and was sleepy. The turtle had a diet based only on pork and poultry meat, and it was housed in a small terrarium arranged by the owner, that was not disposed with a water heating system, thermometer and UV lamp.

Based on clinical signs correlated with the anamnesis, a diagnosis of hypovitaminosis A was established and a treatment plan was instituted.

Results and discussions

Following the clinical examination of the turtle belonging to *Trachemys scripta* species, the next modifications were found: oedema of eyelids (fig. 1), bilateral exophthalmia (fig. 2) and conjunctivitis with mucopurulent discharge, which dries up and sticks the eyelids.

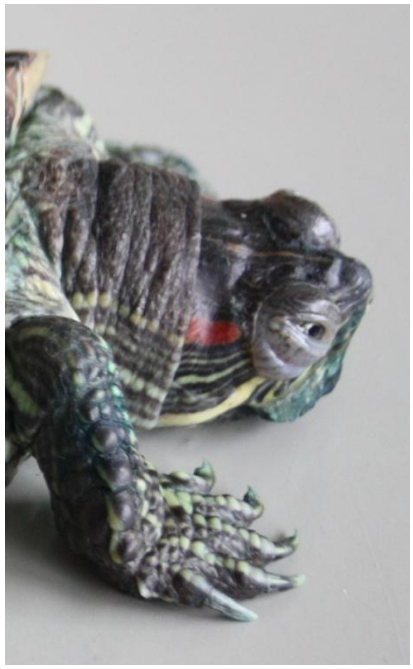

Fig. 1
Oedema of eyelids

Fig. 2
Bilateral exophthalmia

Because of the food refusal for 2 weeks, of vitamins lack and accommodation and hygiene unsuitable conditions, the turtle presented listlessness, dehydration, muscular limbs and neck was flaccid and generally was refusing the movements (fig. 3).

Fig. 3
Dehydration, with the evidence of skin folds in the limbs and neck

The turtle may have the closed eyes following a severe dehydration, which causes the drying of the conjunctiva and eyes clogging in orbits.

After clinical examination and the diagnosis establishing, in the case of this turtle a diet and a drug therapy was settled.

The feeding of turtles bred in captivity must take into account the species (whether is carnivore or vegetarian). Therefore, the turtles will be fed with various feed, in order to provide the necessary quantity of proteins, vitamins and minerals for development and a normal body functioning.

The carnivorous turtles should be feed with meat and organs of domestic animals (pork meat is not indicated), snails, fish, earthworms and special prepared food. It can administered dry food prepared for frogs, dogs and cats.

Generally, it may be given 10 g of feed per 100g weight for each day. The rate of administration is 1-2 rounds per day in young, gradually reducing to half at 2-3 days in adults. It is reccomended that in the case of aquatic carnivorous turtle, the feed shoud be administrated alive, thus conserving the hunting behavior. It is not recommended that food should be left to the turtle in water, because it can alter and infect the pool water, causing conjunctivitis and dermatitis, (Păunescu Ileana Cornelia, 2007).

Periodically (2 times per week), in order to ensure the necessary vitamins and salts, as well as for a regular digestive transit, the carnivorous turtles are feeding with lettuce and carrots, (Ciudin Elena, Burtan Liviu, 2002).

The terrestrial omnivores turtles are feeding with vegetables (carrot, lettuce, cabbage, potatoes), fruits (cherries, sour-cherries, grapes, strawberries, bananas, apples, oranges), vegetables (clover, alfalfa, dandelion) and with food of animal origin (meat finely chopped, cottage cheese, cheese, milk powder). The rate of administration is as frequent as for carnivorous turtles.

Drug treatment consisted of trimming the eyelids and eyes with sterile saline compresses, with the application of an ophthalmic antibiotic ointment (ophthalmic ointment

with kanamicin 1%), twice per day. Per os, oral drops with vitamin A were administrated, calcutating a dose of 40-50 UI/kg. For the basin water, an AD3E drinkable product was added in a dose of 2 ml/L of water. The treatment was applied for a period of 2 months and correlated with the basin hygiene, while the diet correction led to the improvement of the general condition.

Conclusions

Following the clinical examination performed in a turtled submitted to the Infectios Diseases and preventive medicine from Faculty of Veterinary Medicine, Iasi, it might be concluded as follows:

1. The turtle was presenting clinical signs specific for hypovitaminosis A: oedema of eyelids, bilateral exophthalmia, conjunctivitis with mucopurulent discharge, dehydration, listlessness.

2. The diagnosis of hypovitaminosis A was confirmed by the diet mostly with pork and poultry meat and unsuitable environmental conditions.

3. The frequency of this pathology is due to the owners incapacity in generating a microclimate similar to the natural one.

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EXPRESSION PROFILES OF CIRCULATING CYTOKINES IN PIGS IMMUNIZED WITH TWO DIFFERENT RABIES VACCINES

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Abstract
Quantitative suspension Xmap technology allows the simultaneous measurement of different cytokines in small sample volumes. This technology is based on a capture-detection sandwich-type assay using fluorescent microspheres analyzable by Luminex instruments. Ussualy the rabies response is preponderant humoral but we propose the evaluate the biomarkers of cellular respons after immunization. The opportunity of quantification multiple variable is important for us for measurement of rabies antigen-specific cellular response. In this paper we propose the evaluate the biomarkers of cellular respons after immunization of pigs with two different vaccines (a commercial vaccine and a rabies vaccines that use the canine adenovirus type 2 as a vector).In this experiment we measurement the level of IFNγ and other cytokine (IL4, IL6, IL8 and IL10) in conventional pigs aged 6 weeks after immunization with two rabie vaccine (Nobivac and a recombinant product that use the CAV-2 as a vector). For this paper was used the Luminex Xmap and the results reveal us a constant level of IFNγ and cytokine that was evaluated during the experiment in both group of pigs used for this experiment.
Keywords*: CAV-2, cytokine, luminex platform, rabies, vaccine.*

Introduction

Rabies remain a real problem, particularly in the developing countries (Perianu, 2004). The treatment for post exposure prophylaxis in animals is administration of a vaccine. To prevent rabies in domestic animals we need safe and effective vaccines. Usually pig rabies has been reported occasionally in recent years and only in certain geographical areas. Although there are commercial inactivated vaccines in place to ensure protection against rabies in swine, but searching for a more economically viable formulation for use in developed countries is always a priority. Luminex's xMAP Technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology to deliver multiplexed assay capabilities. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately (Bru, 2003; Morales, 1998; Pedreno, 2008).

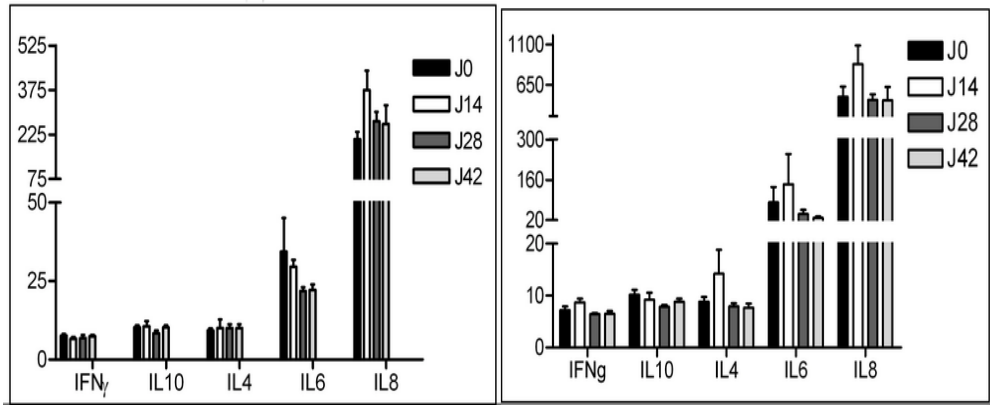
Materials and methods

In this experiment was used 11 commercially available 6 weeks old Large White-Landrace hybrid pigs (5 females and 6 male), identified by microchip and housed together in the high security animal facility at USAMV. The pigs was randomly divided into three groups, were daily monitored for health status, fed and received tap water. Group 1 of 5 animals (3 females and 2 male) were inoculated once with 2 ml of recombinant Cav-G R+ (10^8TCID$_{50\%}$/ml), group 2 of 5 animals (2 females and 3 male) were inoculated once with one dose of inactivated antirabies vaccine Nobivac Rabies (Intervet) and group 3 of one pig (a male) received 2 ml of PBS as negative control. All vaccines were administered by the intramuscular route inhind limbmuscles. Blood samples for serological assays were collected weekly for8 weeks afterthe startof the experiment by epigastric vein puncture and sera were prepared and stored at -20°C. Quantitative suspension Xmap technology allows the simultaneous measurement of different cytokines in small sample volumes. This technology is based on a capture-detection sandwich-type assay using fluorescent microspheres

analyzable by Luminex instruments. The opportunity of quantification multiple variable is important for us for measurement of rabies antigen-specific cellular response. In this paper we measurement the level of IFNγ and other cytokine (IL4, IL6, IL8 and IL10).

Results and Discussion

All the data revealed by Luminex assay using Procarta by Affymetrix shown us a relatively constant level of IFNγ, IL4, IL6, IL8, and IL10 in group of pigs was received one dose of 10^8TCID$_{50}$/ml of CAV-G vector. That constant level it is normal if were consider that immunologic route for increasing rabies antibody is not the same with a conventional humoral response after vaccination with a commercial product that use the all the rabies viral particle



A B

Fig.1 Dosage of cytokines in group of pigs immunized with CAV-G vector (a) and Nobivac Rabies (b)

In group immunized with one dose of Rabies Nobivac (Intervet) were obtained high level of analyzed cytokine in day 14 of the experiment wich correspond with the peak of rabies antibody level. If the humoral rabies response was a protective one we consider that level of cytokines were increased considerable during the experiment.

Conclusion

In the present research work, we demonstrated that the implication of IFNγ, IL4, IL6, IL8, and IL10 in rabies cellular response after vaccination was not decelable (group of pigs was received one dose of CAV-G) using Luminex assay or was determined a very weak level (group of pigs received one dose of Rabies Nobivac vaccine).

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HISTOLOGICAL AUTOFLUORESCENCE STUDY OF THE NICTITATING GLAND IN DOGS

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Abstract
The purpose of this study is to reveal and describe the histological autofluorescence for the nictitating gland in dogs. Nictitating membrane of both eyes were harvested from nine dogs belonging to three different breeds – Pekingese (2), German Shepherd (2) and mongrel dogs (5), 5 males and 4 females, weighing between 6-35 kg, aged 3 to 7 years. Nictitating membranes were placed in 10% neutral buffered formalin. Subsequently, histological specimens were dehydrated, cleared, and then impregnated and embedded in paraffin. Paraffin blocks were sectioned to a thickness of 5 µm and stained with haematoxylin-eosin (HE), waxed and unstained slides were used for the study of natural fluorescence. These slides were analysed under the light microscope, fitted with an epifluorescence module which is operating with UV light and special objectives for fluorescence, using the filter for green fluorescence. In dogs, autofluorescence is distinguished by a uniform fluorescence, low and diffuse intensity, sometimes more intense at the apical pole of all cells or only some glandular epithelial cells. These have, most probably, glycoproteins. Intense fluorescence is observed in the interstitial space between tubuloacini, given by the collagen and elastin from the connective fibers structure.
Keywords: *gland of the nictitating membrane, histological autofluorescence, dogs.*

Introduction

Autofluorescence is based on the stimulation of endogenous fluorophores when are illuminated with ultraviolet light (2, 5, 9).

Natural fluorescence (autofluorescence) is given mostly by substances as:

- flavins (present in Chinese hamster ovary cells, rat hepatocytes, bovine and rat neurons, the inner ear of Goldfish),
- nicotinamide adenine dinucleotide phosphate/(NAD(P)H) (present in the rat cardiomyocytes and hepatocytes, Chinese hamster ovary cells),
- lipofuscin (in the human, rat and Rhesus monkey spinal cord; in the rat heart, liver and retina; in the human muscle, myocardium, hepatocytes and brain),
- Advanced Glycation End-Products (A.G.E.), present in the human and Chinese hamster lens, human cornea, diabetic human skin,
- collagen and elastin (human aorta and coronary artery, human skin),
- porphyrins (psoriatic human skin),
- chlorophyll and lignin (in plants and algae),
- mucins (1, 6, 7, 11).

Autofluorescence images become visible only when the fluorescence is significant on a black background. Lipofuscin is strongly fluorescent, appearing green-yellow-orange or blue under UV excitation. Elastin and collagen occur in green-yellow or green under UV excitation. In large blood vessel wall, the fluorescence may be significant due to the high abundance of collagen fibers (1).

Tissue autofluorescence is used for early diagnosis of tumours (Rigacci *et al*., 2000; Chiang *et al*., 2003; Ell, 2003), study of the human skin changes with aging (Oria *et al*., 2003), using animal cell culture technique for quantitative and qualitative assessments and many other applications (Croce *et al*., 1999).

According to Payne (1994), the presence of autofluorescent material in Harderian gland, suggests the presence of porphyrins.

Autofluorescence has a similar pattern of distribution and intensity both in the Harderian gland stained with haematoxylin-eosin and in the unstained and unfixed histological slides (13). Porphyrin autofluorescence occurs as a result of unbound porphyrin molecules (Strum and Shear, 1982, and Chon, 1995, as cited in Ortiz *et al*., 2001).

Materials and methods

Nictitating membrane of both eyes were harvested from nine dogs belonging to three breeds – Pekingese (2), German Shepherd (2) and mongrel dogs (5), 5 males and 4 females, weighing between 6-35 kg, aged 3 to 7 years. The corpses of dogs were provided by various private veterinary practices (from Iaşi county) and from Department of Forensic Veterinary Medicine, Faculty of Veterinary Medicine, Iaşi.

Bilateral excision of the nictitating membrane was performed by incision and dissection of the bulbar and eyelid conjunctiva and underlying structures to its complete isolation. There were collected only the nictitating membranes with normal clinical appearance.

Nictitating membranes were trimmed and midsagittal sectioned, after which they were placed in 10% neutral buffered formalin. Subsequently, histological specimens were dehydrated, cleared, and then impregnated with paraffin and embedded in paraffin. Paraffin blocks were sectioned to a thickness of 5 µm and then plated on microscope slides, dewaxed, hydrated and subsequently stained with haematoxylin-eosin (HE) and, in addition, for the study of natural fluorescence were used waxed and unstained slides.

Histological slides were examined under the light microscope ***Micros Austria MC300*** with camcorder ***Motic 352*** attached, and histological analysis of autofluorescence of the nictitating gland was performed on the same microscope, fitted with an epifluorescence module which is operating with UV light and special objectives for fluorescence. They were examined under UV light at a wavelength of 420-530 nm, using green fluorescence filter.

Results and Discussions

Secretory tubuloacinar units present a non-fluorescent lumen, dark, sometimes with fluorescent material or peripheral fluorescence, above the apical pole of the epithelial cells (fig. 1 and fig. 2).

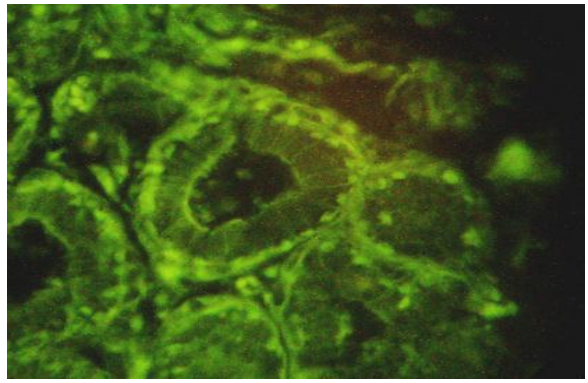

Figure 1. Nictitating gland in dogs: Secretory tubuloacinar units.
Unstained histological slide

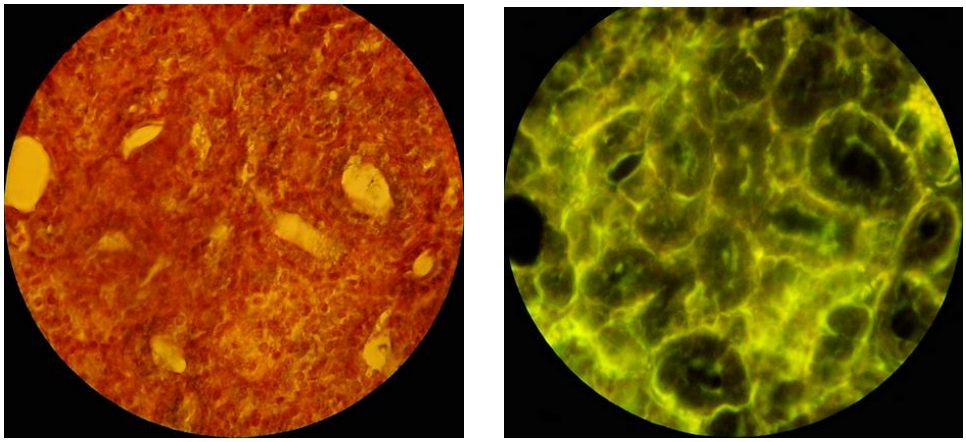

Figure 2. Nictitating gland in dogs: Secretory tubuloacinar units.

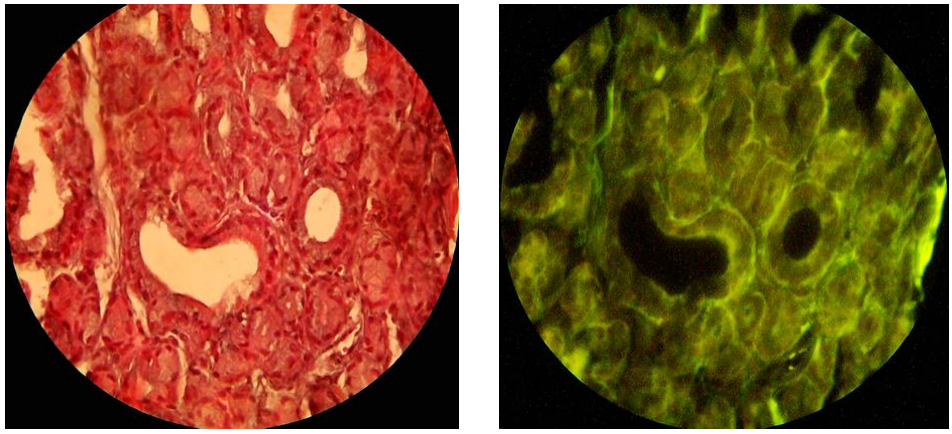

Figure 3. Nictitating gland in dogs: Secretory tubuloacinar units.

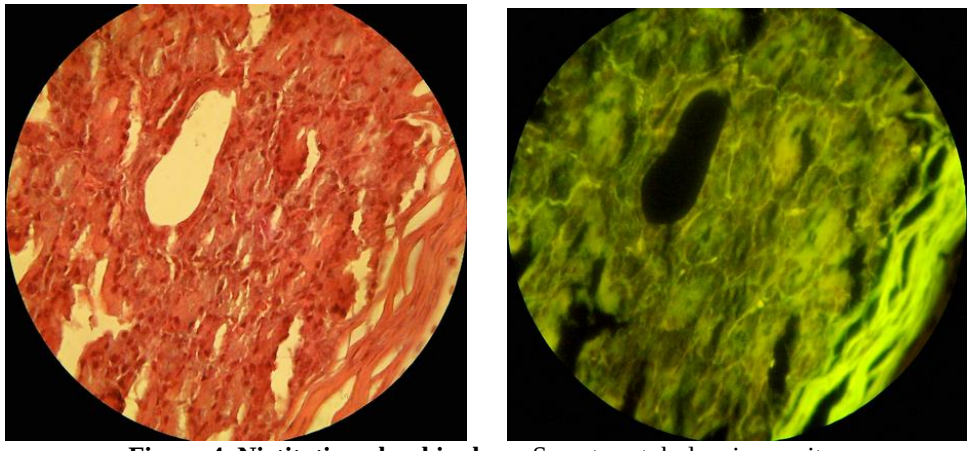

Figure 4. Nictitating gland in dogs: Secretory tubuloacinar units.

Epithelial cells are visible by the presence of a uniform fluorescence with low and diffuse intensity, and sometimes more intense fluorescence in the apical pole of all cells or

only some glandular epithelial cells (fig. 1; fig. 2 and fig. 4). The most intense fluorescence is observed in the space between tubuloacini, given by collagen and elastin from connective fibers structure (Fig. 2, Fig. 3 and Fig. 4).

Regarding Harderian gland and nictitating gland, natural autofluorescence was reported only at Harderian gland, in mice (Derrien and Turchini, 1924, as cited in Ortiz *et al*., 2001; Strum and Shear, 1982, as cited in Payne, 1994), in Syrian Hamster, rat, mouse, gerbil, guinea pig (Ortiz *et al*., 2001), in dolphin (Ortiz *et al*., 2007), in rat and rabbit (Eltony, 2009).

According to Christensen and Dam (1953), Woodhouse and Rhodin (1963), Johnson *et al*. (1983), Payne (1994), the authors cited in Ortiz *et al*. (2001), the material present in the lumen of the secretory units of the Harderian gland is generally devoid of fluorescence. However, when membrane debris is contained in intraluminal material, autofluorescence is present. The presence of cell debris and/or vesicle debris suggests apocrine secretion (15).

Payne (1994) states that the presence of autofluorescent material in Harderian gland, suggests the presence of porphyrins. Porphyrin autofluorescence occurs as a result of unbound porphyrin molecules (Strum and Shear, 1982, and Chon, 1995, as cited in Ortiz *et al*., 2001). Porphyrin appears as an intracytoplasmic fluorescent material, generally located outside to the secretory vesicles and around the nucleus (13).

Histological and cytological aspects of autofluorescence of the nictitating gland have not been described so far in Romanian and international scientific literature, in any species exhibiting this type of gland.

The nictitating gland of the dog is structured by secretory acini with mixed sero-mucous secretion (neutral and acidic glycoproteins) and by serous tubules (neutral glycoproteins) (3). In dogs, autofluorescence is distinguished by a uniform fluorescence, with low and diffuse intensity, sometimes more intense at the apical pole of all or just some glandular epithelial cells. So, autofluorescence is given, most likely, of neutral and acidic glycoproteins secreted by tubuloacinar units of the nictitating gland in various stages of secretory cycle. In addition, in analogy with Harderian gland, the fluorescence expressed by the epithelial cells of the nictitating gland could be the result of the porphyrin presence. Subsequent electron microscopic studies which will focus on identifying porphyrins as intracytoplasmic crystalline formations, should clarify this assumption. However, further studies are necessary to determine fluorophore substances which induce natural fluorescence of the nictitating gland in dogs.

Conclusions

1. Autofluorescence is given, most likely, from neutral and acidic glycoproteins secreted by tubuloacinar units of the nictitating gland in various stages of secretory cycle.
2. Intense fluorescence is observed in the interstitial space between tubuloacini, given by the collagen and elastin from the connective fibers structure.

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ACANTHOMATOUS AMELOBLASTOMA IN A MALE BOXER DOG TREATED WITH INTRALESIONAL BLEOMYCIN INJECTION: CASE REPORT

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Abstract

Objective: Acanthomatous ameloblastoma is a benign gingival tumour that often invades bone. The aim of this case report was to present histopathological features and the efficacy of treatment with intralesional bleomycin injection. Methods: A 9-year-old male Boxer was presented at consultation with a nodular mass in its mandibular gingiva of the left premolars. The mass was biopsied and histologically diagnosed with canine acanthomatous epulides. The treatment with intralesional bleomycin injections was successful with no signs of recurrence one year later. Conclusion: To choose correctly the intralesional bleomycin therapy, an accurate histopathological diagnosis must be done to prevent the recurrence and malignant transformation.

Key words: acanthomatous ameloblastoma, dog, histopathology, bleomycin.

Introduction

Acanthomatous ameloblastoma (AA) is an aggressive benign tumour of the canine jaw, adjacent to the tooth, with papillary or sessile appearance and gray-pink color. Histologically, it is consisting of sheets of no keratinizing odontogenic epithelium, with peripheral palisading epithelium and abundant central acanthocytes with prominent intercellular bridges. AA frequently invades the alveolar bone or recurs after marginal surgical excision (5, 16). Epulides treated with hemimandibulectomy or bleomycin chemotherapy did not recur. Radiation therapy on these epulides is often associated with progression to malignant tumors such as squamous cell carcinoma (10).

Bleomycin is an antitumoral chemotherapeutic agent consisting in a mixture of cytotoxic glycopeptides isolated from a strain of *Streptomyces verticillus*. Bleomycin binds to and causes DNA scission and synthesis suppression resulting in an inhibition of the tumour cell proliferation during the mitotic phases (14).

The purpose of this case report is to highlight the histological diagnosis of canine AA and its intralesional bleomycin successful chemotherapy. Furthermore, the low number of encountered and reported AA cases, successfully treated with bleomycin, justifies this case report.

Case History

A 9-year-old Boxer male was presented to our ambulatory in April 2008 because of a gingival mass (Figure 1A). On physical examination, a 2.5 x 1 cm, pinkish, moderate to firm in consistency gingival nodular mass was seen in adjacent gingiva of the left mandibular premolars (PIII-PIV). There were no obvious ulcers on the tumour surface. No evidence of regional lymph nodes involvement or distant metastasis was observed. Haematological and biochemical parameters were within normal range. Excisional biopsy was performed and tissue samples were fixed in 10% buffered formaldehyde, subjected to routine tissue processing, and embedded in paraffin. Tissue sections obtained from paraffin blocks at a thickness of 5 μm were stained with haematoxylin-eosin-methylene blue (HEMB) techniques and then examined under light microscope. The dog was diagnosed with AA and the treatment with intralesional bleomycin injections, 5 mg once a week for three weeks consecutively, was proposed.

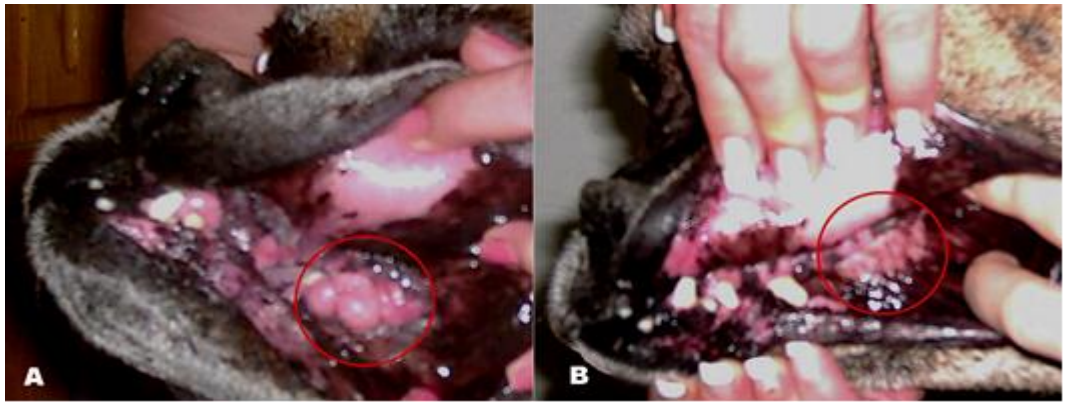

Figure 1. A-B. Macroscopic appearance of the acanthomatous ameloblastoma, before (A) and one year after the treatment (B)

Results and Discussion

On microscopy, an infiltrative growth of epithelial cells, forming cell cords, trabeculae (anastomosis of the networks ridges of hyperplastic mucosa) and islands which invaded the deep submucosal stromal component were seen (Figures 2 and 3A-B). The cell cords, trabeculae and islands consisted of both basal cuboidal to low columnar cells (*stratum basale*/*germinativum*) with a palisade arrangement and prickle cells (*stratum spinosum*), with prominent intercellular bridges. The nuclei of the columnar basal cells were arranged in palisade, with their long axis placed at right angles to the basement membrane with reverse polarity (Figures 3A and 3B). Both types of tumoral cells showed no cellular atypia and the number of mitotic cells was less than 10 per field (400 x).

Because the owner did not agree surgical therapy, the intralesional bleomycin was accepted as therapeutic alternative. Bleomycin was intralesionally injected 5 mg once a week, for three times. Even if, sometimes, because of dense tissue was difficult to inject the bleomycin solution, because solution food pipe outside of the needle, bleomycin was proven to be effective. The tumour volume decreased after first intralesional bleomycin injection, but two other administrations were necessary for complete healing. A one-year follow-up after intralesional bleomycin chemotherapy was performed, with no tumour recurrence (Figure 1B).

Epulis refers to any tumour or tumour-like mass on the gums or gingiva. However, epulis is considered as only a clinical term (7).

Epulides can be classified into four types on the basis of histologic appearance: fibromatous, ossifying, acanthomatous and giant cell epulis (5). According to Dubielzig *et al*. (3), epulides originating from the periodontal ligament may be classified, based on histological criteria, into the following three types: fibromatous, ossifying and acanthomatous. Baker *et al*. (1) consider necessary the description of the following forms: pyogenic granuloma, giant cell epulis, gingival vascular hamartoma, fibrous hyperplasia, fibromatous epulis and acanthomatous epulis.

AA previously has been called *acanthomatous epulis, peripheral ameloblastoma, basal cell carcinoma,* and *adamantinoma* (5).

The animals with AA presented signs of increased salivation, weight loss, halitosis, dysphagia or pain on opening the mouth or mastication (12).

The aetiology of epulis remains unclear. However, the frequency of dental plaques in AA is 52.9% in mild severity form, 23.5% in moderate form and 17.7% in severe form (16).

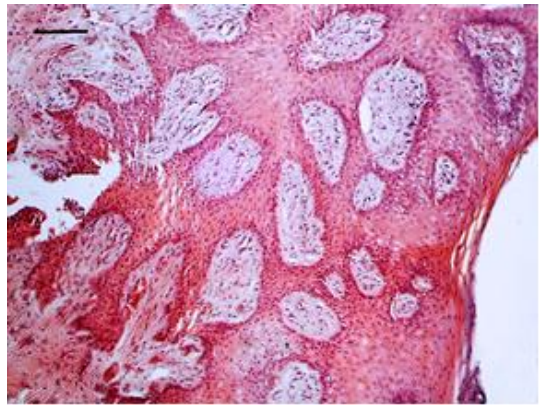

Figure 2. Infiltrative growth of epithelial component of the dog gingiva, forming trabeculae and cell cords which invaded the deep submucosal stromal component. Haematoxylin-eosin-methylene blue stain; x 100. Bar = 200 µm.

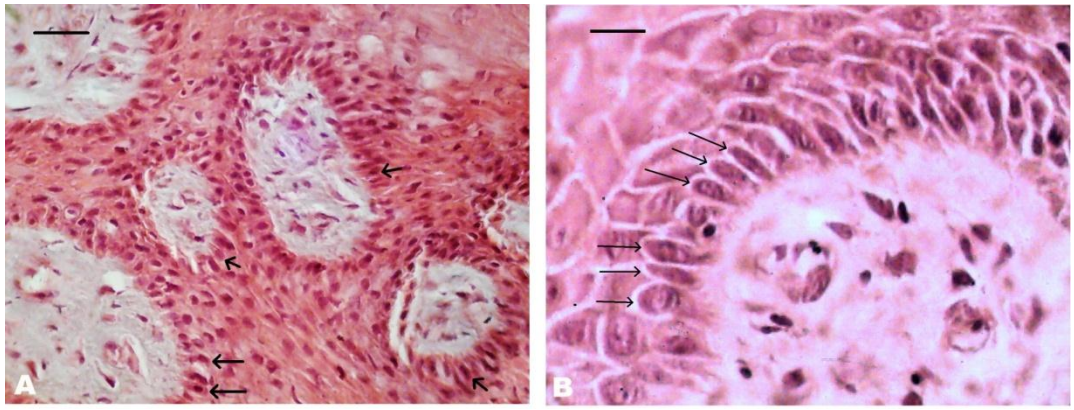

Figure 3. A-B. Infiltrative growth of epithelial component of the dog gingiva, forming trabeculae (A) which consisted of basal columnar cells and prickle cells (B). The nuclei of the columnar basal cells were arranged in palisade with reverse polarity (arrows). Haematoxylin-eosin-methylene blue stain; x 400, bar = 50 µm (A) and x 1600, bar = 15 µm (B)

In a clinicopathological study of 189 canine epulides, Yoshida *et al*. (16), reported that the incidence of the AA were 18%, the average ages of dogs with acanthomatous epulides were 7.8 and the male/female ratio of dogs with the acanthomatous epulis were 15/19 (0.8). The most noticeable result was that 38.2% of the acanthomatous epulis occurred in Shetland sheepdogs, mixed breed 17.6% and Shih-tzu 11.8%. In Boxer, incidence was 2.9%. Approximately 71% of the acanthomatous epulides arose from the gingiva around the maxillary and mandibular canines. The most favoured site of the acanthomatous epulis was the mandibular canines (58.8%). In the mandibular premolars, AA was 5.9%. In another clinicopathological study of 174 canine epulides, peripheral ameloblastoma incidence was 11.5%, and the average ages of dogs were 7 years (4).

Clinical aspects have a low value of diagnosis for setting the type of epulides.

AA were histologically characterized by the presence of interconnected sheets of odontogenic epithelium and by infiltrative growth of epithelial cells, forming cell cords or solid clusters which invaded the deep submucosa (5, 7, 16). The epithelial component of AA consisted both of basal and prickle cells with prominent intercellular bridges. The nuclei of the basal cells were distinctly palisaded, with their long axis arranged at right angles to the basement membrane (16). The proliferating cells showed no cellular atypia, and mitotic figures were uncommon. Unusual multilayered basal cells were found in the border of ameloblastic islands, in a case of acanthomatous ameloblastoma in a female Spitz dog. The nuclei of these columnar cells remain in opposite direction of basement membrane (2).

In the fibromatous epulides, dense collagenous tissue with stellate cells was found in the submucosa with proliferating strands of overlying epithelium (15). The branching cell cords in the fibromatous and ossifying epulides were formed solely from basal cells, while in the acanthomatous epulis the epithelial component consisted both of basal and prickle cells with prominent intercellular bridges (16).

In the ossifying epulides, osteoid formation was a prominent feature in the submucosal stromal component, and osteoids were made up of stellate cells and dense fibrillar collagen (15).

Peripheral giant cell granuloma or giant cell epulis in dogs, with gingival location, microscopically shows hyperplasia of the gingival epithelium, ulcerations, and an extremely well vascularized stroma. The presence of giant cells, with numerous central nuclei, often ten to twenty visible nuclei, and abundant eosinophilic cytoplasm, situated in a dense stromal cell mass, is characteristic (5, 11).

In conclusion, the clinical and histopathological aspects of the case reported here were consistent with diagnosis of canine acanthomatous ameloblastoma.

Current treatments for AA include surgery and radiation therapy. Surgery with bone removal has a recurrence rate of less than 5%, but is a mutilating procedure (13). Marginal excision without resection of bone results in a high rate of local recurrence, with a mean time to recurrence of 32 days (16). The use of rim excision appears to be a viable option for AA and results in improved dental occlusion, cosmetics, and no evidence of epulis recurrence (8). Radiation therapy has a survival rate of 80% for 3 years after treatment and local recurrence was reported in 8-18% of dogs. Furthermore, malignant transformations were reported in 5-18 % of dogs (10).

In this study, the use of bleomycin injected directly into the tumour was successful.

Intralesional chemotherapy induces high local chemotherapeutic concentrations and minimal systemic toxicity. Although intralesional chemotherapy appears to be effective, there are few studies and reports in veterinary medicine (6).

Bleomycin is an antineoplastic antibiotic that has been effectively used to treat basal cell carcinomas, squamous cell carcinomas, Sticker sarcoma, adenoma and carcinoma of the perianal glands tumors and AA (9, 14). In AA, intralesional bleomycin causes vacuolar degeneration and tumour cell necrosis (14).

Yoshida *et al*. (14), only in four dogs, indicates that intralesional bleomycin is an effective treatment for dogs with AA. Three to ten treatments were administered. Through the administration period of bleomycin, no adverse reactions were reported in any case. Our patient was treated with bleomycin in 2008, according to Yoshida *et al*. (14). Three administrations of intralesional bleomycin were required for the disappearance of tumors, without any side effects (Figure 1B). Recently, seven dogs were treated with intralesional bleomycin (one of them presented with advanced, nonresectable AA was palliatively treated).

One to sixteen treatments were administered (median, 5). Six of the seven dogs had a complete response within 4 months from initial intralesional injection, whereas the palliative case had approximately 25% decrease in tumour volume 14 days from initial injection. Adverse effects were limited to wound formation with bone exposure, mild tissue reactions, local swelling and local infection (6).

In conclusion, intralesional bleomycin is an effective treatment in canine AA, it is a safe, simple, aesthetic and cost-effective method. Histopathological diagnosis of AA is compulsory before the initiation of therapy with intralesional bleomycin.

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ROE DEER (CAPREOLUS CAPREOLUS) AGE-DENTITION-TROPHY CORRELATION

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Abstract

One of the species of cynegetic interest well represented numerically in the north of Moldavia, the roe deer (Capreolus capreolus) (Linnaeus, 1758) is a hunt species with high demand because of the trophy obtained in the harvest periods from the adult males. Researches developed in 2013-2014 on 8 roe deer skulls harvested in the north area of Moldavia (counties Suceava, Neamț and Iaşi) have shown a correlation between the ages of males and the development of the horns (trophies). The age of the roe deer included in the study was between 3 and 4 years, presenting premolars and molars with flat edges and smooth surfaces, the enamel ridges and infundibulum almost disappeared. The length of the horns was between 17 and 23 centimeters, and the weight of the dried trophy between 390 and 430 grams.

Introduction

Along the time there have been numerous researches about establishing the age of wild ungulates after dentition. Some measurements were based on counting lines in dental cementum, on species like fallow deer *Dama dama* (Moore et al. 1995), deer *Capreolus capreolus* (Aitken 1975). This method has been characterized as an objective method for establishing the age in wild ruminants. However, there are studies performed on large samples of mandibles from the deer *Capreolus capreolus* who claim that the method of counting rings in dental cementum is not suitable for this species (Grue and Jensen 1979, Cederlund et al. 1992, T. T. Hoye 2006).

A particular influence in establishing the age based on dentition is also the way they feed.

Material and methods

The study material was represented by 8 deer skulls shot during organized hunting period, in hunting seasons of 2013 and 2014, on hunting funds from Iaşi, Suceava and Neamț and examined in the Pathological Anatomy and Forensic Veterinary Medicine laboratory from the Faculty of Veterinary Medicine Iași.

The skulls were examined both fresh and processed. Processing was performed by boiling, removing the soft tissues (skin, muscle, buccal cavity organs, eyes, brain), bleaching with 10% aqueous solution of hydrogen peroxide, washing under running water and drying.

The antlers were assessed according to the official score sheets for deer trophy used by national forestry department regarding the length of main beams, weight of the trophy, volume of antlers, inside span, color, pearling, burrs, tine ends, structure and quality of the tines. (Annex 1).

Results and discussions

Measurements on antlers were performed in order to establish the age of 4 deer skulls, harvested throw shooting. The measurements were made in the Forensic Veterinary Medicine Laboratory from the Faculty of Veterinary Medicine Iași, using a Nikon D 5000 camera, roulette meter, electronic scale and a necropsy kit. The dental formula registered on studied individuals enframe in the formula cited in the specialty literature, as follows:

The age of studied deer was between 3 to 4 years, showing mandibular incisors placed almost horizontally, being arranged in fan shape. The crown is shaped as a pallet flattened vestibulolingual, with the occlusal surface transformed into a masticatory edge. With age the masticatory surface narrows in a transversal direction.

Premolars and molars are hypsodont teeth with an extended growth, but limited in time. Their anatomic crown, mostly included in the dental alveoli, has longitudinal enamel grooves on vestibular and lingual surfaces. From the appearance of the masticatory surface they belong to the selenodont type. Enamel folds and grooves are distinct. In adult animals the enamel ridges and infundibulum can disappear as a result of blunting. (Fig.1, Fig.2).

The age of the deer included in the study was between 3 and 4 years, presenting premolars and molars with flat edges and smooth surfaces, the enamel ridges and infundibulum almost disappeared.

The evaluation of the deer trophy *(Capreolus capreolus)* was performed according to ministry decision no. 418 / 2005, following three main categories: measurements on trophy, additions for beauty and penalties.

Fig. 1. Deer. The occlusal surface of the molars (M1-2-3)

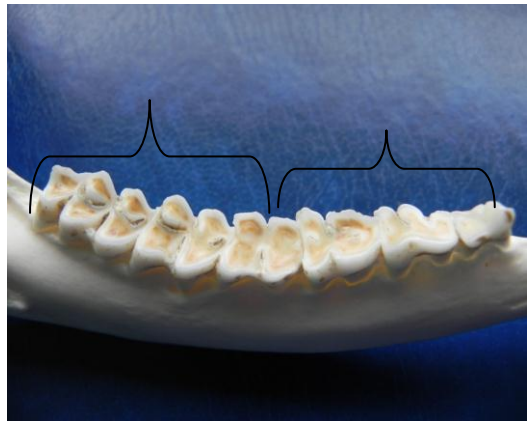

Fig. 2. Deer. Lateral aspect of mandibular arcade: premolars and molars

We measured the length of main beams, in cm, with a precision of a 1.0 mm, on the outer curvature, starting to the base of the burr, up to the highest tip of the antler, which can be the behind tine end, without pressing the roulette in the angle formed by the burr and antler. The results of our measurements were rated between 17-23 cm, the highest score being evaluated as 11,75 (Annex 1).

The weight of dry antlers was determined in grams, at least three months from the date of processing the trophy, with a precision of 5 g. From the resulting mass by weighing, at the trophy with the entire upper jaw are decreased 90 g, at the one with the jaw bone with teeth removed we subtract 65 g, and at the one cut short, on nasal bones, the mass obtained

by weighing is the one taken in consideration, in our case the dried trophy mass enframed between 390-410g. The best score of 72 points were obtained on the 410g trophy (Annex 1).

The volume of antlers was established through the difference between the mass of the trophy weighed in air and submerged in distilled water, in cm^3.

In addition for beauty was measured the inside span, between the antlers, on a perpendicular trajectory with the median line lifted of the skull, were the distance between them is at a maximum, estimated the color, based on the overall appearance of the surface color of antlers, considering that to the inside of the antlers the color is darker and to the outside the color is lighter, this variation registering also from base to tip, the pearling accorded after the aspect of the antlers, from antlers almost without pearling to antlers with big pearls over the entire length of the antlers. The obtained score of the trophies taken in study were included between 3-4 points (Annex 1).

The points for the burrs are granted depending on their height and diameter, considering the next scale of assessment:

- small, shaped like a thin cord, slightly pearled;
- medium, shaped like a thick cord, medium pearled;
- normal, high enough and prominent around the antler;
- big, tall with a big diameter, well pealed;
- very big, both in terms of height and diameter, representing at least 1½ from the diameter of the antler, extremely pearled.

Tine ends were evaluated on their roundness and color. For the quality of the tines, points are given based on their length from normal tines (3.6-5.0 cm length) up to very big tines (at least 6.1 cm length). The points for structure are assessed according to the shape, massiveness (volume), symmetry and uniformity of the trophy. Maximum points may be given only if the trophies whose form is crossing from the "V" shape to "heart" shape, massive, symmetric, and well-proportioned constitutive elements. Penalties are given for short tines and for irregularities, for asymmetries in the shape of the beams and tines, as well as for other defects, regarding undesirable shape of the antlers and of the two main beams (asymmetrical, abnormally positioned), as well as the porous mass of the antlers or other abnormalities that make them to be unconventional (atypical). The maximum score registered on the 410g trophy (Annex 1). (Fig.3, Fig.4)

Fig. 3. Deer. Measuring the antlers

Fig. 4. Deer trophy

Conclusions

As a result of the conducted studies on the 8 trophies we have established the age of animals from which they originated, and can be able to give information and relevant data about each trophy. This shows that it can be made a correlation between the value of the trophy and the age that the animal had from which it was harvested. The range in which we placed after finishing the investigations was between 3 and 4 years.

Acknowledgments. This paper was *published under the frame of* European Social Fund. *Human Resources Development Operational Programme 2007-2013*. *project no*. POSDRU/159/1.5/S/132765.

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Annex 1

Official score sheets for deer trophy

DIRECȚIA SILVICĂ IAȘI

OCOLUL SILVIC PADUREN
NT 4250 din 06.06.2014

R.N.P. REGIA NAȚIONALĂ A PĂDURILOR

FIȘĂ DE EVALUARE
a trofeului de

CĂPRIOR
(Capreolus capreolus)

Trofeul de vânat deținut de

[redacted]

obținut în
ROMÂNIA

în baza autorizației de vânătoare Individuală
seria A1 nr. 174426/ 31.05.2014
eliberată de :
OCOLUL SILVIC PADUREN
pe fondul de vânătoare SI GRUPURI
JUDEȚUL IAȘI

este evaluat pe contrapagină.

Sigiliu nr DE 0147789

Evaluarea trofeului de căprior după formula oficială C.I.C.

Nr. crt.	Elemente		Rezultat măsurători	Suma	Medie	Coeficient	Punctaj
A. Măsurători							
1	Lungimea prăjinilor (cm)	dr.	23,4	47,0	23,5	0,5	11,75
		st.	23,6				
2	Masa trofeului uscat (g)		Brut 450		masa netă 320	0,25	72,00
3	Volumul coarnelor (cm³)		Scăderi 90			~~0,25~~	
B. Adaosuri pentru frumusețe							
4	Deschiderea trofeului		(0-4 puncte) $\frac{13,1}{23,5} \times 100 = 55,75$				4,00
5	Culoarea		(0-4 puncte)				4,00
6	Perlajul		(0-4 puncte)				4,00
7	Rozetele		(0-2 puncte)				2,00
8	Vârfurile ramurilor		(0-2 puncte)				2,00
9	Conformație și calitatea ramurilor		(0-5 puncte)				5,00
	Suma punctajului						105,75
C. Penalizări			(0-5 puncte)				0
Punctaj final (A+B-C)							105,75

Comisia de evaluare:

Nume, prenume: Semnătura: Data: 06.06.2014

1. ing. [redacted]
2. ing. [redacted]
3. [redacted]
4. [redacted]
5. _____

REGIA NAȚIONALĂ A PĂDURILOR ROMSILVA DIRECȚIA SILVICĂ IAȘI

Tipografia **Vicador** Reghin, tel.: 0265-512628

RADIOLOGIC DIAGNOSIS OF CARDIAC CHANGES IN THE DOG USING QUANTITATIVE METHODS. RETROSPECTIVE STUDY FEBRUARY 2013 – FEBRUARY 2014

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Abstract

The radiological diagnosis provides important information regarding the heart's volume and shape changes, its position changes and also the ones of the great vessels. In order to correctly read a radiographic image it is recommended the use of a qualitative exam and a quantitative one, the latest being represented by the quantification of cardiac changes using measurements or diagnosis methods. The aim of the present study is to establish from a radiological point of view, the changes of the heart and great vessels, using a quantitative examination of the heart, of the patients to whom a cardio-thoracic radiological exam was recommended, after a presumptive cardiac affection diagnosis. The radiological examination of 72 dogs was interpreted by 3 different examiners. Following the radiological exams' interpretation, in 48 patients the VHS was calculated, with an average of 10,46v with limits between 8,6 and 13,5v , whilst examination using the clock face analogy had the next results: 4 normal images, 6 inconclusive images, 14 images with unique localized changes and 48 images with multiple changes. The patients who were clinically diagnosed with cardiac affections have proved the development of visible cardiac changes from a radiological point of view, in a proportion of over 85%. In this study, the vertebral heart score (VHS) was calculated in 48 patients , having an average of 10,46v , the group presenting a statistic difference ($p<0.001$) in regard to the reference interval. The image examination using the clock face analogy led to the observation of unique and multiple changes.

Key words*: cardiomegaly, radiological diagnosis, VHS*

Introduction

The radiological diagnosis provides important information regarding the heart's volume and shape changes, its position's changes and also the ones of the great vessels. In order to correctly read a radiographic image it is recommended the use of a qualitative exam and a quantitative one, the latest being represented by the quantification of cardiac changes using measurements or diagnosis methods. The quantitative examination of the heart on a radiographic film implies the calculation of the vertebral heart score (VHS) and applying the clock face analogy both in lateral and dorsal radiographic views. The cardiac silhouette can be normal on a radiographic film, although a cardiac affection is present. Amongst the radiological changes in cardiac diseases, there is the alteration of the normal position of the heart, the decrease and augmentation of the heart's dimensions, as well as the presence of a cardiac abnormal formation.

The aim of the present study is to establish from a radiological point of view, the changes of the heart and great vessels, using a quantitative examination of the heart, of the patients to whom a cardio-thoracic radiological exam has been recommended, after a presumptive cardiac affection diagnosis.

Material and method

The study contains the radiological exam of 72 dogs who were presented for a cardio-thoracic radiological exam within the Radiology Diagnostic Laboratory of the Faculty of Veterinary Medicine Iaşi between February 2013 and February 2014. The patients included in the study were selected based on the clinical examination's presumptive cardiac

affection diagnosis and were represented by 30 males and 42 females of different breeds and statures, having the age between 10 months and 18 years. The radiological examination was realised on alert patients, in lateral and dorsal radiographic views, with the Roentgen Eltex 400 machine and the film developing has been done with the authomatic machine HQ 350 XT. In 38 patients (53,4%) 2 radiographic views were performed, meaning the lateral and dorsal, in 30 patients (41%) only the lateral view was performed and in 4 patients (5,4%) only the dorsal view was performed.

Following the patients' selection for this study, there were excluded the patients who were under 10 months old, patients of whom the radiographic films were inconclusive, patients of whom the radiographic films presented other pathologies which didn't allow the examination of the cardiac silhouette and patients who were suspected of pericardial effusion, the evaluation of the cardiac silhouette being impossible.

The methods used for the interpretation of the radiographs were the clock face analogy [Tobias Schwarz, BSAVA – Manual of Canine and Feline Thoracic Imaging, 2008] and the mesurement of the VHS - Vertebral Heart Score [Buchanan, 1995, Vertebral scale system to measure canine heart size in radiographs]. The interpretation of the radiological films of the patients was realised using the same methods, by 3 examiners, a Phd student in the imaging field, a Phd student in the cardiology field and a bachelor student, having as information the charachteristics of the animals, the medical history and the clinical presumptive diagnosis.

Results and discussions

The radiographs of 72 patients with a clinical presumptive cardiac affection diagnosis were examined, out of which 30 males, representing 41% and 42 females, representing 59%. The patients had the age between 10 months and 18 years, with an average of 9,4 years and were distributed on 3 age categories: Ist category – young patients with age < 1,5 years, 4 patients (n=4), IInd category – adult patients, with age between 1,5 and 8 years, 20 patients (n=20), IIIrd category – geriatric patients, with age > 8 years, 48 patients (n=48). The radiologic examination was performed in both radiographic views (right lateral and dorsal) in 38 patients, representing 53,4%, only in the lateral view in 30 patients, representing 41% and only in the dorsal view in 4 patients, representing 5,4%. The VHS was calculated for 48 patients, with an average of 10,46v, with limits between 8,6 and 13,5v.

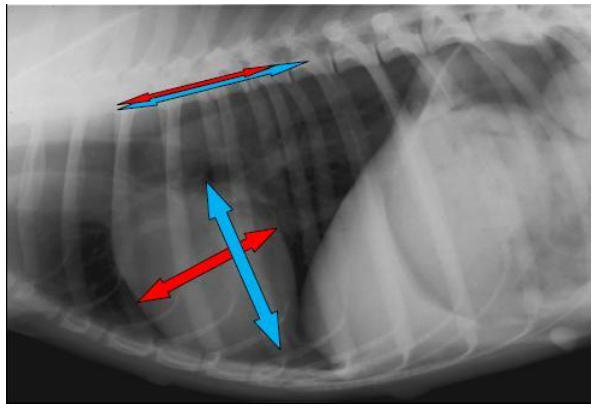

Fig. 1 The vertebral heart score method by calculating the long and short axis of the heart and relating them to the vertebral bodies starting with T4.

The results of clock face analogy examination of the radiographic images are: 4 normal images, 6 inconclusive images, 14 global cardiomegaly images, 1 image with global cardiomegaly and dilation of the aortic base, 15 images with left atrial and vetricular cardiomegaly, 4 images with left atrial cardiomegaly, 3 images with left ventricular cardiomegaly, 8 images with right atrial and ventricular cardiomegaly, 5 images with right atrial cardiomegaly, 3 images with left atrial cardiomegaly and aortic dilation, 1 image with bilateral atrial dilation, 1 image with left atrial dilation and right ventricular cardiomegaly, 2 images with bilateral atrial dilation and left ventricular cardiomegaly, 1 image with left atrial dilation and bilateral ventricular cardiomegaly, 1 image with left atrioventricular cardiomegaly and aortic dilation, 1 image with bilateral atrial dilation and main pulmonary artery dilation and 1 image with the aortic base dilation.

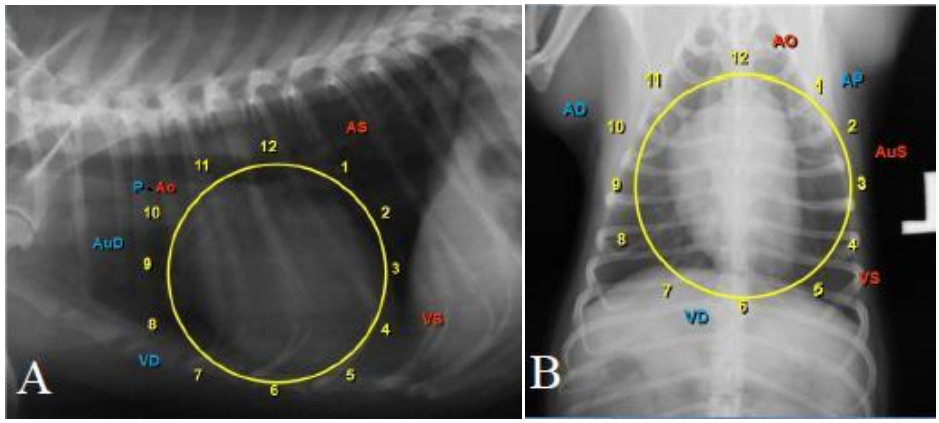

Fig. 2 A. Aplying the clock face analogy method in order to evaluate the shape changes of the heart on the radiography in the lateral view. B. Aplying the same method on the radiography in the dorsal view

Overall there were diagnosticated 44 images with radiographic changes of the left atrium, 32 images with changes of the right atrium, 36 images with changes of the left ventricule, 25 images with changes of the right ventricule, 7 images with changes of the aorta, 1 image with the change of the main pulmonary artery, 13 images with changes of all cardiac chambers and 1 image with global cardiomegaly and the dilation of the aortic base.

Table no 1. Types of changes, unique and associated, diagnosed from a radiological point of view in the patients assisting in the study

UNIQUE CHANGES		**ASSOCIATED CHANGES**	
Change	No. of cases	Change	No. of cases
LA	4	GCM	14
RA	5	GCM + Ao	1
LV	3	LA+LV	15
Ao	2	RA+RV	8
Overall unique	14	LA+RA	1

	LA+RV	1
	LA+Ao	3
	LA+RA+LV	2
	LA+LV+Ao	1
	LA+LV+RV	1
	LA+RA+MPA	1
	Overall associated	48
Overall cases = 62		

The study has followed the radiological diagnostic of 72 patients with cardiac affection suspicion, established through a clinical examination, by independent summing of cardiac dimensions by 3 examiners, using the VHS method and the clock face analogy. The patients who were clinically diagnosed with cardiac affections have proved the development of visible cardiac changes from a radiological point of view, in a proportion of over 85%. Six radiographs were not available for interpretation for various reasons and in 4 patients, representing 5,5% there were no changes from a radiological point of view. A quantitative exam, represented by the quantification of changes, using cardiac measurements on the radiographic film, such as Vertebral Heart Score and Clock Face Analogy, offers precise information and significantly raises the diagnosis' value. [Buchanan, Hamlin].

The calculation of the vertebral coefficient is a global manner of assessment of the cardiac silhouette, without giving details on the localized changes. In this study, the VHS was calculated in 48 patients, with an average of 10,46v , the group presenting a statistical difference ($p<0.001$) in regard to the reference interval determined by Buchanan for healthy dogs, from a cardiac point of view (9.7±0.5v) [Buchanan].

There was no statistical correlation ($r=0.190$) between the age of the patients taken into study and the vertebral coefficient obtained from the radiological examinations. The examination of the images using the clock face analogy [Ruth Dennis, 2010, Handbook of small animal radiology and ultrasound], in both lateral and dorsal radiographic views has led to the detection of some unique changes such as left atrium, right atrium, left ventricule or aorta and associated changes same as left atrium and left ventricule, right atrium and right ventricule, left and right atrium, left heart and the aorta, right heart and the main pulmonary artery and global cardiomegalies. These two quantitative methods, VHS and clock face analogy, can offer additional information to the clinical exam and bring contributions to the final diagnosis. At the same time, the quantification of the heart's dimensions and shape using the two methods, can be practiced for the follow-up in time of a cardiac affection that evolves with changes of the heart's shape and dimensions. Some cardiac diseases can evolve without visible changes from a radiological point of view (examples: restrictive cardiomyopathy, concentric hypertrophic cardiopathy), reason for which the sensitivity of the radiological exam is not high and the specificity is reduced because the degree of the cardiac change can be determined, but the differentiation between pathologies can not be realised. [Kealy, 2000, Diagnostic radiology and ultrasonography of the dog and cat]. The radiological examination can detect the presence of a cardiac pathology, but it cannot infirm it and must always be consolidated with the clinical exam and special exams used in the diagnostic of cardiac pathologies.

Conclusions

1. The two quantitative methods, VHS and clock face analogy, can offer additional information to the clinical exam and bring contributions to the final diagnosis. At the same time, but can also be used for the follow-up in time of a cardiac affection that evolves with changes of the heart's shape and dimensions.
2. The radiological examination can detect the presence of a cardiac pathology, but it cannot infirm it and must always be consolidated with the clinical exam and special exams used in the diagnostic of cardiac pathologies.

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IMAGISTIC ASPECTS IN INTEGRITY DISORDERS OF THE URETHRA IN DOGS

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Abstract

During a period of 1 year, four dogs were brought in the Roentgen diagnostic Laboratory of the Veterinary Medicine Faculty, Iaşi, that were showing signs of dysuria, straining, abdominal pain and a general altered state. It was proven, after examination, that all the dogs had urethral lesions.Although difficult to examine, the lesions that are affecting the urethral lumen are important because, by disrupting the animal's ability to pass urine, can lead to more severe changes in the general state of the animal. The difficulty of obtaining a certain diagnosis can, often, bring a false image of the medical problem. A thorough examination and the usage of urethral catheterization and contrast substance is necessary to ascertain the true nature of the urinary problem and, to establish a certain diagnosis, this method is still the main one used.

Introduction

Although difficult to examine, the lesions that are affecting the integrity of the urethral lumen are important because, by disrupting the animal's ability to pass urine, can lead to more severe changes in the general state of the animal, such as the uremic syndrome that could end in the death of the patient if left unresolved. The difficulty of obtaining a certain diagnosis can often bring a false image of the medical problem. A thorough examination and the usage of urethral catheterization and contrast substance are necessary to ascertain the true nature of the urinary problem. To establish a certain diagnosis when it comes to the integrity of the urethra, this method is still the main one used.

The urethra is the main conduit through which the urine is eliminated from the urinary bladder and it is found between the internal urethral orifice and the external urethral orifice.

In females, the urethra is short, in dogs being only 5 to 6 centimeters long, and its opening is located at the limit between the vagina and the vaginal vestibule.

In males, the urethra is longer, and it is also a conduit for the seminal material during ejaculation. Topographically, it can be divided in 2 segments, one pelvis segment and a penile segment.

Method and materials

The study included 4 dogs, of various breeds, aged between 6 and 14 years that were showing signs of dysuria or urinary retention.

The radiological examination was performed on the alert dogs, in lateral and ventral decubitus, to attain the latero-lateral and dorso-ventral recumbencies. As a technical material, for the X-ray examination, a Roentgen Eltex 400 and a mobile Roentgen Intermedical Basic 4006 devices were used. For a contrast urethrography, a non-ionic solution was used, based on ioversol (OptiRay 350).

Fig. 1. Materials used in urethral catheterization

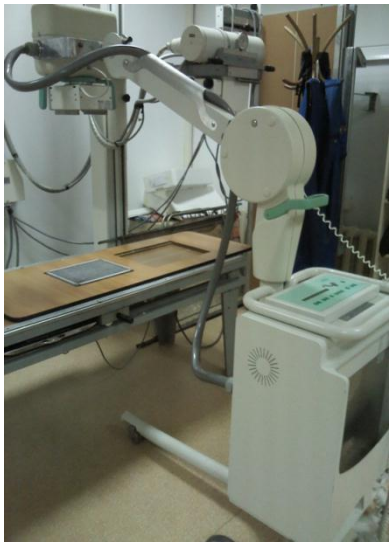

Fig. 2. The Roentgen Intermedical Basic 4006

For the urethral catheterization, a urinary catheter was used, along with sterile gloves, vaseline, a recipient for the urine that could be expressed, syringe and the contrast substance, Optiray 350. The radiological films used were in a green specter.

Fig. 3. Urethral catheterization

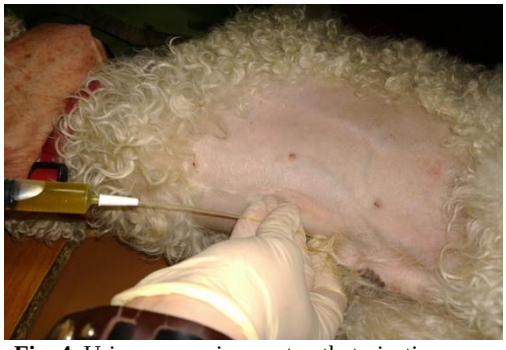

Fig. 4. Urine expression post-catheterization

Results and discussions

One of these dogs had previously been seen at a private practice that suspected the presence of an urethral calculus. In the lateral view taken, there was a small lesion that could be observed caudally of the penile bone, on the projection area of the urethra. The owners refused further examinations using contrast substances.

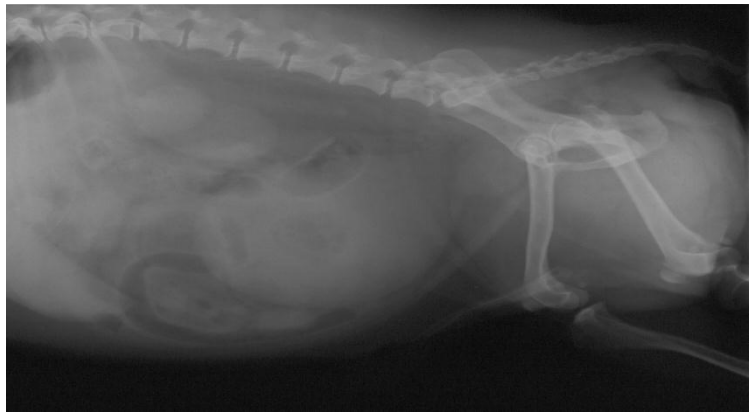

Fig. 5. Dog, whole male, Pekingese, 14 years – suspected urolithiasis and resisting catheterization. Latero-lateral abdominal recumbency, native x-ray.

In the radiological image, a small lesioned spot can be seen in the region caudally of the penile bone, as a slightly more radiopaque area. Also, in the abdominal area, the urinary bladder can be seen as a large mass, suggesting a case of urine retention.

A second dog was brought in the clinic showing signs of dysuria, an altered general state and a rectal prolapse. In the radiographic images that were taken, the urinary bladder could be seen attached to the prolapsed mass. The urethral lumen seemed to be strangulated. After the surgical intervention to replace the herniated tissue, a new series of x-rays was taken, using contrast substance – Optiray 350. Unfortunately, the urethral lumen, while permitting the access of the urethral catheter, did not allow the urine to pass. A strangulation was suspected that persisted post-operatory.

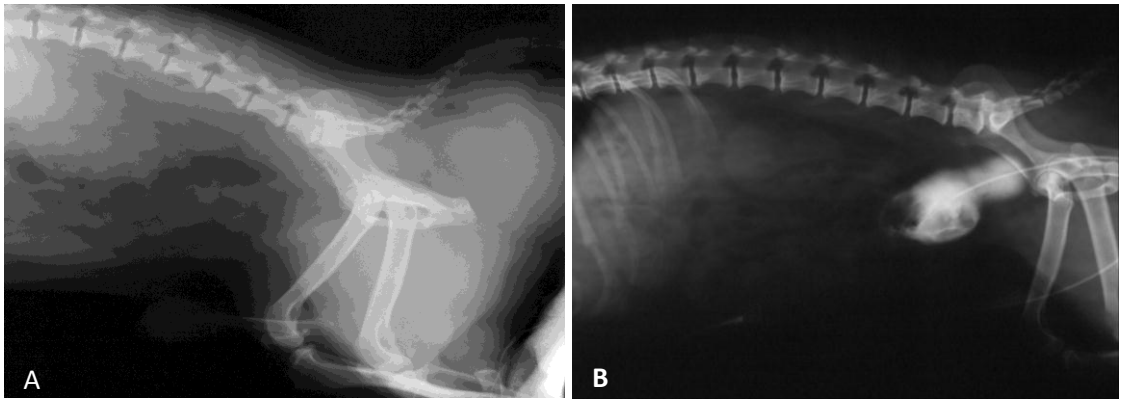

Fig. 6. A. Dog, whole male, Pekingese, 9 years, perianal hernia with urinary bladder involvement. Latero-lateral recumbency. **B.** Dog, whole male, Pekingese, 9 years, perianal hernia with urinary bladder involvement – urethral permeability verification post-operatory. Latero-lateral recumbency

A third patient, a mixed breed dog was brought in the clinic with signs of uremia and a generally altered state. The native x-ray images proved to be inconclusive. The images taken using a contrast medium showed the presence of a urethral fissure that allowed the contrast substance to escape in the abdominal cavity.

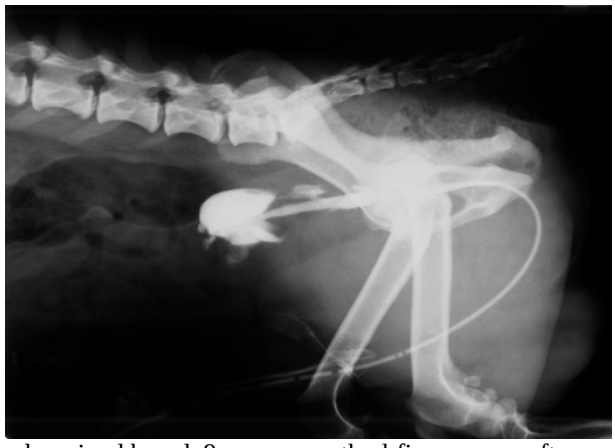

Fig. 8. Dog, whole male, mixed breed, 9 years – urethral fissure seen after urethral catheterization and injecting the contrast substance. Latero-lateral recumbency.

The fourth case, a small sized dog, was brought in the clinic after a car accident showing signs of extreme lumbar pain. The radiographic images taken while using urethral catheterization and contrast substance revealed that the pelvis was fractured and the urethra was sectioned caudally of the pubic bone. The contrast substance escaped the urethral lumen and could be seen spreading in the adjacent tissues. On the abdominal region, the urinary bladder could be seen in a distended state suggesting a disruption in the dogs' ability to pass urine.

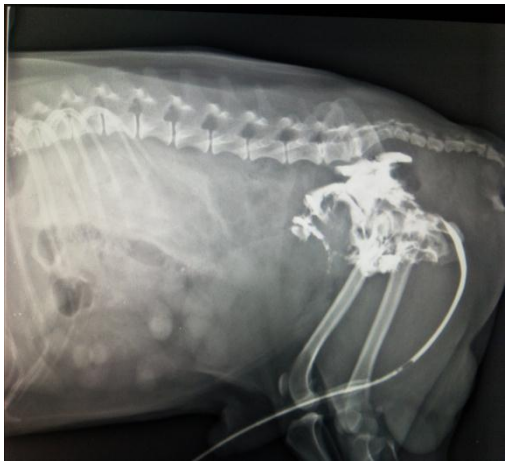

Fig. 9. Dog, whole male, Pekingese, 6 years – post-traumatic x-ray. Latero-lateral recumbency. The pelvic bones can be seen in the fractured state.

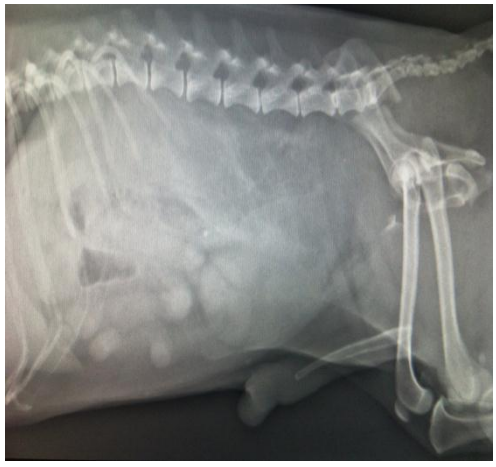

Fig. 10. Dog, whole male, Pekingese, 6 years – post-traumatic x-ray using substance contrast to determine the integrity of the urethra

Conclusions

The first and most important conclusion is that, even with the advancements that the medical science has been doing lately, the urethral examination methods are still scarce and that the radiological examination using contrast substance remains the best solution for verifying the integrity of the urethral lumen.

Also, when faced with possible surgery techniques that could involve displacing or replacing adjacent tissues to the urinary bladder or the pelvic segment of the urethra, it is indicated to verify the permeability of the urethra after the operation is done.

The pelvis fractures have the possibility to cause lesions that can impair the animal's ability to pass urine so, when suspected, urethral catheterization to verify the permeability of the lumen is indicated.

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A PRELIMINARY STATISTIC STUDY REGARDING VERTEBRAL DISORDERS, ACCOMPANIED BY DYSURIA, IN DOGS

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Abstract

During a period of 18 months, 2370 patients were brought in the Radiology Clinic, requiring a radiographic examination. Out of these, 440 were presented with disorders that required an examination of the vertebral column. 93 of these patients also showed signs of dysuria, giving a percentage of 21.13% out of the total number of cases. In many cases, the pathology was complex, including several pathological signs, such as lameness, paralysis, lumbar pain, stranguria. The radiographic lesions that could be observed included osteophitosis, increased radioopacity in the intervertebral spaces, vertebral fractures or other vertebral body modifications, alterations in the vertebral spaces, vertebral subluxation or luxation, disk hernias or mineralization. In many cases that showed signs of dysuria, there were also signs of medullar compressions visible on the x-rays taken using contrast substance. The purpose of this paper was to correlate the apparition of the urinary symptoms with the existing vertebral disorders and to establish the percentage of patients with this clinical sign in relation to the respective vertebral lesion, a feat that we consider that we achieved: osteophitosis – 17.24%, increased radioopacity in the intervertebral spaces – 28.57%, vertebral fractures – 20.83%, or other vertebral body modifications – 20%, alterations in the vertebral spaces – 17.69%, vertebral subluxation or luxation – 20%, disk hernias or mineralization – 32.65%.

Keywords*: radiographic examination, vertebral column, dysuria, statistic*

Introduction

The following paper tries to offer a preliminary overview of the main vertebral disorders that are accompanied by dysuria in dogs and to define the first steps towards a statistical approach. Vertebral disorders can, depending on their location, induce urinary disorders such as dysuria or incontinence. The purpose of this paper was to correlate the apparition of the urinary symptoms with the existing vertebral disorders and to establish the percentage of patients with this clinical sign in relation to the respective vertebral lesion.

Method and material

Over a period of 18 months, 2370 patients were received at the Roentgen diagnostic Laboratory at FMV Iasi. Out of these, 440 were dogs requiring a vertebral column imagistic examination with or without contrast substance. For each dog, a complete clinical examination was made, before being redirected to one or more paraclinical examinations, in this case, the radiological examination of the vertebral column.

The radiological examination was performed on the alert dogs, in lateral and dorsal decubitus, to attain the latero-lateral and ventro-dorsal recumbencies. As a technical material, for the X-ray examination, a Roentgen Eltex 400 and a mobile Roentgen Intermedical Basic 4006 devices were used. For the contrast examination of the spine – mielography, the dogs were sedated and a non-ionic contrast solution was used, based on ioversol (OptiRay 350).

Very often, the dogs were brought in for a radiological examination of the spine after being redirected from the clinical examination that showed changes in the spine and hind limbs position. The modifications included signs of strong pain, kyphosis, the refusal of stationary positions, partial or total paralysis and sometimes colic-like symptoms.

The patients were aged between 11 months and 17 years and the breeds examined were varied.

Results and discussions

Out of the 2370 patients seen in the Radiology Clinic over a period of 18 months, only 440 of these were dogs brought in with spine disorders. 93 of these were also showing signs of dysuria, stranguria or other signs of urinary dysfunctions. Quite often, the general state of the animal can be changed, being expressed through a lack of appetite, apathy, and even prostration or paralysis when the pathology has a long evolution and the dog becomes organically exhausted.

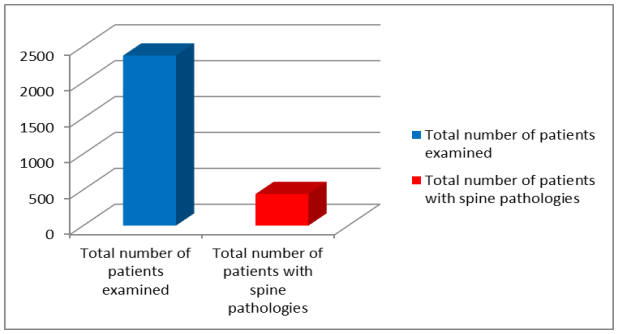

Chart no. 1. The ratio between the total number of patients examined and the total number of patients brought in with spine disorders

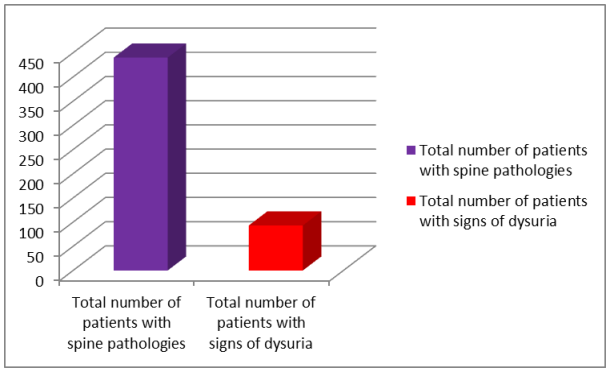

Chart no. 2. The ratio between the total number of patients with spine disorders and the total number of patients that were showing signs of dysuria.

For a thorough analysis, the patients that were diagnosed with different vertebral column disorders were then systematized in a table using the primary lesion identified on the radiological images as the main criteria of differentiation.

Table no. 1. Patient systematization based on the main lesion differentiated on the radiological images and the number of patients that also showed signs of dysuria.

No.	Type of lesion that could be observed on the radiological image	Number of patients examined	Number of patients that showed signs of dysuria	Percentage
1.	Osteophytes	116	20	17.24%
2.	Increased radioopacity in the intervertebral spaces	56	16	28.57%
3.	Vertebral fractures	24	5	20.83%
4.	Other vertebral body modifications	35	7	20%
5.	Alterations in the vertebral spaces	130	23	17.69%
6.	Vertebral subluxation or luxation	30	6	20%
7.	Disk hernias or mineralization	49	16	32.65%

As seen in the table above, the main vertebral column disorder observed in the radiological images were the alterations in the vertebral spaces. However, out of 130 patients examined, only 23 showed signs of dysuria, reaching a percentage of 17.69% out of the total with this lesion.

The osteophytes were observed in 116 patients, 20 of these showing urinary difficulties and reaching a percentage of 17.24%.

The highest percentage of patients that showed signs of dysuria was reached by the Disk hernias and mineralization category that showed that 32.65% of the patients were affected. The increased opacity in the intervertebral spaces – inflammations – induced signs of dysuria in 28.57% of the dogs with this lesion observable on the radiological image.

In 20% of the patients with vertebral subluxation/luxation and in those with vertebral body modifications, the signs of dysuria were present. The vertebral fractures were accompanied by urinary symptoms in 20.83% of the cases.

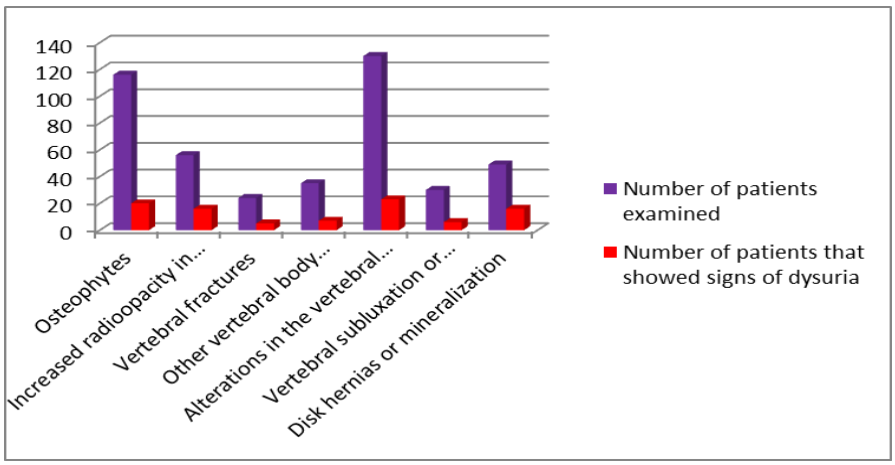

Chart no. 3. The ratio between the number of patients examined for each type of lesion identified on the radiological image and the number of patients that also showed signs of dysuria.

Conclusions

This study, even though it is in a preliminary form, shows at a statistical level that, although intervertebral disk disorders are not so commonly seen, they are the lesions that tend to be accompanied the most often by signs of dysuria. Aside from the mineralization, the disk hernia is more difficult to diagnose due to the fact that it requires the usage of contrast substance, the procedure bringing some risks to the patients' life that sometimes the owners are not willing to take. Therefore, we can't exclude the existence of vertebral disk hernias in patients with signs of medullar compression for which the contrast substance examination could not be achieved.

The alterations of the intervertebral spaces and the presence of osteophytes seem to be the most common lesions that can be observed on the native radiological film, along with the increased radioopacity in the intervertebral spaces and the vertebral disk disorders. Vertebral body fractures might not be so rare, but due to the gravity and their traumatic nature, the possibility of a radiological examination might be negative.

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DIAGNOSTIC IMAGING TECHNIQUES USED IN SUSPICIOUS ABDOMINAL MASSES IN CATS

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Abstract

The rate of tumors in cats is increasing, and their observed location is more common in the abdomen. In old cats with clinically diagnosed mammary tumors, radiology and ultrasound revealed intraabdominal masses that were confirmed during the necropsic examination. Pathological findings that were diagnosed on survey radiographs included: increased radiopacity in the splenic radiographic projection area, radiopaque structure defined caudally to the hepatic radiographic projection area, ascitis. Ultrasound examinations findings were: diffuse decrease in echogenicity of the liver internal structure, with nodular hypoechoic appearance. Also, during the necropsy, there were diagnosed hepatic and splenic tumors that were evolving simoultaneously with the primary clinically seen tumor.

Keywords: *tumor, cat, liver, spleen, radiography, ultrasound*

Introduction:

The rate of tumors in cats is increasing, and their observed location is more common in the abdomen. In old cats with clinically diagnosed mammary tumors, radiology and ultrasound revealed intraabdominal masses, that were comfirmed during the necropsic examination.

Material and Method:

Twenty-two female cats were included in the study with ages between ten and fourteen years, during a period lasting twelve months (January 2013-January 2014), during clinical examination, in the Veterinary Radiological Laboratory of Iasi. For all the patients, both the x-ray and the ultrasound examinations were made. All the female cats were diagnosed with breast tumors.

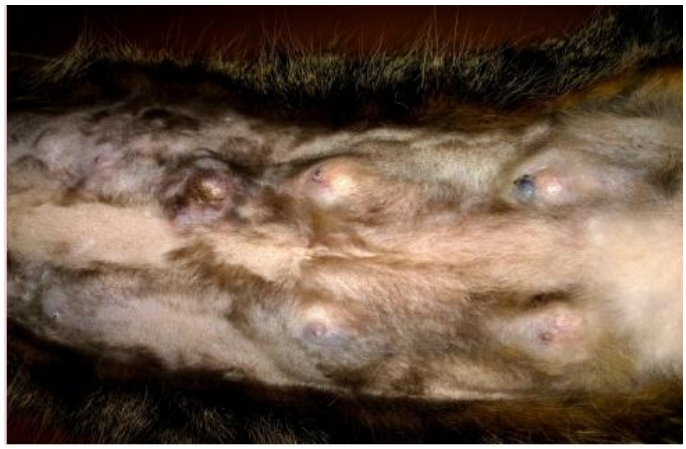

Fig. 1. Cat. Mamary nodules. False lactation. FMV Iași.

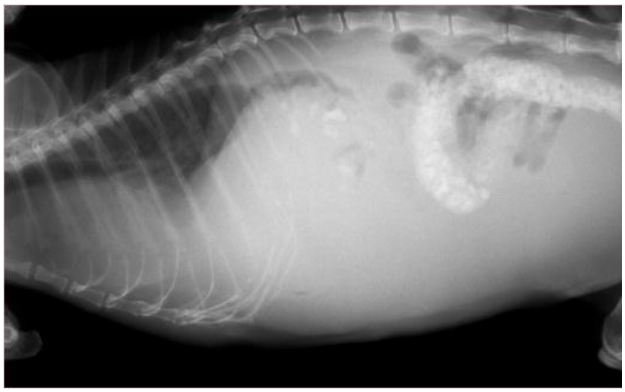

Fig. 2. X-ray lateral abdominal view. Pleural and abdominal fluid. FMV Iaşi.

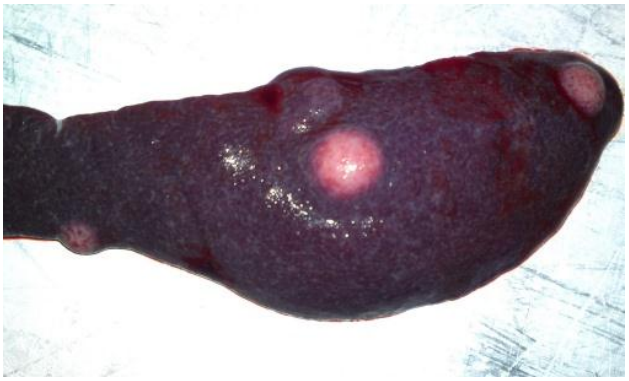

Fig. 3. Cat spleen. Necropsic examination.
Tumoral asymmetric nodules. Increased spleen size.

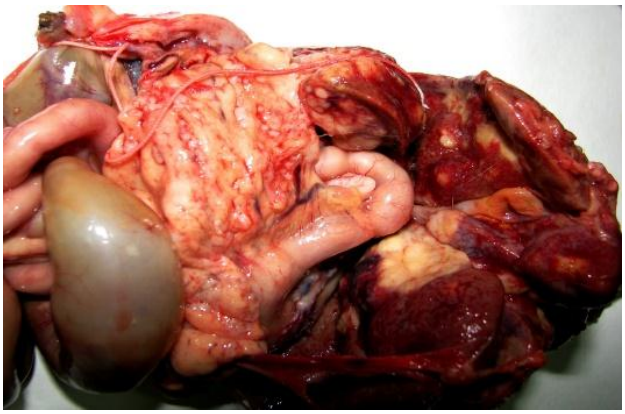

Fig. 4. Necropsic examination. Hepatic and mesenteric metastases.
FMV Iasi.

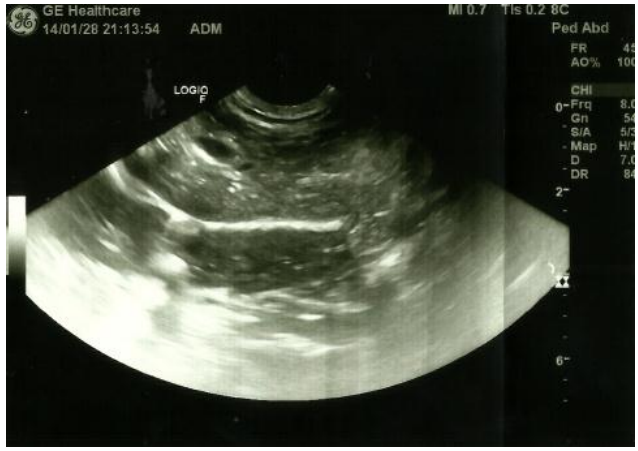

Fig. 5. Longitudinal hepatic ultrasonography. Structural change of echogenity. FMV Iași.

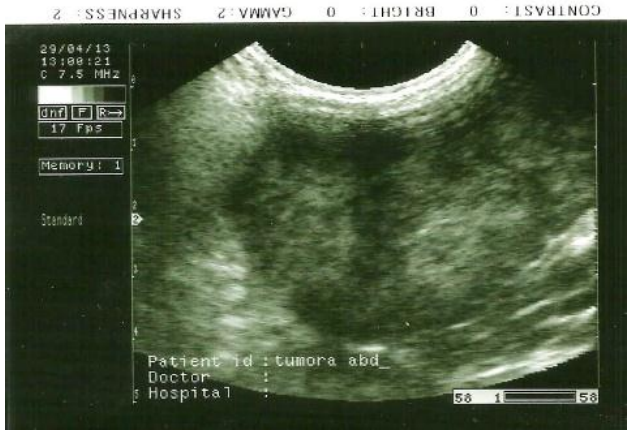

Fig. 6. Longitudinal hepatic ultrasonography.
Structural change of echogenity. FMV Iași.

Results

Pathological findings that were diagnosed on survey radiographs included: increased radiopacity in the splenic radiographic projection area, radiopaque structure defined caudally of the hepatic radiographic projection area, ascitis. Ultrasound examinations findings were: diffuse decrease in echogenicity of the liver internal structure, with nodular hypoechoic appearance. Also, during the necropsy, there were hepatic and splenic tumors diagnosed that were evolving simultaneously with the primary clinically seen tumor.

According to the clinical symptoms observed, recommendations may direct to one or both imaging methods of investigation. In the case of advanced slimming and poor general condition, without other clinical localized abdominal changes, the solution is turning the patient to ultrasonographic examination, that could confer structural data for the internal abdominal organs. When the clinical examination focuses on localized breast tumors, it is recommended to perform a radiological examination to evaluate the existence of any metastases.

Conclusions

The diagnosis for any suspect tumoral formations must be formed on the basis of the clinical changes of the affected organ.

The origins of clinical organ changes must be confirmed by further analysis and imaging and laboratory tests.

The incidence of primary tumors located on the abdominal organs, in cats, has a low rate.

Standalone ultrasonography or radiological examination as the sole method, are not sufficient to establish a diagnosis of tumoral origin with certainty.

Necropsic and histopathological examination give certain data in the type and origin of the primary tumor.

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THE RADIOLOGICAL EVALUATION OF RADIUS AND ULNA FRACTURES IN CATS

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Abstract

The radius and ulna fracture in cats is very common in cats and may be considered a triviality in veterinary trauma, but the inappropriate therapeutical approach may easily result in failure. The radiological evaluation is fundamental for the diagnostic of these types of fractures. The type of fracture (proximal, diaphyseal, distal) requires a default protocol that respects the A O Vet principles. This study presents the case of Kinsha, a 4 year old neutered male cat, that suffered a fall trauma from approximately 8 meters (fourth floor). The consequence was a reducible diaphyseal fracture in the distal radius and ulna. External fixator osteosintesis was a good approach to this type of fracture, but owner and pet compliance was difficult and resulted in complications.

Key words: *Therapeutical approach, radiological evaluation, failure, fracture, cat, pet/owner compliance.*

Introduction

Anatomical and physiological particularities of the feline radius and ulna are frequently incriminated in conditions that have as a primary symptom lameness. Topographical particularities of the bone segments along with their reduced size make the exploration and diagnostic difficult to realize using only base semiological methods. The radiological examination increases the accuracy of the diagnostic and, by default, orients the vet in using the best option for the therapeutical approach.

Materials and methods

The study has been realized on a neutered male cat, longhair crossbred, 4 years old, 4.4 kg. The radiological examination was possible due to a Senecal roentgen with Primax G400F films. The positions used for the best view where the cranio-caudal and medio-lateral. The pictures where taken with a Sony Cybershot camera.

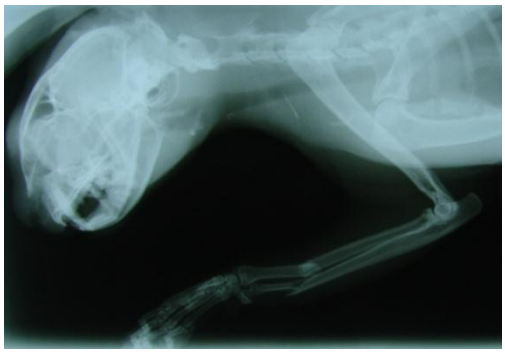
The available treatment options where either a gypsum splinter that had to be maintained 45 days or osteosintesis with a internal or external fixator.

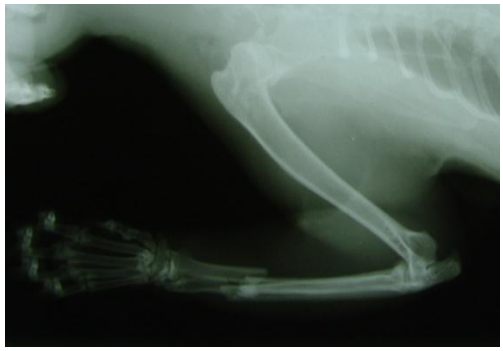
The first option was refused by the owner, and the only option accepted was the surgical internal fixation of the bones using a compression plate and a centro-medular brooch.

The anesthesia was inhalatory, using isoflurane, and the centro-medular brooch used was a 1,2 mm Kirshner. The compression plate had 6 slots with 2,5 mm diameter drywall screws. The splint used for the foreleg was a standard plastic one destined for small animals on wich was applied a movement blocking bandage.

The post-operative antibiotic used was a mix of amoxicillin and clavulanic acid (Clavaseptin 50mg) and meloxicam for pain management (Metacam, 2mg/ml)

Results and discussions





Fracture type – reducible diaphyseal in the distal region.

A close evaluation of the radius reveals a longitudinal fissure in the proximal region of the fracture. The ulna also presents an oblique fissure.

Patient Score

Biological factors:

- Tissue trauma
- Time of occurrence/surgery
- Surgycal approach
- Age/health status

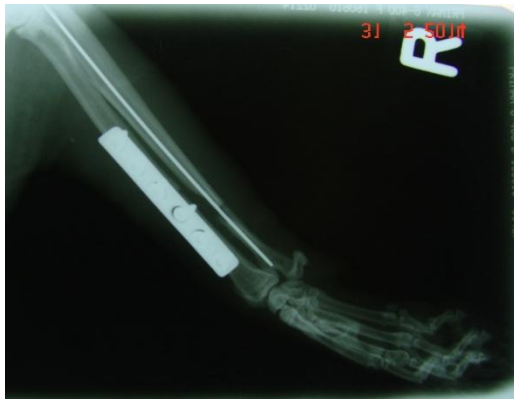
Mechanical factors:

- Body weight sharing
- Weight of the animal
- Movement

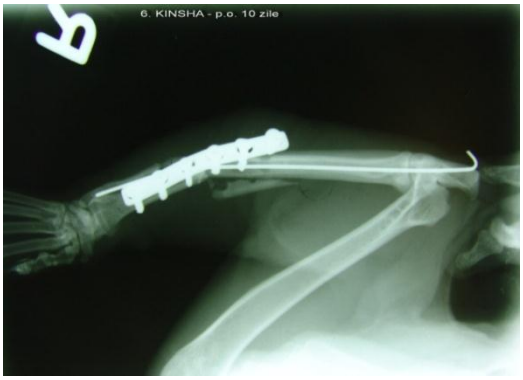
Factori clinici:

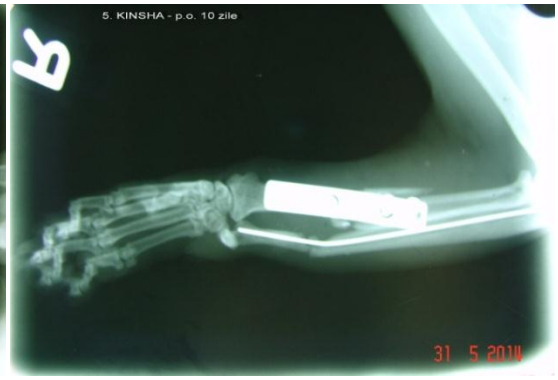
- Post-operative attention
- Pacient
- Disponibilitate
- Complianța proprietarului

Score is between 1 and 10; Kinsha has 7.

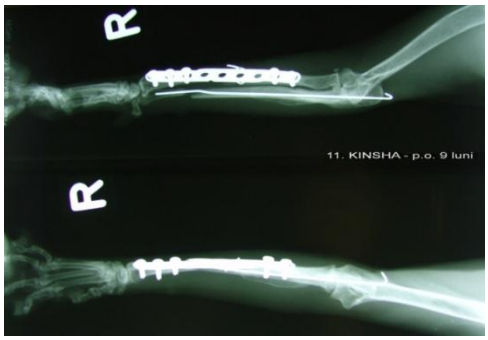


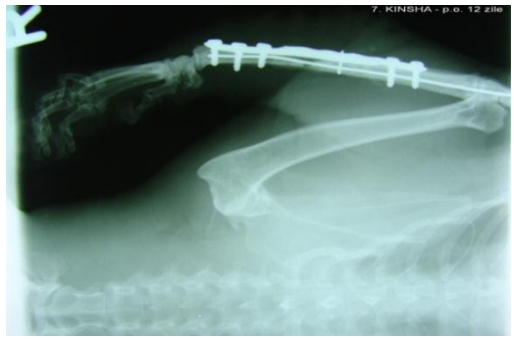
Two days after surgery, the patient started using the leg and the owner refused the splinter due to the stress it could cause. The downside of this decision was continuous usage of the leg only after a few days from surgery. After a 1.5 m jump the compression plate and the brooch bent.



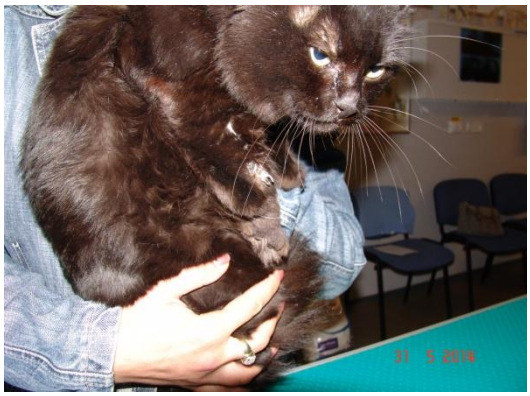


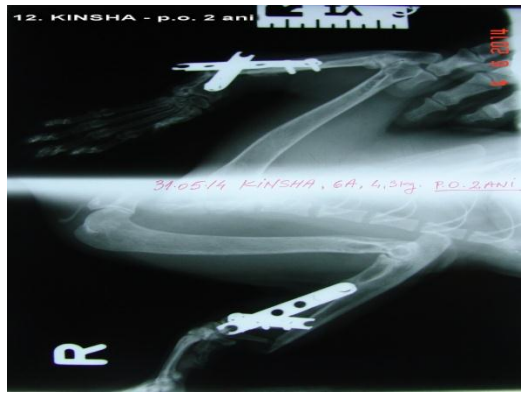
The patient was called in for a second surgery, this time with a splinter for about 40 days.

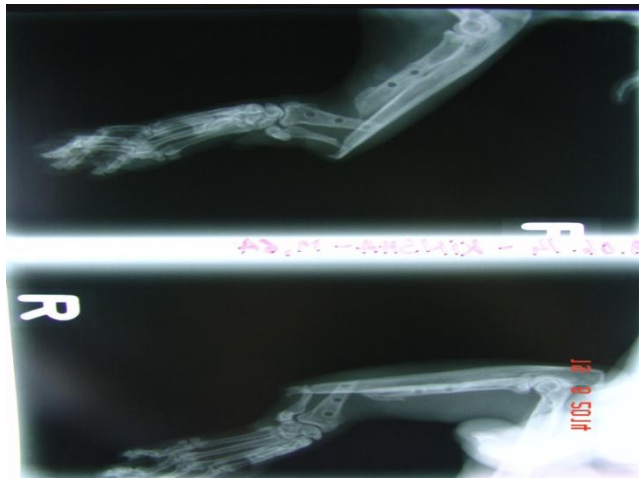




During the period september 2013 and april 2014 (7 months) the owner hasnțt presented the patient for a reexamination. After 1 year and 10 months from the intial fracture, the leg was completely compromised.







Conclusions

1. The absence of the splinter for 40 days from the first surgery predisposes the fracture to other traumas or prevents the bone from forming a solid callus.
2. Poor vascularization at the second surgery results in the forming of a poor callus.
3. Owner and pet compliance is very important for the prevention of further traumas that may affect the callus.
4. Reexamination at strict time intervals is necessary to appreciate the healing time of the bone.

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INTERVERTEBRAL DISC HERNIATION IN DOGS: RADIOLOGICAL, MYELOGRAPHIC AND COMPUTED TOMOGRAPHIC STUDY

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Abstract
Intervertebral disc herniation is one of the most common neurological affection of the spinal cord and the main indication for spinal diagnostic imaging in dogs. Chondrodystrophic breed dogs are prone to develop Hansen type I disc degeneration (extrusion), while the large, nonchondrodystrophic breed dogs are prone to develop Hansen type II disc degeneration (protrusion). The main diagnostic imaging methods for examination of vertebral column and spinal cord in dogs are radiography, myelography and computed tomography. The main features of these diagnostic imaging techniques are related in the present study. Radiography represents the first diagnostic imaging method for dogs suspected with spinal cord lesions for trauma or neoplasia exclusion; in the same time, radiography provide important information related to the degenerative process affecting the intervertebral disc, which is a specific sign of disc protrusion occurrence. Myelography remains the "gold standard" where is no access to advanced diagnostic imaging techniques. With a great sensibility for evidence of extradural spinal cord compression, myelography provides important aspects also for the lateralization of disc herniation in dogs. As a sectional imaging technique, computed tomography is an accurate imaging procedure for dogs with suspected disc herniation, providing the accurate assessment of spinal cord.
Key words: *intervertebral disc herniation, dog, diagnostic imaging*

Introduction

Intervertebral disc disease is the most encountered pathology of the spine in dogs, characterized by degenerative changes and disc herniation into the vertebral canal, causing spinal cord compression (Priester, 1976; Sharp and Wheeler, 2005).

In 1952 Hansen described 2 different types of intervertebral disc herniation, each one being characteristic to various breeds of dog (Hansen, 1952). Chondrodystrophic breed dogs are prone to develop Hansen type I disc degeneration (extrusion), while the large, nonchondrodystrophic breed dogs are prone to develop Hansen type II disc degeneration (protrusion) (Brisson, 2010; Goggin et al., 1970).

The main diagnostic imaging methods for examination of vertebral column and spinal cord in dogs are radiography, myelography and Computed Tomography (CT). The purpose of this study was to relate the main features of these diagnostic imaging techniques used to characterize the intervertebral disc herniation.

Radiography represents the first diagnostic imaging method for dogs suspected with spinal cord lesions for trauma or neoplasia exclusion; in the same time, radiography provide important information related to the degenerative process affecting the intervertebral disc, which is a specific sign of disc protrusion occurrence. The sensibility of radiographic survey for intervertebral disc herniation is related to be between 60-78% (Schulz et al., 1998). It is important to take 2 views of the suspected vertebral segment: lateral and ventro-dorsal. The main radiographic features of intervertebral disc herniation are: collapse of the intervertebral disc space, calcification of the intervertebral disc, narrowing of the space between articular facets and presence of disc material at the level of intervertebral foramen (fig. 1, fig.2).

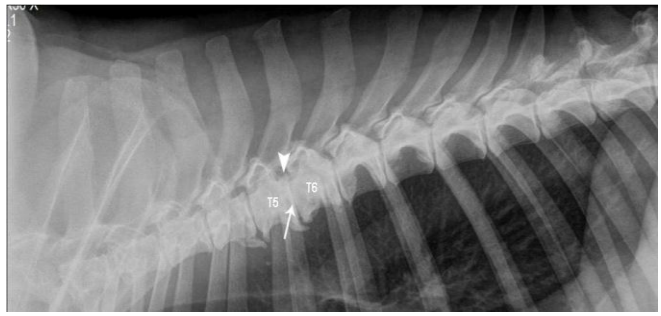


Fig. 1 Radiography of thoracic spine, lateral view; note the narrowing of T5-T6 intervertebral space (white arrow) with the presence of a radiopaque zone at the level of T5-T6 intervertebral foramen (white arrow head)

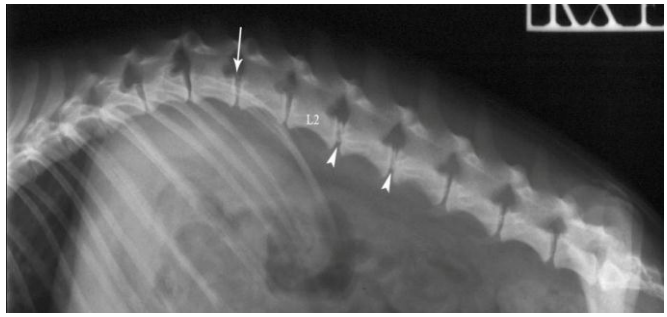


Fig. 2 Radiography of lumbar spine- lateral view, note the presence of disc mineralization at L2-L3 and L3-L4 intervertebral spaces (white arrow heads) and radiopaque zones at the level of T13-L1 intervertebral foramen (white arrow)

The main advantages of the radiographic exam are: providing a useful diagnosis, short processing time and low cost processing.

Myelography is a type of radiographic examination that uses a contrast medium to detect different pathology of the spinal cord, including the location of a spinal cord injury, such as intervertebral disc herniation. When a contrast material is injected into the subarachnoid space surrounding the nerve roots and spinal cord, it allows the radiologist to view outlines of the different areas of the spine that usually are not visible or distinguishable on X-rays.

The normal myelographic pattern is characterized by two contrast line, ventral and dorsal, which are parallel each other and have the same thickness (fig. 3).

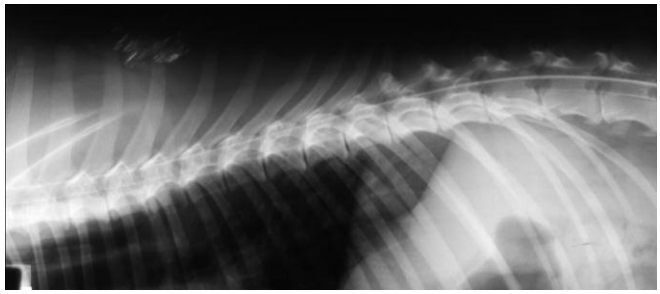


Fig. 3 Myelography of thoracolumbar spine-lateral view; normal aspect of those two contrast lines, ventral and dorsal

With a great sensibility for evidence of extradural spinal cord compression (94-100%) (Hosgood, 1992; Kirberger et al., 1992; McKee, 1992), myelography provides important aspects also for the lateralization of disc herniation in dogs. For this, it is very important to perform also the oblique views (right and left) to assess the lateralization of the extruded disc material (fig. 4).

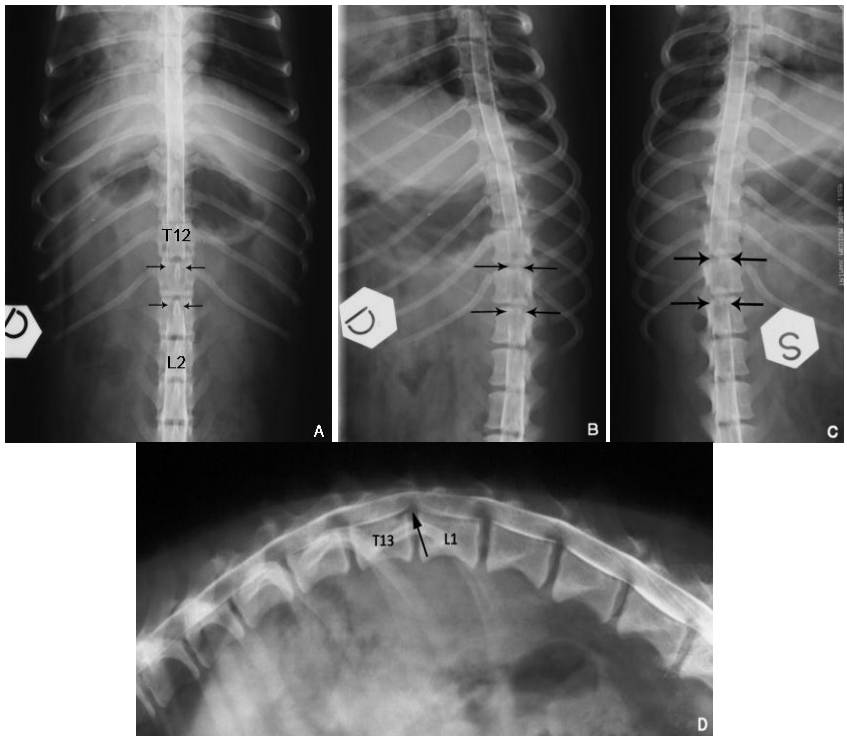


Fig. 4 Thoracolumbar myelography, VD (A), right oblique (B), left oblique (C) and lateral (D) views; note the loss of contrast line at the level of T13-L1 vertebrae (A,B,C) and dorsal deviation of the contrast line (D) (black arrows), which indicates a ventro-lateral extradural compression

Myelography is a very safe procedure that is well tolerated in the dogs. While it has fallen out of favor because of the ease of CT, myelography still remains a valuable tool in assessing patients with spinal cord disorders.

In veterinary medicine, CT is one of the most commonly used methods for imaging the spine because it provides a detailed examination of the spinal cord and surrounding tissues. CT provides more accurate information and takes less time than myelography, particularly in chondrodystrophoid breeds, and is usually much easier to decide which side the disc material is located from CT images than from myelograms (Lim et al., 2010).

Advantages of CT for evaluating canine disc extrusions include elimination of superimposition, fast image acquisition, and low risk of morbidity due to procedure-related complications. On a CT transverse scan, the spinal cord is round-shaped, surrounded dorso-laterally by epidural fat and ventrally, by two nerve roots (Fig. 5).

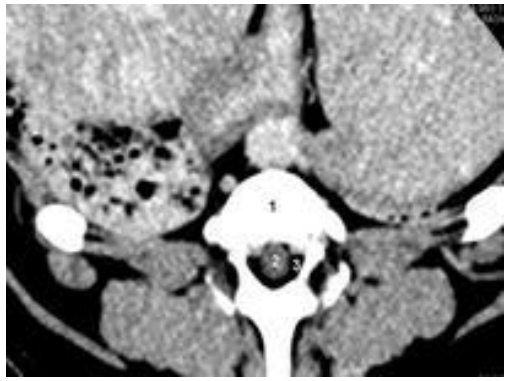


Fig. 5 Computed tomography cross-section at the level of T10-T11 intervertebral disc-normal aspect of thoracic vertebra and spinal cord,
1- intervertebral disc, 2- spinal cord, 3- epidural fat

Another advantage of the CT is the possibility of multiplanar image reformatting (fig. 5), 3D reconstruction of the images and achievement of virtual endoscopy (fig. 6). These techniques allows the radiologist to clearly visualize and characterize the distribution pattern of disc herniation.

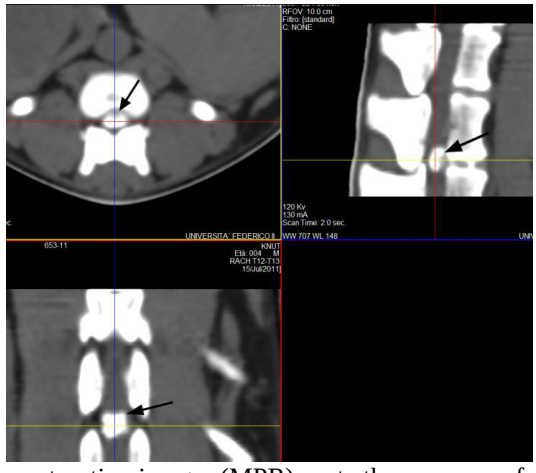
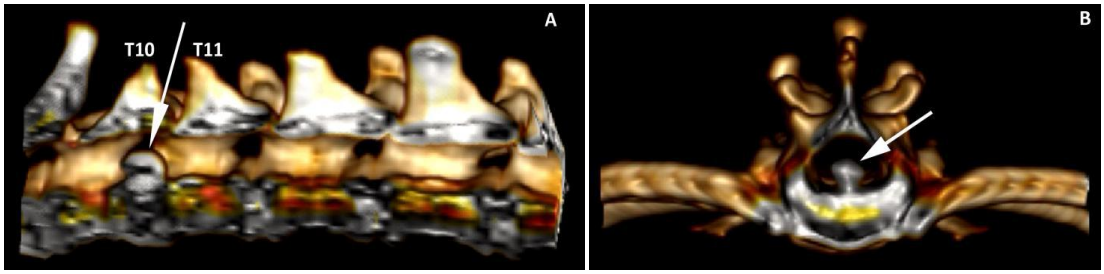


Fig. 5 Multiplanar reconstruction images (MPR)- note the presence of one hyperdense areas (black arrows), with different dimensions which exert a mass effect on thoracolumbar spinal cord, characteristic features for moderate severe disc herniation



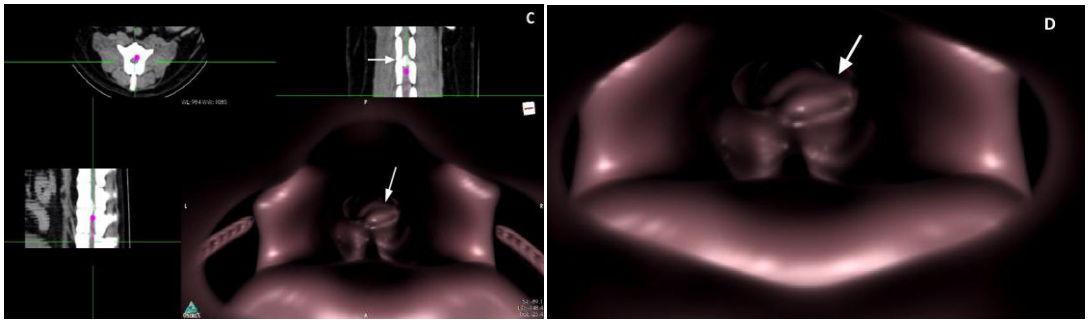


Fig. 6 3D reconstructions (A- sagittal section; B- cross section) and virtual endoscopy (C, D- details), outlining a Hansen type I disc herniation (white arrow) at the level of T10-T11 intervertebral disc

Several advantages of using CT to identify disc herniation includes the lack of negative side-effects associated with the procedure as compared with myelography, the ability to determine lateralization of disk material, and the time required for imaging.

In summary, both myelography and CT are reasonable diagnostic imaging modalities for locating the site of disc herniation with a relative sensitivity of slightly >90%., while radiography still remain the method which allows only to suspect the intrevertebral disc herniation without any information regarding the lateralization of the disc material.

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IMAGING APROACH OF VESICOURETHERAL REFLUX IN CATS

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Abstract

Vesicoureteral reflux (VUR) represents the backward flow of urine from the bladder into the kidneys. Normally, urine flows from the kidneys through the ureters to the bladder. The muscles of the bladder and ureters, along with the pressure of urine in the bladder, prevent urine from flowing backward through the ureters. Both in human and veterinary medicine, a combination of imaging methods are used to diagnose this disease. The present study reveals the imaging methods used in the diagnosis of this disorder in two cats with vesicouretheral reflux.

Key words: *Vesico-uretheral, reflux, diagnostic, imaging*

Introduction

Vesicoureteral reflux (VUR) represents the backward flow of urine from the bladder into the kidneys. Normally, urine flows from the kidneys through the ureters to the bladder. The muscles of the bladder and ureters, along with the pressure of urine in the bladder, prevent urine from flowing backward through the ureters. VUR can be primary, or secondary. The most common type is the primary VUR which is usually detected shortly after birth (Grebeldinger, et al.2009). The secondary VUR is associated with or caused by, high intravesical pressures, usually in urinary tract obstruction. The presence of VUR rises the risk of urinary tract infections (UTI), and of so called reflux nephropaty.

Materials and methods:

One 13 years old male domestic short hair cat and one 5 yeas old male Persian cat were included in the study. To avoid the exposure to unnecessary and injurious x-rays, a patient selection based on the presence or absence of distal ureteral dilatation was done, and on the time elapsed between typical or atypical symptoms of urinary tract infection and fever. After ultrasound selection, retrograde cystouretrography was performed to confirm the reflux. performed. Also the Power Doppler was used to evaluate the vesicouretheral reflux.

Results and discussions:

After ultrasound, dilatation of the distal urether was observed for both cats included in the study (fig.1.).

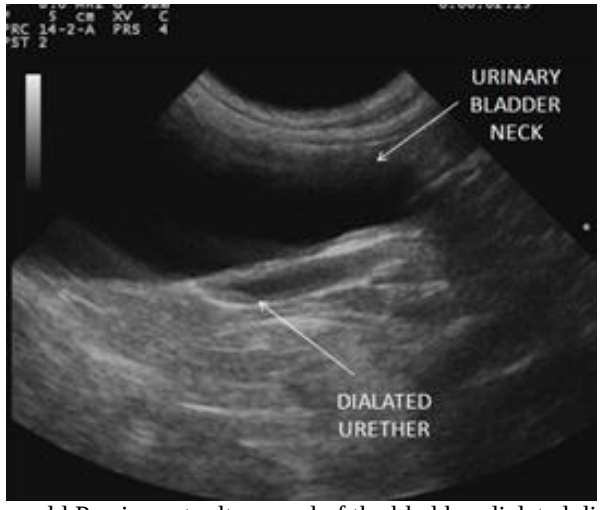
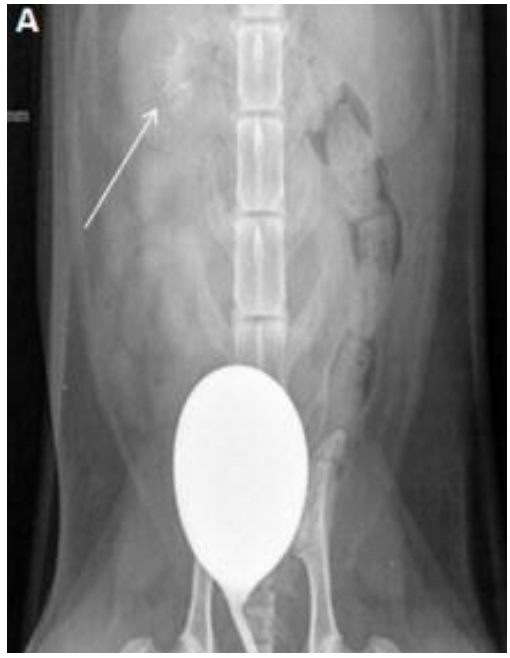


Fig.1. 5 years old Persian cat, ultrasound of the bladder, dialated distal urether

The use of the retrograde cystography revealed the filling with contrast of the affected urether, the contrast reaching also the renal pelvis (fig. 2).



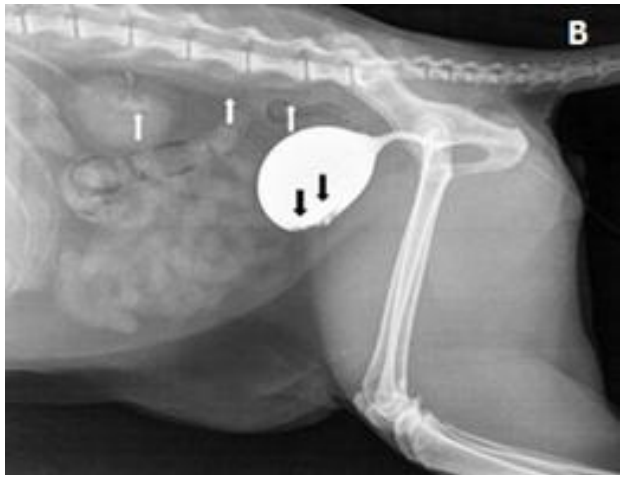


Fig.2. 13 years old neutered male cat, retrograde cystography, A -VD recumbency, the right pelvis is filled by contrast medium due to the ureteral incompetence, B-Lateral retrograde positive cystography of the same subject in the previous figure. The right pelvis and ureter are filled by contrast medium (white arrows). There is also a slight filling defects of the urinary bladder due to an initial carcinoma (black arrows).

The use of Power Doppler heplt in the identification of the uretheral jet,(fig.3).

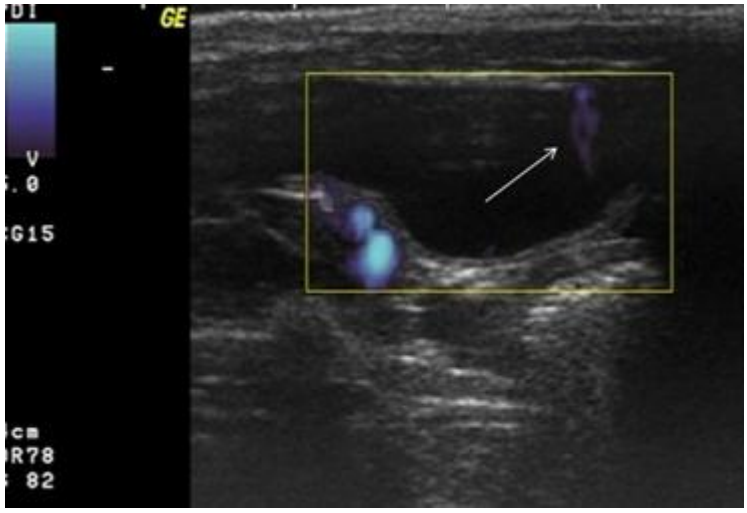


Fig.3. Power Doppler view of the uretheral jet, arrowed.

The identification of V.U.R. is very important to avoid reflux nephropathy and it's complications. To set a good diagnosis of this disease diagnostic imaging is needed.

An abdominal ultrasound might suggest the presence of V.U.R., if ureteral dilatation is present; however, in many circumstances of V.U.R. of low to moderate severity, the sonogram may be completely normal, thus providing insufficient utility as a single diagnostic test in the evaluation of the patient (Park, 1974; Feeney et al., 1983, Papadopoulou et al.2014).

Vesico-uretheral cystography (VCUG) is the method of choice for grading and initial workup. A high index of suspicion should be attached to any case where the patient presents with a urinary tract infection, and anatomical causes should be excluded. A VCUG and abdominal ultrasound should be performed in these cases

Early diagnosis is crucial as studies have shown that patients with V.U.R. who present with a UTI and associated acute pyelonephritis are more likely to develop permanent renal cortical scarring than those without VUR. Thus VUR not only increases the frequency of UTI's, but also the risk of damage to upper urinary structures.

Conclusions

The use of both ultrasonography and contrast cystography is crucial for the diagnosis of vesicouretheral reflux. It is important to diagnose vesicouretheral reflux to avoid complications like pyelonephritis or reflux nephropaty.

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MOST COMMON ORTHOPAEDIC DISORDERS IN THE FORELIMB OF YOUNG AND GROWING DOGS. DIFFERENTIAL DIAGNOSIS.

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Abstract
In this paper we describe the radiological signs of most frequent pathologies of front limbs in dogs and cats. All of them can be diagnosed through radiographic examination. In some occasions the Computed Tomography (CT) can help elucidate the diagnostic. The disorders that we describe are Panosteitis (Enostosis) and Hypertrophic osteodystrophy – primarily affecting bones – and Osteochondrosis and Elbow dysplasia – primarily affecting joints. The techniques used with conventional X-ray are also discussed. The radiologists can choose between using 2 intensifying screens (orto screens) with green light – film sensitive – and working parameters such as kV 45-60 and 2 mAs or, for elbow, knee or distal foot they can choose mammography (1 intensifying screen) and also green light – film sensitive. This gives much better quality to the image BUT they need to use 6mAs (more radiation!). It is unnecessary to use grid or Potter-Bucky for the forelimb.
Key words: *panosteitis, hypertrophic osteodystrophy, osteochondrosis, elbow dysplasia.*

The disorders primary affecting bones are panosteitis and hypertrophic osteodistrophy.

Panosteitis (enostosis) – is a self-limiting disease that affects the long bones of young large-breed dogs. Male are more affected. Very common in German Shepherd dogs. Dogs between 5 and 18 months are most often affected. The disease may be solitary or affect to multiple bones. The term of panosteitis is a misnomer because there is no evidence of an inflammatory response. Clinical signs are sudden lameness without trauma. The pain is elicited by pressure of the medullar cavity of the long bones.

X-ray: Mediolateral view of long bones. We observe an increase in the opacity (sclerosis) in the diaphysis of the long bones (humerus, ulnae, radius, femur and tibia). Often originate near the nutrient foramen. Bone involvement is often sequential and the disease may be protracted overall several months, with lesions resolving in some areas while developing in others. Sometimes it is possible to see a smooth periostal reaction of the bone.

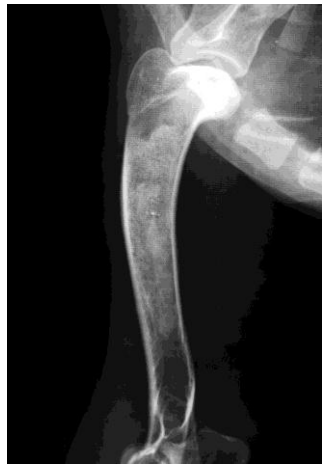

Fig 1 – Dog, M. Sclerosis of the diaphysis of the humerus, with a smoke-like appearance. Panosteitis

Hypertrophic osteodystrophy – is a systemic illness that usually affects large- and giant-breed dogs between the ages of 2 and 7 months. The cause is unknown. Clinical signs include pyrexia and bilateral symmetric limb swellings and limp. The bone disease involves the metaphysis of the long bones, particularly the distal radius, ulna, and tibia. Although the disease is usually self-limiting and resolves after a few weeks, more severe involvement can result in abnormal physeal closure and skeletal deformity.

Radiological signs appear in radius, ulna and tibia as a transversely oriented lucent zone within the metaphysis that are parallel and adjacent to the physes (appearance of "double physis" sign). It is possible to see a large sclerosis of the metaphysyses and a diffuse soft-tissue swelling centred on the metaphyseal region.

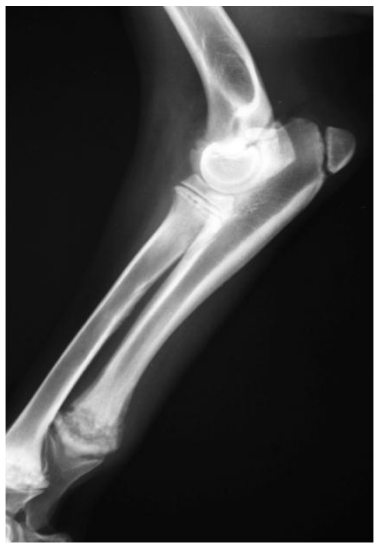

Fig 2 – Young large breed dog with double physis sign in the metaphysic, in proximal radius and more severe with thick, unsmooth radiolucent line, in distal radius and cubitus.

The disorders primarily affecting joints are osteochondrosis of the shoulder and the elbow dysplasias.

Osteochondrosis of the Shoulder – is a common cause of lameness in young, rapidly growing dogs. Clinical signs usually develop between 6 and 9 months of age. You can look for lameness and pain in the rotation of the shoulder.

It is a failure of normal endochondral ossification. When the osteochondral fragment separates from adjacent to subcondral bone should be referred to as *osteochondritis dissecans*.

Anatomical location: Caudal aspect of the proximal humeral head. It may be bilateral but only one limb with clinical signs. X-ray: Mediolateral view. If the trachea is under the head you can have a better contrast.

Except in the chondrodistrophic breeds, the head of the humerus must be spherical in the caudal aspect. In the osteocondrosis you can see a radiolucent area in the caudal aspect (osteochondrosis).

The caudocraneal view may offer another view of the lesion but in general it is not necessary. With computed tomography (CT) is possible to find very small lesions.

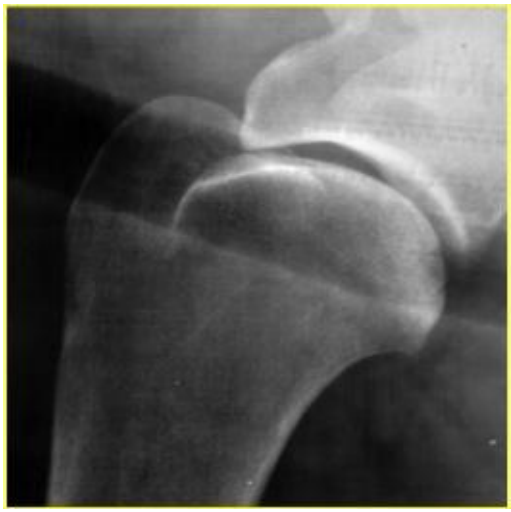

Fig. 3 – Radiolucent area in the caudal aspect of the humeral head – osteochondrosis.

The elbow dysplasia is a nonspecific term to a group of developmental lesions that include: Ununited anconeal process, fragmented medial coronoid process of the ulna and osteochondrosis of the medial humeral condyle.

You can see lameness and a painful elbow, especially in the hyperflexion, hyperextension, external rotation, or pressure in the medial aspect of the elbow**.** Be careful not to move the shoulder at the same time!

The Ununited Anconeal Process.

The anconeal process should normally be fused to the olecranon of the ulna by 150 days. It is necessary to look for a radiolucent line separating the anconeal process from the olecranon.

X-ray: A flexed mediolateral view of the elbow. This view displaces the medial epicondylar physis away from the anconeus. Additionally a craniocaudal view can help to evaluate better the elbow. CT scan is unnecessary in this case.

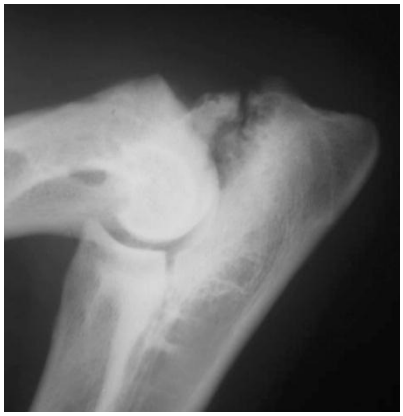

Fig. 4 – Radiolucent line on ulnar anconeal process. Sclerosis on trochlear notch of the ulna

Fragmented medial coronoid process.

This is the most common developmental disorder in the elbow joint. It affects medium and large-breed dogs with a higher influence in males. Clinical signs may be apparent as early as 4 to 6 months.

X-ray. Radiographic visualization of the coronoid fragment is usually not possible because of superimposition of the radius. Generally the diagnosis is *indirectly* made by recognising the secondary degenerative changes (osteoarthritis).

A flexed mediolateral view facilitates visualization of new bone formation on the proximal margin of the anconeal process (osteophyte). Usually this is the first x-ray sign.

A craniolateral-caudomedial view highlights the medial coronoid region and fragmented coronoid process (sometimes).

Mediolateral view facilitates the evaluation of joint congruency between humerus, ulna and cubitus. You can also see sclerosis in the trochlear notch of the cubitus.

CT is the most sensitive technique to diagnose the fragmented medial coronoid process if you have pain in the elbow but you can see anything in the x-ray. If you can not see anything is necessary and exploratory arthroscopy to evaluate the cartilage.

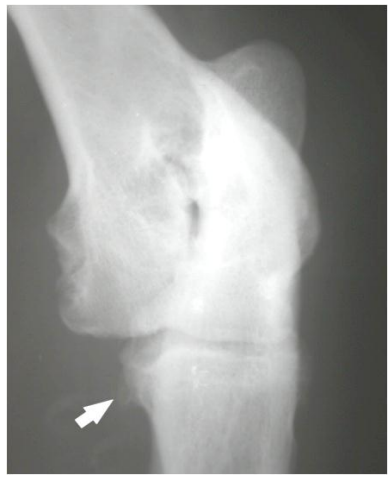

Fig. 5 –Fragmented medial coronoid process with secondary osteoarthritis on proximal aspect of the radius, medial humeral condyle and anconeal process

Osteocondrosis of the Medial Humeral Condyle**.**

This pathology really affects the distomedial aspect of the humeral trochlea, with similar signs to the other elbow diseases.

X-ray. A lucent concavity in the subcondral bone of the medial humeral condyle is seen. Sometimes you can see a fragment (flap) separated from adjacent subcondral bone (osteochondritis dissecans).

CT: Sometimes is difficult to differentiate the OC in the humeral condyle from a kissing lesion produced by the fracture of the medial coronoid process.

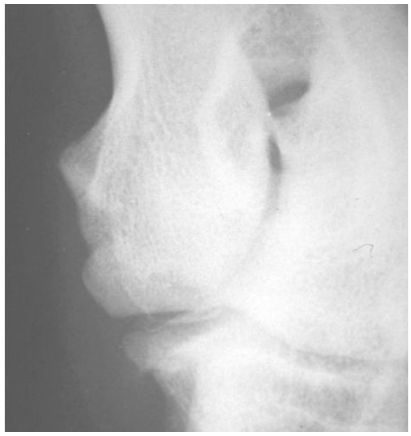

Fig. 6 – Osseous fragment separated from the subchondral bone of the medial humeral condyle. Osteochondritis dissecans in elbow

All the cases of young dogs accusing lameness should be checked out for all these affections, because one dog having a panosteitis or a hypertrophic osteodistrophy doesn't necesarely exclude having a joint disease like elbow displasia or shoulder osteochondrosis or even both. When a dog arrives limping, a thorough examination is mandatory and finding one lesion must not put an end to the radiologic exam.

CT is a valuable tool in some of these affections, most of them are well diagnosed by radiology, with less invasive tool, less expensive and quicker.

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THORACIC HANSEN TYPE I HERNIATED DISCS: RADIOLOGY VS COMPUTED TOMOGRAPHY FINDINGS.

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This paper describes two mineralized Hansen Type I hernias in a dog using digital radiography surveys and CT studies.

***Key words**: disk hernia, digital radiography, computer tomograph*

Clinical case:

A three year old teckel entire female dog, was presented 12 hours after the acute onset of pelvic limbs paresia that evolved to paraplegia four hours later. The superficial sensorial and spinal reflexes were normal. Initially the pannicular reflex was normal but after two hours it changed to absent at the spinal cord L1 segment.

A survey digital radiographic study of the spine was performed. On lateral views two mineralized intervertebral discs were seen at T11-T12 and T12-T13 levels, respectively. The intervertebral T12-T13 space was collapsed but no hernia signs could be detected at T11-T12 levels.

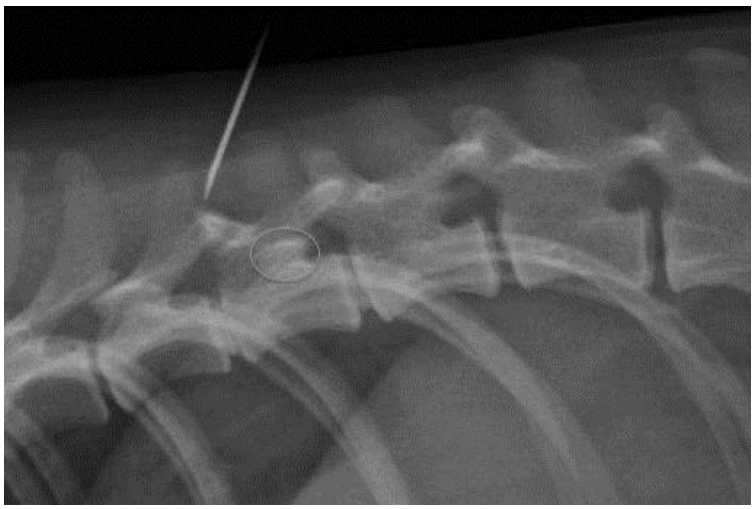

Fig1.- Left-Right lateral radiographs showing an opacity inside the vertebral canal.

A computed tomography (CT) scan of the vertebral column was then performed.

This study included thoracolumbar segments (T3-L3) and images were reconstructed using bone and soft tissue algorithms. On CT images mineralized extruded disc material (450H) was observed at T12-T13 level.

This material was placed craneal up to the middle of T13 vertebra invading out of the 80% of the vertebral canal. At T11-T12 intervertebral space a light mineralized and extruded disc material was observed at the left ventrolateral side but without medullar compromise.

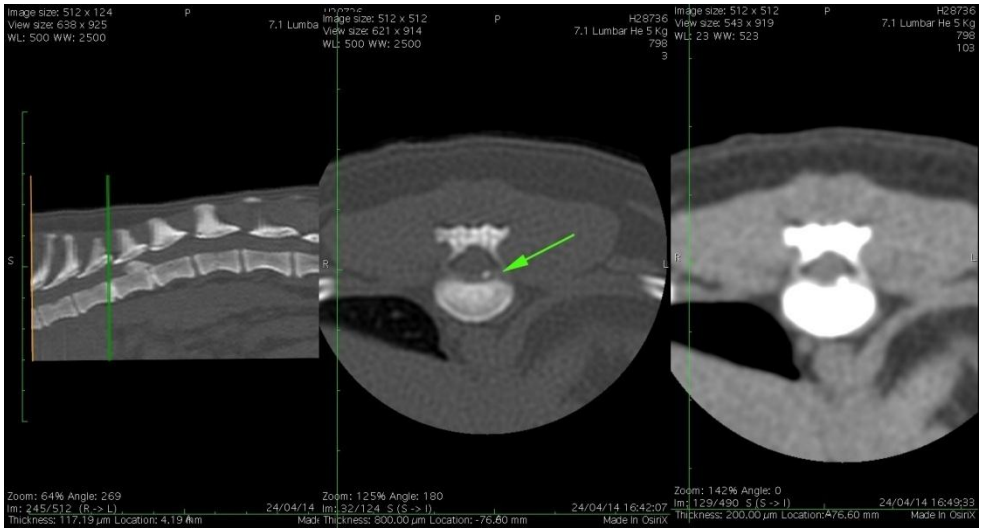

Fig 2.- Transverse, bone and soft tissue algorithms, plain CT images from case at the level of T11-T12. A small mineralization of the disc can be seen without spinal cord compromise.

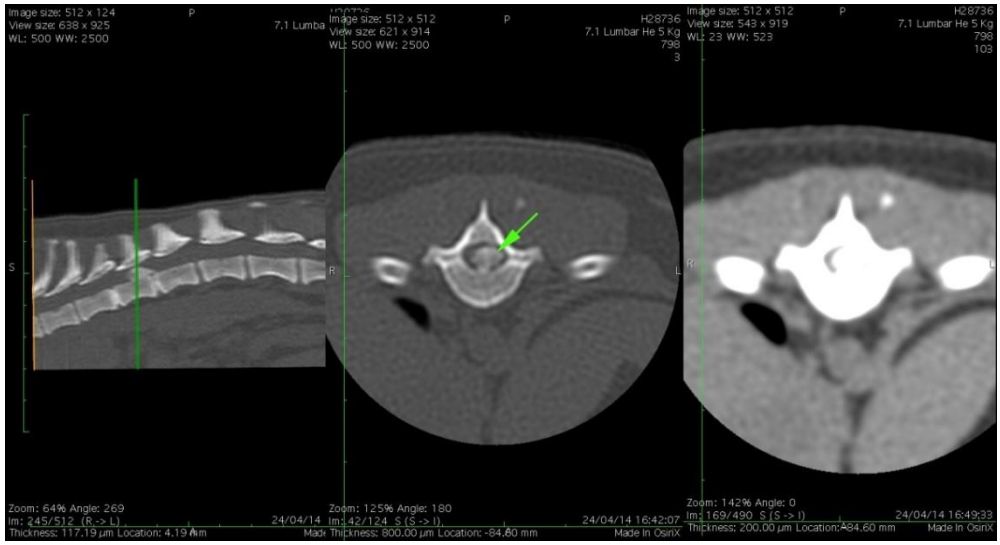

Fig 3.- Transverse, bone and soft tissue algorithms, plain CT images from case at the level of T12-T13 showing the extruded disc material inside the canal eliciting severe spinal cord compression.

Results and discussion

On plain radiographs extruded material can keep hidden. In these cases a myelography is usually needed. The use of CT provides an excellent depiction of Hansen

type 1 hernias making not necessary more invasive contrast technics such as myelography or mieloCT. On the other hand volume rendering technics provide a sensation of three-dimensionality offering a good view of the real situation inside the spinal cord and thus making surgery planning much easier.

Conclusions.

The Hansen Type I disc disease in dogs can be difficult to diagnose using survey radiographies. In the case we present only after viewing CT images, the extruded disc material could be suspected in the survey radiographs. CT studies represent a more reliable method to detect Hansen type I hernias in dogs.

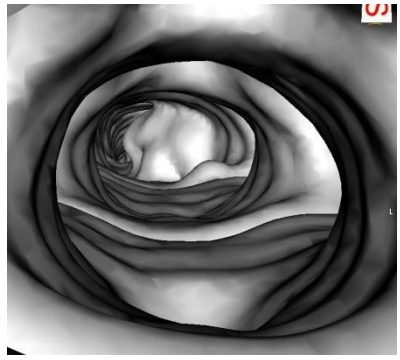

Fig 4.- Volume rendering of the vertebral canal (craneal view). The small (T11-T12) hernia on the left ventrolateral surface of the canal can be seen and behind that the huge T12-T13 hernia invading 80 % of the vertebral canal is evident.

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THE HEAD: CLINICAL CASES

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Abstract
Aims: To show specific radiographies for different pathologies. Clinical cases comparing x-rays vs. computed tomography (CT) images. Techniques. With conventional x-ray the radiologist may use 2 intensifying screens (Orto screens) with green light- sensitive film and working parameters between 5O-75 kV and 2 mAs OR a mammography film (with 1 intensifying screen), also green light- sensitive film. The last gives excellent quality but it requires an increase up to 6 mAs (more radiation!). In large-breed dogs there might be necessary to use grids or Potter-Bucky for dorsoventral or ventrodorsal views. Comparation: X-ray gives two-dimensional planes images with superposition of different structures. Computed Tomograpy (CT) gives thin slices of topographical anatomy.
Key words*: head disease, radiology*

For the nasal cavities we use an x-ray technique depending on the suspected pathology and the segment we wish to visualise. We use two orthogonal images, one right lateral and one dorsoventral or ventrodorsal view (as both give similar information).

The first one ensures a more stable position for the head, which is really important (we must use sedation or anaesthesia).

Intraoral dorsoventral view (of canine nasal cavity) – it is preferable to avoid the superposition of the jaw sowe must introduce the cassette in the mouth. (Fig 1)

Ventrodorsal view (maxilar region) - open mouth: this radiography produces a shorter nasal cavity but it is possible to see more caudal aspect of the nasal cavity and maxilla. It is also a preferred method for brachycephalic dogs.

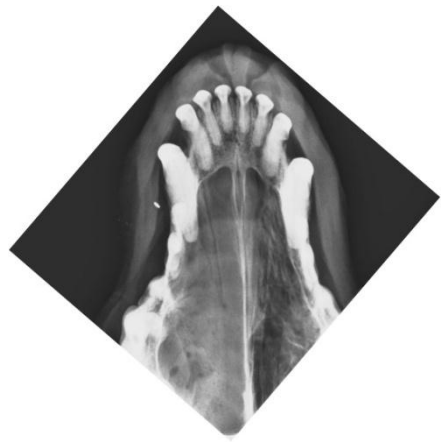

Fig 1 – Soft tissue opacity within the right nasal cavity. Dog, intraoral DV view

For the frontal sinuses, we must take the Rostrocaudal Tangential View of the Frontal Sinuses. With the head in vertical position the x-ray beam is centred dorsally to the level of

the eyes between the frontal sinuses, so it is possible to see the mass occupying the sinuses (Fig 2.)

Nasal aspergillosis is a destructive rhinitis involving the nasal cavity and paranasal sinuses of the dog. It affects younger, nonbrachyceplahlic breeds more frequently than other breeds. The most common radiographic appearance includes the lyses of conchae, increased localized soft-tissue opacity of the nasal cavity and sinus opacity. Destructive rhinitis can be difficult to differentiate from radiological point of view from neoplasia. Both diseases cause loss of conchae detail, but a nasal cavity mass with an effect of invasion over the bones surrounding the nasal cavity are more common features of nasal cavity neoplasia.

Regarding the nasal rhinitis, depending on the chronicity and severity of the rhinitis, evidence of destruction of conchae and of bony erosion may be present. Radiographic changes can range from none in mild infection to increased opacity of the nasal cavity and frontal sinuses with conchae and vomer destruction in severe infections.

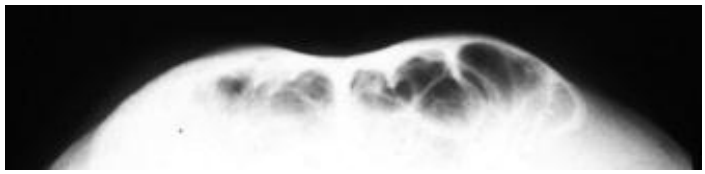

Fig 2 – Rostrocaudal view of the frontal sinuses. Fluid opacity within the right frontal sinus

For the teeth, we must take an intraoral ventrodorsal view. When it comes to dogs with large heads it is possible to have an excellent view of the incisive and some premolar teeth. Squamous cell carcinoma is frequently in this region.

Periapical infections have the appearance of a radiolucent halo around the affected root with destruction of alveolar bone. Other signs include widening of the periodontal space surrounding the apex, bone lysis or sclerosis adjacent to the apex, loss of the lamina dura and resorption of the tooth root. In dogs, infections of the fourth maxillary premolar (carnassial tooth) often result in a draining fistula below the eye. For teeth, the open-mouth view is preferred, showing better images of this area.

Craniomandibular osteopathy is a proliferative bone disease that occurs mainly in young West Highland White Terrier (Westie). On radiographic evaluation of the skull, an increased bony opacity is present in affected areas, primarily in the mandible, the tympanic bulla, and the petrous temporal bone. The changes may be symmetrical or not.

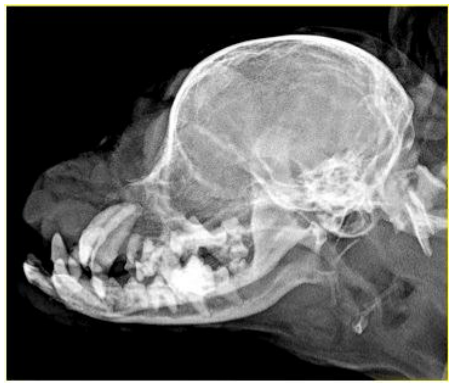

Fig. 3 – Right lateral open mouth view of a dog with lysis in the incisive and

maxilar bones – dental roots mass (neoplasia)

The temporomanbidular joint is preferably to be evaluated with a lateral view, dorsoventral or oblique views. In the case of degenerative joint disease it is possible to see osteophytes. Osseous cyst-like lesions are difficult to see with x-rays.

As for the ear, it is possible to evaluate this area with a right lateral, dorsoventral, oblique view or more specific with a rostroventral-caudodorsal oblique (open-mouth) view of the tympanic bullae. With this view it is also possible to evaluate the dens of the axis (odontoid process).

In the dorso-ventral view we may see the external ear canal stenosis and mineralization of the soft tissues. It is important to evaluate the tympanic bullae for the presence of increased opacity of thickening of the osseous bulla indicating otitis media, which is possible to see with lateral oblique view and open-mouth radiographic projections. When the disease is unilateral the diagnosis is simplified by a comparison between the two tympanic bullae.

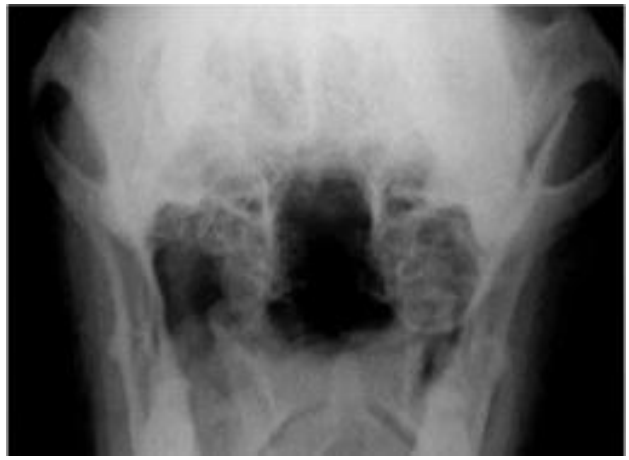

Fig. 4 – Rostrocaudal open mouth view of the timpanic bullae. Note the marked asymmetry

In the case of the calvaria and associated structures, the lateral view is the best for the evaluation of sever hydrocephalus. With horizontal x-ray beam it is possible to see level of liquid.

Regarding the hydrocephalus, doming of the calvaria and cortical thinning, persistent fontanellas and homogeneous appearance of the brain, all these are radiological signs of hydrocephalus. Still, radiographs aren't always sensitive for its detection, much more easy to see it with CT or MR imaging. Ultrasound can be used to assess ventricular size.

Occipital dysplasia is a normal dorsal extension of the foramen magnum as a result of a developmental defect in the occipital bone. Foramen magnum size and shape can be evaluated in the rostrodorsal-caudoventral skull radiograph. Patient is placed in dorsal recumbency with the neck flexed so that the nose is angled toward the sternum.

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CLINICAL ASSESSEMENT OF NON-CARDIAC THORACIC ULTRASONOGRAPHY IN DOG AND CAT

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Abstract
Currently the thoracic non-cardiac ultrasonography (US) is getting more and more used in small animal practice for the assessment of thoracic structures, not including the heart. The best results of thoracic US are obtained when performed in conjunction with thoracic radiography, giving the fact that US can see where radiographs cannot. The presence of pleural fluid that masks intra-thoracic lesions on radiographs maximizes ultrasound progression and therefore the visualization of intra-thoracic organs. US is useful to detect and determine the characteristics of pleural fluid, to visualize mediastinal, pleural and pulmonary lesions (unless underneath pulmonary aerated lobe) and to assess the integrity of the diaphragm. Ultrasound exam is also used to safely perform interventional manoeuvres. Thoracic US is limited by the barriers of aerated lung, bony thorax, and pneumothorax.
Key words: thoracic ultrasound, pleural nodules, pleural effusion, pulmonary masses.

Introduction

Until very recently the radiographic exam was considered the golden standard technique for the evaluation of the thoracic lesions. It was considered to be quick and easy to perform, easy to access and most important – it offered great contrast with the air inside lungs. On the other hand, it had some interpreting issues - low contrast resolution for soft tissues versus fluid, worsened by the pleural effusions and the impossibility to differentiate between solid and fluid lesions.

In the last few years, the CT techniques became more accessible. It had many advantages (much higher resolution contrast and volumetric detail, plus it made it possible to perform interventional manouvres) but it was and it still is expensive, not easy to perform and it requires anaesthesia (1).

The CT is not widely used, it has a much higher radiation dose so it is used only in complicated cases, this is the reason why it became the "second level technique" as the radiography became "first level technique".

The ultrasonography is the technique that provides real time sectional anatomy of body structures. It uses the wave's propriety to better propagate through fluids and water rich tissues so it is particularly useful for soft tissues studies(5).

So it is clearly that the ultrasonography can help where the radiography is limited – in differentiating the soft tissue masses from fluid ones, as long as bones, air and gases don't interfere with waves propagation.

The so called "lung paradox" states that normal lung parenchyma is not evaluable through ultrasonography, the only time you can actually see the lung is when there is a pathology that fills the alveoli or the pleural space with fluid/soft tissue masses (6).

The technique indicates the hair to be clipped and a coupling gel to be used. Generally intercostal, parasternal and subcostal windows are used, in the last one using the liver as acoustic window. The patient can be put in dorsal, or sternal recumbency if the condition indicates, the lateral recumbency is not recommended as it may induce

physiological changes in the lung – decubital atelectasis – that cannot be differentiated from pathological changes.

If the patient is not cooperative or the disease doesn't allow to stay in dorsal decubitus, it can be examined standing (6).

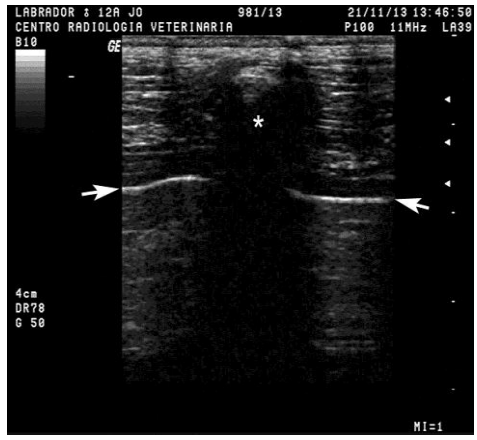


Fig 1 –pleural hyperechoic interface.

Matherial and method

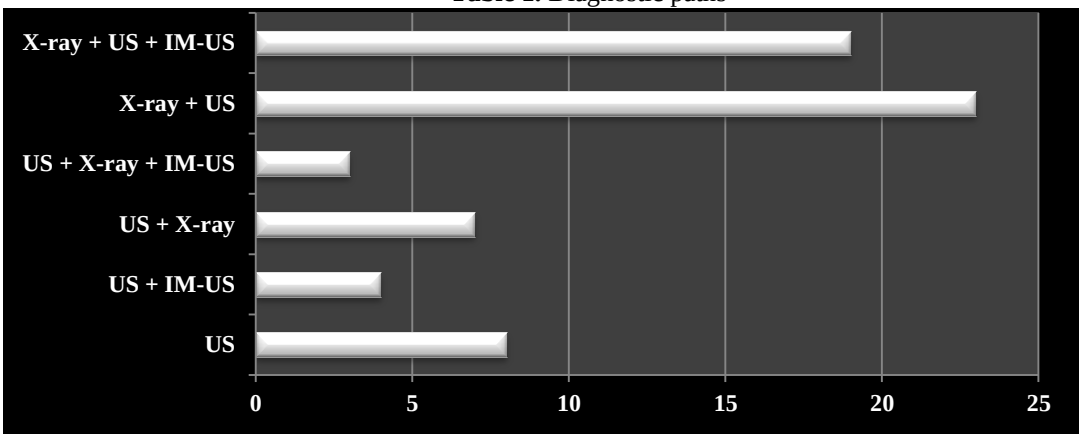
In the present study, a series of 64 subjects (39 dogs an 25 cats) were retrospectively reviewed from the database of the Interdepartmental Centre of Veterinary Radiology of Naples. All the subjects underwent at least one ultrasound exam of the thorax, often in conjunction to a radiographic and/or a CT study. All the studies were analysed and, based of the information furnished by US compared to radiography and/or CT, they were classified as "not important", "important", and "very important". On that basis, in 8 cases US was considered as being "not important", in 20 cases "important", and in 36 cases "very important".

Results and discussions.

This was a retrospective study made from the Interdepartamental Radiology Centre Database. The inclusion criteria were: 64 dogs and cats submitted to non-cardiac thoracic ultrasonography, alone or associated with radiographic exam, from January 2003 until December 2013. For all the cases the following data were recorded: species, sex, exam performed (US or US+R), diagnostic paths and the execution of interventional manoeuvres.

Based on the provided findings, we classified the US as "not important" – it didn't provide any new information, "important " – it provided information but not the diagnostic, and "very important" – it provided fundamental information for further diagnostic.

Table 1. Diagnostic paths



In 22 cases ultrasonography (US) was the first exam

In 42 cases radiography (X-ray) was the first exam

In 26 cases interventional manouvres ultrasound-guided (IM-US) were performed.

The "not important" information were collected from 8 cases – 7 diaphragmatic hernias and one thymoma suspicion – for the diaphragmatic hernias the diagnosis were already made by radiography, and the ultrasound was made only to see which organs had herniated. The owner of the dog refused other diagnostic procedures so the cranial mediastinal mass wasn't aspirated. By location and radiological and US appearance it was compatible with a thymoma (2).

The US was considered "important" in 20 cases. In 2 cases US-guided fine needle aspiration (FNA) was performed. The information in those cases was considered important but only in two cases the diagnostic was made – a lymphoma and an endo-pericardium lipoma. It was considered important because it helped the diagnostic but the radiograph was the main imagistic tool that first identified the condition.

The "very important" category had 36 cases. In 24 of them there were performed interventional manoeuvres. This category included the cases that came for a totally different exam – abdominal ultrasound, and during this study the operator observed through the hepatic window the presence of lesions in the thoracic cavity – pleural effusion, diaphragmatic or pleural nodules (4).

In those cases the ultrasound was the most useful tool in the diagnostic process, leading to the diagnostic of thoracic pathologies like haemothorax, lung abcess, lung carcinoma, chylothorax, foreign body, FIP granuloma, lymphoma (1 thymic), lipomas (one endopericardial), lung metastasis, pyothorax, suspect lung methastasis and thymoma (3,4).

Almost all the cases in which US were classified as "not important" were represented by diaphragmatic hernias. US was considered "important" or "very important" when visualizing lesions not visible on radiographs, as pleural lesions or lung nodules adjacent to the thoracic wall or better characterized lesions that were already visible on radiographs, in distinguishing solid versus fluid lesions. In 26 cases, the US exam was completed by an interventional procedure (fine needle aspiration, biopsy, thoracocentesis etc.).

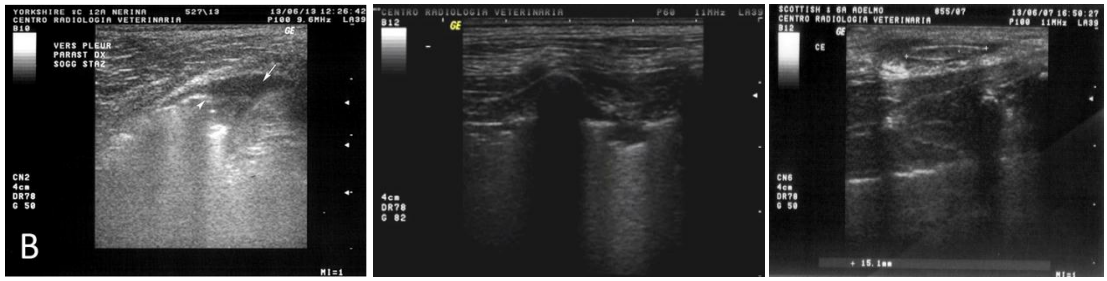


Fig. 2 – A-fluid pocket seen in the right costo-phrenic aspect – pleural effusion. B-small hypoechogenous subpleural nodule - metastasis. C-migrating grass awn and small fluid pocket.

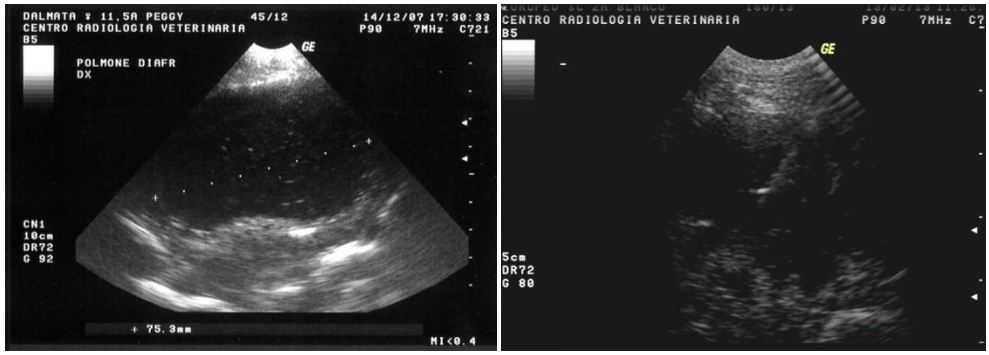


Fig. 3 – A-big fluid pocket with twinkle appearing – cells within fluid, abscess. B-mediastinal mass and US guided FNA – thymic lymphoma.

The most important aspect of this work is the fact that none of the patients came directly for thoracic noncardiac ultrasound. They were either sent to further investigations (US or US-IM) due to abnormal observations during radiographic examination or to abdominal US and during this procedure abnormalities were found within the pleural space thus transforming the abdominal examination into a thoracic one.

In this last situation there were the cases in which the US was considered to be "very important". They were the cases with general signs like fever of unknown origin, anorexia and weight loss and some digestive signs like regurgitation, so no particular sign of thoracic illness.

The pathologies thus discovered were severe ones and the US was the main tool in the diagnostic procedure.

Conclusions

1. Only a few years ago the ultrasound was considered manly a cardiac evaluation tool, nowadays it is a complementary tool to radiography and CT.
2. Our results confirmed that ultrasound exam is a valuable tool in studying the characteristics of the thoracic wall, pleural effusion, pulmonary and mediastinal lesions in dogs and cats. Moreover, it is essential when interventional manoeuvres are considered.
3. However it is still difficult to perform it if lesions are located beyond acoustic thresholds (hair, aerated lung, bones).
4. Considering that US technique is not detrimental for the patient nor for the

operator, it's not expensive, not stressful for the dyspnoeic patient, it usually doesn't require sedation or anaesthesia and, last but not least, it permits to perform interventional manoeuvres, we strongly recommend to add an ultrasound exam to the radiographic and/or CT studies of the thorax in dog and cat.

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HEMATOLOGICAL, BIOCHEMICAL AND MICROBIOLOGICAL STUDIES IN DOGS TREATED WITH ERYTHROMYCIN 10%

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Abstract

This paper aimed to highlight changes in erythrocytes, leukocytes and biochemical parameters in clinically healthy dogs treated with erythromycin 10% administrated by the intramuscular route. We also followed the behavior of intestinal microbial flora to the treatment with erythromycin. For the experiment were used three groups of dogs treated with various doses of erythromycin 10%. Administration were made daily for three consecutive days. Onwards and in day 3 of treatment blood samples were collected for hematological and biochemical analysis. From these groups strains of Staphylococcus spp., Streptococcus spp., Bacillus cereus, Arcanobacterium spp. and Escherichia coli were isolated after the treatment. These strains were tested for resistance to erythromycin. The results of erythrocyte parameters showed statistically insignificant results ($p \geq 0,05$) in group that receiving double dose of erythromycin in 3 days after the first administration. Mean leukocyte parameters were slightly increased during treatment but had no significant values between the two sampling and compared to the control group. Biochemical profile in dogs treated varied with significant mean values ($p < 0.05$) only in aspartate aminotransferase and alkaline phosphatase between the two blood sampling. In case of the other erythrocyte, leukocyte and biochemical values, oscillations were insignificant ($p \geq 0,05$). Erythromycin resistance was found in only 7.14% of the strains of staphylococci and 20% to those of E. coli. In strains of Streptococcus spp. Bacillus cereus and Arcanobacterium spp. no resistance was observed. The results from tests with erythromycin show that this product may change some hematological and biochemical evolution without their clinical expression. Due to good antimicrobial efficiency of this product, we recommend its use in microbial diseases (especially Gram-positive bacteria) in dogs where these types of microorganisms are involved.

Key words: *erythromycin, dog, hematology, biochemistry, antibiotic resistance*

Introduction

Antimicrobial resistance is a very complex issue involving different species of bacteria, mechanisms of resistance and transfer of resistance mechanisms. Several studies have shown that uncontrolled use of antibiotics in animals contribute to the selection of strains with antimicrobial resistance, thus presenting a risk for other animals and humans (Guardabassi et al., 2004; Kruse 1999).

Microbial resistance in macrolids gradually installs especially after repeated and prolonged treatments. This resistance is due to exposure to the antibiotic and is plasmid mediated. The emergence of resistance occurs due to active transport system deficiency or decrease membrane permeability, which makes the antibiotic concentrations not high enough in bacterial cells. Plasmids can also be carriers of genes responsible for resistance to other antibiotics - aminoglycosides, chloramphenicol, sulfonamides. Plasmids can be transferred to other bacteria by conjugation or transduction phenomenon (Weisblum, 1995).

This paper is aiming the haematological and biochemical status evaluation in dogs treated with 10% erythromycin and erythromycin susceptibility assessment of bacterial strains isolated from dogs.

Material and methods

The experiment was conducted on three groups of dogs grouped as follows:

- Group 1 (n = 5) - dogs that received subcutaneous (SC) for 3 days in daily dose of 1 ml/10 kg body weight;
- Group 2 (n = 5) - dogs that received SC for 3 consecutive days, the daily dose of 2 ml/10 kg body weight;
- Group 3 (n = 5) - dogs that received SC for 3 consecutive days, the daily dose of 2 ml/10 kg 0.85% saline solution;

Clinically healthy dogs of common breed were weighing between 10 and 35 kg and aged between 2.5 and 5 years.

Medication management protocol respected the following steps:

Day 0, before administration of the medicinal product and included:

- Supervision of animal behavior and observing food and water consumption in groups;
- clinical examination.
- blood sampling for hematologic and metabolic profiling;

Day 1 the administration of the medicinal product, including:

- clinical examination;
- administration of the first dose depending on the lot;

Day 2 second administration of the medicinal product, including:

- clinical examination;
- doses readministration depending on the group;

Day 3 third administration of the medicinal product, including:

- clinical examination;
- doses readministration depending on the group;

Day 4, final examination (post-treatment) were made in 10 to 24 hours after the last administration of the products and consisted of clinical, haematological and biochemical tests.

In terms of hematological parameters the following erythrocyte parameters were investigated: Hematocrit, Hemoglobin, RBC, MCV, MCH, MCHC and the following leukocyte parameters: leukocytes, neutrophils, eosinophils, basophils, lymphocytes, monocytes. Biochemical parameters investigated in groups of dogs were: AST - aspartate aminotransferase; ALP- alkaline phosphatase; CK - creatine phosphokinase, CA - calcium; PHOS - phosphorus; TP - total protein; GGT- glumatil gamma-transferase; BUN- blood urea nitrogen; ALB - albumin; GLOB - globulin; Mg - magnesium.

All groups were kept under clinical observation for 10 days after completion of administrations.

On all groups, protocol was conducted after the steps described above.

Testing for resistance to erythromycin 10% of bacterial species was achieved by isolating strains from groups treated with erythromycin and testing by disc diffusion method. 5 "American Type Culture Collection" (ATCC) were also tested.

Statistical evaluation of haematological and biochemical test results was done by calculating the mean, standard deviation and statistic correlation index by T-Test.

Results and discussions

Following the tests conducted with erythromycin 10% in group 1 it can be seen that between individual values and the average pre-administration parameters and post-therapeutic treatment we found no statistically significant differences ($p > 0.05$).

Evolution of red blood cell parameters are presented in Table 1, where it can be seen

that the mean values of hematocrit and hemoglobin were within the physiological limits, post treatment hematocrit evolution was ascendent, whereas hemoglobin decreased slightly after the treatment. For the total number of erythrocytes, the mean values recorded were within physiological limits ante and post therapeutic treatment. Mean erythrocyte constants showed less significant average levels fluctuations compared to the mean reference values, within physiological limits for this specie.

Table 1. Mean erythrocyte parameters for Erythromycin 10% in dose of 1 ml /10 kg

Sample nr.	Hematocrit	Hemoglobin	Erytrocytes	Mean erythrocyte values		
	(%)	(g/dl)	(T/l)	VEM/(fl)	HEM(pg)	CHEM(g/dl)
Ante-therapeutic						
Mean	41.45	16.01	6.81	68.70	23.02	32.76
Standard deviation	4.62	1.95	1.18	3.65	0.59	2.03
Post-therapeutic						
Mean	42.60	15.89	6.86	69.80	23.94	33.06
Standard deviation	0.50	0.80	0.75	5.94	0.94	1.01
R[1]	*37-55*	*12.0-18.0*	*5.50-8.50*	*60-77*	*19.5-24.5*	*31-34*

R[1] Ghergariu et al., 1999.

In case of *leukocyte parameters*, mean values decreased after the administration, post-treatment from the average value of 12,16 g/l. In neutrophils was observed a decreased after treatment with erythromycin, from initial average value of 62.4 ± 4.93 up to an average of 56.4 ± 12.07%, both values were within normal limits for the target species. The average values of eosinophils did not exceed the upper limit of reference. Similar lymphocyte population showed minor variations, within the physiological limits. For monocytes population, slightly elevated values ante and post-therapeutic were observed, values that have exceeded physiological specie limit (Table 2).

Table 2. Mean values of leukocyte parameters registered for erythromycin 10% 1 ml / 10 kg

Sample nr.	Leucocytes	Leucocitary foormula (%)				
	(G/l)	Neutrophils	Eosinophils	Basophils	Limphocytes	Monocytes
Ante-therapeutic						
Mean	14.08	62.40	4.40	0.40	22.20	10.60
Standard deviation	4.65	4.93	2.51	0.55	1.92	3.97
Post-therapeutic						
Mean	12.16	56.40	5.20	0.40	28.20	9.80
Standard deviation	1.81	12.07	1.64	0.55	9.65	2.59
R[2]	6.0-17.0	60-70	2.0-10.0	0	12.0-30.0	3.0-10.0

R[2] Mehner et al., 1983; Wallach et al., 1983.

Blood biochemical parameters in dogs of group 1 showed no significant variations ante and post-treatment. In albumin there was a slight increase post-treatment but within physiological limits for dogs. For aspartate aminotransferase, gamma-glumatil transferase, total proteins, globulins and urea nitrogen, calcium, magnesium and phosphorus the values were within normal limits corresponding species. Creatine phosphokinase was over the reference values both ante and post treatment (Table 3).

Table 3. Mean blood biochemical parameters for administration of erythromycin 10% in dose 1 ml/10 kg

Nr.	ALB	ALP	AST	CA	GGT	TP	GLOB	BUN	CK	PHOS	MG
Sample	(%)	(U/l)	(U/l)	(mg/dl)	(U/l)	(g/dl)	(%)	(mg/dl)	(U/l)	(mg/dl)	(mg/dl)
Ante-therapeutic											
Meana	3.46	79.60	35.00	10.30		5.66	3.26	21.20	154.80	4.52	2.00
Standard deviation	0.26	18.32	4.30	1.41		0.49	0.36	3.90	85.00	0.95	0.22
Post – therapeutic											
Mean	3.58	56.20	37.80	10.62		6.18	3.36	23.20	131.00	4.94	2.10
Standard devation	0.33	19.69	3.42	1.70		0.77	0.46	3.96	86.14	1.15	0.35
Reference values	*2.6-4.0*	*10.6-101*	*8.9-49*	*8.7-11.8*	*1.0-9.7*	*5.5-7.5*	*2.1-3.7*	*8.8-26*	*14-120*	*2.9-6.2*	*1.7-2.7*

AST - aspartate aminotransferase; ALP- alkaline phosphatase; CK - creatine phosphokinase; CA - calcium; PHOS - phosphorus; TP - total protein; GGT- glumatil gamma-transferase; BUN- blood urea nitrogen; ALB - albumin; GLOB - globulin; MG - magnesium-

In group 2 treated with erythromycin 10% dose 2 ml/10 kg has been observed that erytrocyte, leukocyte and biochemical parameters variations were nonsignificant between collections, being classified within physiological limits as shown below in Tables 4.5 and 6.

Table 4. Average values of red blood cell parameters for erythromycin 10% in dose of 2 ml/10 kg.

Sample nr.	Hematocrit	Hemoglobin	Erytrocytes	Mean erythrocyte constants		
	(%)	(g/dl)	(T/l)	VEM/(fl)	HEM(pg)	CHEM(g/dl)
Ante-therapeutic						
Mean	41.00	15.39	8.33	66.60	23.48	33.57
Standard deviation	5.83	1.59	1.00	4.66	0.60	1.64
Post-therapeutic						
Mean	42.28	15.18	8.01	69.40	23.17	33.75
Standard deviation	4.39	1.85	1.12	4.93	1.04	0.75
R[1]	*37-55*	*12.0-18.0*	*5.50-8.50*	*60-77*	*19.5-24.5*	*31-34*

Table 5. Mean leukocyte parameters for erythromycin 10% in dose of 2 ml/10 kg

Sample nr.	Leucocytes	Leucocitary formula (%)				
	(G/l)	Neutrophils	Eosinophils	Basophils	Limphocytes	Monocytes
Ante-therapeutic						
Meana	9.41	64.80	5.80	0.20	22.20	7.00
Standard deviation	2.73	3.63	3.42	0.45	1.79	3.00
Post-therapeutic						
Mean	10.41	59.80	4.80	0.40	26.60	8.40
Standard deviation	3.20	2.17	1.10	0.55	3.21	1.14
R^2	*6.0-17.0*	60-70	2.0-10.0	0	12.0-30.0	3.0-10.0

Table 6. Mean values of blood biochemical parameters for erythromycin 10% in dose of 2 ml/10 kg

Nr.	ALB	ALP	AST	CA	GGT	TP	GLOB	BUN	CK	PHOS	MG
Sample	(%)	(U/l)	(U/l)	(mg/dl)	(U/l)	(g/dl)	(%)	(mg/dl)	(U/l)	(mg/dl)	(mg/dl)
Ante-therapeutic											
Mean	3.50	98.20	39.00	9.18		5.70	2.78	15.00	118.60	6.02	1.94
Standard deviation	0.31	7.05	4.00	1.03		0.54	0.36	3.00	17.42	0.90	0.19
Post – therapeutic											
Mean	3.86	101.40	41.40	9.74		6.30	2.86	15.00	108.60	6.12	2.04
Standard deviation	0.55	4.62	2.07	1.30		0.66	0.45	3.39	24.91	0.79	0.29
Reference values	*2.6-4.0*	*10.6-101*	*8.9-49*	*8.7-11.8*	*1.0-9.7*	*5.5-7.5*	*2.1-3.7*	*8.8-26*	*14-120*	*2.9-6.2*	*1.7-2.7*

In control group 3 were obtained the following results shown in tables 7, 8 and 9.

Table 7. Mean erythrocyte parameters for the administration of saline solution.

Sample nr.	Hematocrit	Hemoglobin	Erytrocytes	Mean eryrocyrte values		
	(%)	(g/dl)	(T/l)	VEM/(fl)	HEM(pg)	CHEM(g/dl)
Ante-therapeutic						
Mean	38.90	12.71	6.66	65.30	22.30	32.17
Standard deviation	1.90	1.70	0.88	3.91	1.27	1.19
Post – therapeutic						
Mean	37.90	12.90	7.12	67.70	22.34	32.28
Standard deviation	0.73	1.22	0.59	5.43	1.72	1.14
R^1	*37-55*	*12.0-18.0*	*5.50-8.50*	*60-77*	*19.5-24.5*	*31-34*

Table 8. Mean leukocyte parameters for the administration of saline solution.

Sample nr.	Leucocytes	Leucocitary formula (%)				
	(G/l)	Neutrophils	Eosinophils	Basophils	Limphocytes	Monocytes
Ante-therapeutic						
Mean	13.08	65.40	5.60	0.40	20.20	8.40
Standard deviation	5.71	14.50	5.46	0.55	7.46	3.85
Post – therapeutic						
Mean	12.31	61.20	4.60	0.60	25.00	8.60
Standard deviation	4.06	12.32	2.97	0.55	8.92	2.61
R^2	6.0-17.0	60-70	2.0-10.0	0	12.0-30.0	3.0-10

Table 9. Mean blood biochemical parameters for the administration of saline solution

Nr.	ALB	ALP	AST	CA	GGT	TP	GLOB	BUN	CK	PHOS	MG
Sample	(%)	(U/l)	(U/l)	(mg/dl)	(U/l)	(g/dl)	(%)	(mg/dl)	(U/l)	(mg/dl)	(mg/dl)
Ante-therapeutic											
Mean	3.20	80.00	32.20	10.46		6.26	2.88	18.80	107.40	6.18	2.16
Standard deviation	0.25	8.94	6.06	0.91		0.73	0.43	2.59	13.90	0.72	0.18
Post – therapeutic											
Mean	3.28	84.80	34.40	10.36		6.38	2.88	19.00	111.20	5.22	2.10
Standard deviation	0.26	8.35	1.82	1.09		0.59	0.41	2.55	16.15	1.11	0.14
Reference values	*2.6-4.0*	*10.6-101*	*8.9-49*	*8.7-11.8*	*1.0-9.7*	*5.5-7.5*	*2.1-3.7*	*8.8-26*	*14-120*	*2.9-6.2*	*1.7-2.7*

Overall analysis of the data resulting from statistical processing parameters of differences between mean values with large oscillations are presented in Table 10 for dogs, reveals that vast majority of monitored parameters by haematological and biochemical tests, showed values within species limits, confirming the absence of any kind of side effects, general or local in the animals investigated.

Table 10. Statistical significance of differences in mean values reported for lymphocytes and monocytes pre- and post-treatment

Group	Parameter (ante-/post-therapeutic)		p	Significant/nonsignificant
1	Limphocytes	G/l	0.0945	p≥0,05, statistical insignificant
	Monocytes	%	0.4264	p≥0,05, statistical insignificant
2	Limphocytes	G/l	0.7651	p≥0,05, statistical insignificant
	Monocytes	%	0.0875	p≥0,05, statistical insignificant
3	Limphocytes	G/l	0.5260	p≥0,05, statistical insignificant
	Monocytes	%	0.4698	p≥0,05, statistical insignificant

Testing the resistance of microorganisms to treatment with erythromycin 10% showed that the phenomenon of resistance of microorganisms to erythromycin had no marked trend against microbial agents isolated from the groups studied.

In support to this, are of major importance the aspects presented in tables 11 and 12 which show data obtained from tests of resistance to erythromycin of bacterial strains isolated from dogs and the standard ATCC strains.

Table 11. Erythromycin resistance of strains tested

Tulpini testate	Resistant (nr.)	Moderate sensitive (nr.)	Sensitive (nr.)	Total tested strains (nr.)	Resistant (%)	Moderate sensitive (%)	Sensitive (%)
Staphylococcus intermedius	1	3	8	**12**	8,33	25	66,66
Staphylococcus spp.	1	5	10	**16**	6,25	31,25	62,5
Streptococcus spp.	0	1	9	**10**	0	10	90
Listeria spp.	0	1	5	**6**	0	16,66	83,33
Bacillus cereus	0	1	4	**5**	0	20	80
Arcanobacterium pyogenes	0	1	3	**4**	0	25	75
Pasteurella multocida	1	1	6	**8**	12,5	12,5	75

It is thus observed for *Staphylococcus intermedius* a resistance of 8.33% and a sensitivity of 66.66% of the strains tested. Out of the 10 strains of *Staphylococcus spp*., a percentage of 62.5% were sensitive to erythromycin, 31.25% were moderate sensitive and 6.25% were resistant. In streptococci, from 9 strains tested none was resistant, 90% sensitive and 10% moderate sensitive to erythromycin. From the strains of *Listeria spp*. none was resistant 16.6% were instead classified as moderate susceptible and 83.33% were susceptible to erythromycin. A similar situation was observed for *Bacillus cereus* where there were no strains resistant to erythromycin. In *Arcanobacterium pyogenes* strains have been found a number of sensitive strains of 75%, 25% moderate sensitive and no resistant strain. *Pasteurella multocida* strains had 12.5% resistant strains, and a sensitivity of 75% of them.

An overall assessment of the results obtained for erythromycin resistance can that from 7 bacterial types tested only three resistant strains have been reported ie *Staphylococcus intermedius, Staphylococcus spp*. and *Pasteurella multocida* and four other species (*Streptococcus spp. , Listeria spp., Bacillus cereus, Arcanobacterium pyogenes*) have not been found resistant strains. Resistance variation was between 6.25% and 8.33% in staphylococci (for Gram-positive) and 12.5% of Gram-negative bacteria. Sensitivity of strains tested ranged between 62.5% and 90%. Percentage of moderate susceptible strains varied between 10 and 31.25%.

Erythromycin resistance was tested in laboratory conditions and for 5 different species of bacterial strains, from the standard strains from American Type Culture Collection. Laboratory results obtained and processed by CLSI standards - 2009 revealed that only strains of *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 6538P were classified as moderate susceptible while others were resistant to erythromycin.

Comparing the data obtained by us on erythromycin resistance of pathogens isolated from animals with other data obtained by other researchers in the field on pathogens isolated from mammals in various areas, the resistance levels obtained in the tests are considered satisfactory. In this context we mention some results obtained by some researchers in the

country, a study of 89*Staphylococcus aureus* strains isolated from north eastern Romania in 2006 were tested by determining resistance to erythromycin using MIC. There has been resistance to this product at a rate of 19%, being slightly higher as determined by our current studies (Tudor, 2008).

Table 12. Erythromycin resistance for American Type Culture Collection strains *(ATCC)*

Antibiotic	*Bacillus cereus ATCC 14579*	*Pseudomonas aeruginosa ATCC 27853*	*E. coli ATCC10536*	*Salmonella enteritidis ATCC 13076*	*Staphilococcus aureus 6538P*
Eritromycin (15µg)	15mm (I)	0 (R)	0 (R)	0 (R)	20mm (I)

S-susceptible; I-moderate sensitive; R-resistant;

Other studies conducted in Belgium on 33 strains of *Streptococcus gallolyticus* isolated from birds showed a 45% erythromycin resistance (De Kimpe et al., 2002). A larger study of 95 strains of *Staphylococcus aureus* isolated from animals were tested in terms of sensitivity to antibiotics in Argentina where there was a resistance of these strains to erythromycin of 2.1% (Russi et al. , 2008).

Studies in the United States on 110 strains of staphylococci isolated from chickens, prove that for this specie erythromycin resistance rate is much higher, reaching 71%. Tests performed on 90 strains of enterococci isolated from poultry in the United States showed a high percentage of resistance to erythromycin, which is 50% (Shabbir et al 2007).

It can thus be seen that the resistance of microorganisms to antibiotics is different depending on bibliographical studies, the studied area and microbial strains and species of animal from which the organisms were isolated.

Conclusions

Based on the overall analysis of results obtained from haematological and biochemical tests of erythromycin 10% following administration at a dose of 1ml/10kg and 2 ml/10 kg was found that erythromycin did not have any type of general or local side effects.

The study reveals that erythromycin resistance is still low in most micro-organisms and that it varies from one bacterial specie or strain to another. We can estimate that for situations involving Gram-negative bacteria as *E. coli* and *Pseudomonas aeruginosa* should be considered more carefully, erythromycin administration being only recommended when the outbreak strains were sensitive to this product.

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THERAPEUTIC STRATEGIES OF UROLITHIASIS IN MALE GOATS

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Abstract

Urolithiasis is the most economically significant urinary tract disease of goats, especially struvite uroliths (magnesium ammonium phosphate). Male small ruminants are particularly predisposed, whereas females are rarely clinically affected. The purpose of the current study was to investigate the therapeutic strategies of urolithiasis in male goats. Ten naturally-occurring cases of urolithiasis in male goats had been presented for treatment at the clinics between 2007 and 2014. All animals were between 6 and 18 months of age and weighing (15–45 kg), from the North-East of Romania. Clinical signs depended on the degree of obstruction, location of obstruction, and duration of disease. Most cases of obstructive urolithiasis required surgical intervention at some point during the treatment process. Identifying the urethral obstruction location was necessary to ensure proper incision placement. The distal penis was exteriorized from the sheath by an assistant, the urethra was incised and a retrograde urinary catheter introduced into the urethral orifice. Fluid, anti-inflammatory, antibiotic, and acidifying therapies were used in support of surgical intervention. Surgical technique was chosen based on the characteristics of the individual case, including site of obstruction and value of the animal. Prevention remains the mainstay of urolithiasis management.

Keywords: *male goat, therapy, urethral obstruction process, urolithiasis*

Introduction

Urolithiasis is the most important urinary tract problem of domestic animals, especially struvite uroliths (magnesium ammonium phosphate, MAP) in feedlot cattle, sheep, goat, cats and dogs. Urolithiasis due to MAP crystal formation is a common condition of young male ruminants (George et al., 2007). Obstructive urolithiasis is a well recognized, highly prevalent, and costly disease of small ruminant population. Diet and animal management are considered important risk factors in the formation of uroliths in ruminants (Kahn et al., 2005, George et al., 2007, Janke et al., 2009). If the animal is diagnosed with urolithiasis pursuant of medical therapy depends on the severity of the disease, the stage of the disease, the nature and extent of the uroliths, the intended long term use of the animal, the frequency of the disease, and most importantly the financial constraints of the owner. The purpose of the current study was to investigate the therapeutic strategies of urolithiasis in male goats.

Materials and method

Ten naturally-occurring cases of urolithiasis in male goats that had been presented for treatment at the clinics of Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine Iaşi between 2007 and 2014 were identified. All animals were between 6 and 18 months of age and weighing (15–45 kg), from the North-East of Romania. All of the male goats had been fed concentrates, although the type and quantity were not recorded and one had been fed commercial forage. Water had been provided ad libitum. The protocol of the study included clinical exams, urinalysis, leukogram, serum biochemistry, ultrasonography, medical and surgical treatment. Ultrasonographic imaging was performed on goats in standing position using Aquila Esaote ultrasonographic apparatus with 5MHZ

linear array probe. To visualize the right kidney longitudinally, the transducer was placed behind the last rib. To visualize the left kidney longitudinally, the transducer was placed parallel to the 3rd lumbar vertebrae. The echogenicities of renal cortex, medullary pyramids and renal sinus were assessed. The kidney length and width, cortex thickness, medullary thickness and renal sinus length and width were evaluated.

Results and discussion

Certain signalment and historical data can lend important clues for evaluation of specific causes. For example, urethral calculi should be suspected immediately in castrated ruminants on high grain diets. (Smith, 2009). In our study, details of the duration of clinical signs (as reported by the owners) ranged from 1 to 52h. Clinically, they were presenting simptoms as: anorexia, depression, mild bloat (secondary to ruminal stasis), restless, swish their tails, grind their teeth, stranguria, tenesmus, colic, dehydration 5-8%, tachycardia, tachypnea and crystals on the hairs of the preputial tuft. The differential diagnosis included: gastrointestinal obstruction (tachycardia, colic, bloat, anorexia), grain overload (vocalize), encephalopathies, coccidiosis, trauma (tenesmus), urinary tract infection (dysuria) and congenital abnormalities. Regarding urinalysis we observed erythrocytes, crystals (struvite), proteins, ph 6,5-7,5. Calculi had a sand-like in consistency or, a combination of sand-like and granular consistency. Those found in urethral process were granular in nature dark-grey in colour, had a chalky texture and were 0.5–3 mm in diameter. Hematological evaluation revealed a stress leukogram. At serum analysis, the goats presented hyperglicemia, but urea nitrogen (18 mg/dL) and creatinine (1,5 mg/dL) were unremarkable in ruminants with acute urethral obstruction. Ultrasonographic findings revealed thickening of the bladder wall, echogenic material within the bladder lumen (figure 1).

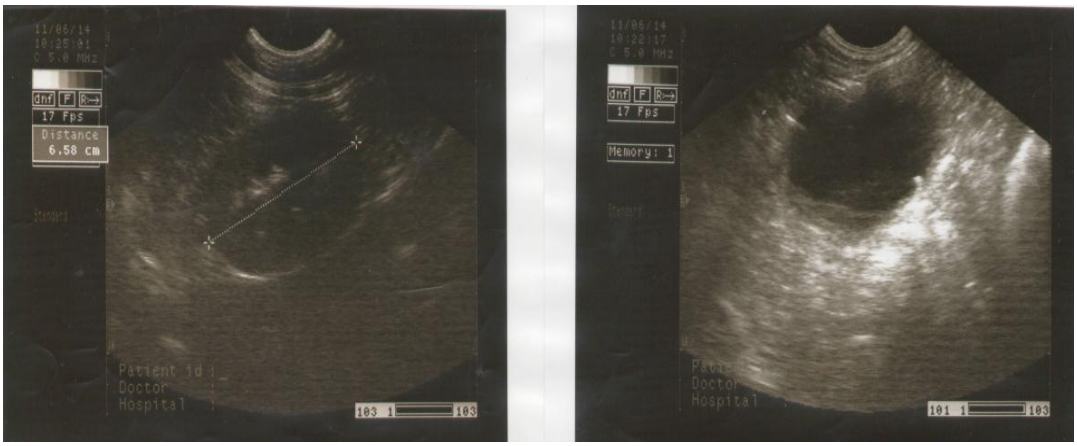

Fig.1 Sonogram of the right kidney (left side) and urinary bladder (right side) at a 18 months goat.

Medical management can only be pursued in the affected animal if the bladder wall is intact. Chemical sedation using diazepam (0.1–0.5 mg/kg) intravenously, acepromazine (0.05–0.1 mg/kg) intravenously and a lumbosacral epidural using 2% lidocaine (0.1–0.2 ml/kg, not to exceed 15mls) which provide temporary paralysis of the retractor penis muscle, allow manipulation of the penis. We used Alpha 2 agonists (Xylazine) 0,05-0,1 mg/kg IV or IM. Xylazine has to be used with caution because of the diuretic effect. As antimicrobial therapy we used Beta-lactam antimicrobials (penicillins and cephalosporins) dependig on the

specifics of the individual case and route of therapy. We used the retrograde/normograde hydropulsion with NaCl 0,9% with a short catheter which gave good results in acute urolithiasis. Normally, the catheterization and retrograde flushing are therefore not useful in ruminants for resolution of obstructive urolithiasis and may result in urethral rupture if too much force is used (Anderson and Rings, 2009).Also, ruminants, cannot be catheterized to relieve obstructive urolithiasis because their urethra has a diverticulum in the region of the ischial portion of the urethra (Palmer at al., 2002).

Acidification of urine can increase the solubility of the uroliths composed of magnesium ammonium phosphate (struvite), calcium phosphate (apatite), and calcium carbonate and thereby inhibit precipitation in the urine. Reducing urine pH by the addition of 0.5% ammonium chloride to the diet has been shown to reduce the incidence of urolith formation; however, long-term, metabolic acidosis, such as that created by feeding ammonium chloride, has been shown to decrease bone mineral density (MacLeay et al., 2004). Irrigation of the bladder through the cystotomy catheter with the chemolytic solution hemiacidrin (acidic gluconocitrate solution) was reported for dissolution of calculi in human medicine (Streeter et al., 2002). 30 ml of hemiacidrin was infused into the bladder via the cystostomy catheter and then the catheter can be occluded for 30 minutes, after which the infusion was allowed to drain out. This can be repeated 4 times a day for three days and appeared to be effective in dissolving existing calculi based on ultrasound evaluation. The use of acetic acid (Walpole's solution) with a pH of 4.5 has been used for medical management and dissolution of struvite crystals, stones, and bladder sludge (Tibary and Van Metre, 2004). The use of this solution requires either and indwelling tube cystotomy catheter, a percutaneous poly propylene catheter, or multiple needle cystocentesis procedures. In one case series the use of acetic acid resolved the urinary obstruction in 80% of the affected animals. Unfortunately medical therapy often fails in over 50% of the affected animals leaving surgical correction, slaughter, or euthanasia as the only other options.

Surgical options include amputation of the obstructed urethral process, penectomy, perineal urethrostomy, prepubic urethrostomy, urethrotomy, cystotomy, tube cystostomy, and bladder marsupialization. The most used in goats is the amputation of the obstructed urethral process. Urethral process amputation in small ruminants is the simplest procedure for relief of obstructive urolithiasis. The uroliths must be present in the process itself or in the distal penile urethra to pass. Examination of the urethral process should be performed in every animal with urolithiasis, and often the process is removed as a precautionary measure. The procedure is performed quickly with scissors or scalpel and is easiest with the animal restrained in a sitting position on its rump. These methods often involved resection of the penis and experienced numerous complications, most commonly stricture formation at the site of entry into the urethra. Success rates varied and were dependent on the duration of obstruction before surgery; condition of the animal and urethral tissue at the time of treatment; concurrent urethral rupture; and location of the urolith itself. Approximately half of the animals treated with amputation will have urine flow restored (Haven et al., 1993). Unfortunately, urethral process amputation usually results in reobstruction within a short period of time because there are more uroliths further proximal in the urethra. Also, urethral stricture at the surgery site is a serious potential complication. In our study, we identified the urethral obstruction location (distal) to ensure proper incision placement. The distal penis was exteriorized from the sheath by an assistant, the urethra was incised and a retrograde urinary catheter introduced into the urethral orifice. The incision was not sutured. Anti-inflammatory drugs (flunixin meglumine 1,1 mg/kg bw, IV for 3 to 5 days) post surgically was used to

facilitate urethral healing.This surgically technique gave good results in acute urethral obstruction of male goats. 6 months after the surgical intervention the male goats had no symptoms of urolithiasis.

Conclusion

Surgical corection of urethral obstruction was a salvage procedure. In small ruminants which have multiple calculi, incision of the urethra may restore urine flow but usually provides only temporary relief. Long term prognosis in goats is poor because there is a high rate of recurrence of obstruction. Prevention remains the mainstay of urolithiasis management.

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ORFUL FROM THE DERMATOLOGIST PERSPECTIVE: A SERIES OF 11 CASES AND REVIEW OF THE LITERATURE

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Abstract

Orf is a parapoxvirus infection. It is widespread in sheep and goats, especially affecting lambs and kids. In humans, it occurs rarely and is often underdiagnosed due to the self-limiting evolution. Transmission is achieved through direct contact with sick animals or indirectly through contaminated objects and food. The clinical picture of human orf is dominated by the presence of single inflammatory nodule, exudation, appeared at the site of inoculation and evolving into 6 stages: maculopapular (erythematous papule from debut) in the target (the nodule with centrally bulla, concentric aspect), exudative (acute exudative nodule), regenerative (surface crust), papillomatous (papillomatous-looking surface), regression (removing the crust and healing), self-limiting evolving in 6-8 weeks. The diagnosis of orf is eminently clinical and based on correct history. If possibe, we must perform an electron microscopy of biopsy material.Treatment is symptomatic and prevents complications. Orf is not an disabling disease. The amputation of the finger is very rarely necessary. The orf is a disease that strengthens the belief of doctors that cooperation between the veterinary doctor and dermatologist is highly beneficial to all our patients.

Keywords: *ectima contagiosum, erythema multiforme, goats, Japanese serow, lymphangitis, necrosis, orf, parapoxvirus, reindeer, sheep*

Introduction

Orf is a viral disease of sheep and goats present in the first year of life, but rarely seen in humans. The etiologic agent is stable in external environment and it is viable on objects contaminated at low temperatures, long time. The frequency of cases in Caucasians in Europe and New Zealand seems to be higher than in other parts of the world, but there are not series of large number of cases. No mortality was reported, but it is important to know and recognize disease to all veterinarians, family doctors and dermatologists to prevent the spread of disease and to treat correctly the patients.

Material and Methods

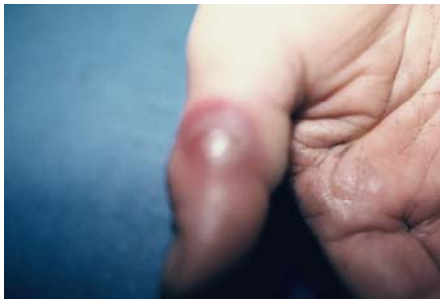
Our study includes 11 cases diagnosed in DermaMed office in Braşov, Derma Lux office in Iași and Derma Clinique office in Iași, between the years 1998-2014; the ages between of 4 and 62 years. Gender distribution was F: B = 3: 8. Only 4 patients were from urban areas and 7 of the countryside. Patients were assessed clinically, it was made a thorough history and in 7 cases nodular lesions were biopsied (belonging to adults). Philips electron microscope CM 100 examination revealed the characteristic intracellular viral particles.

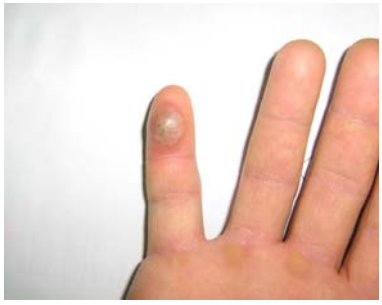
No PCR (polymerase chain reaction) was performed for technical reasons, but it would be more specific diagnostic method.

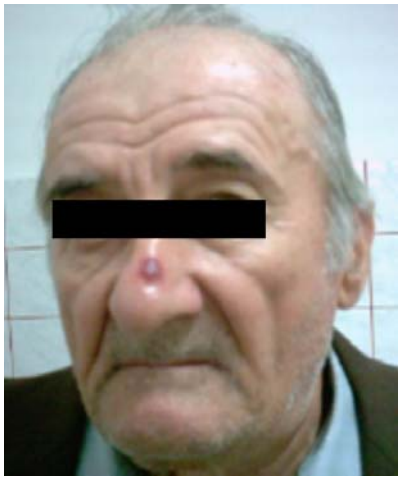
Results

The 11 patients were two children and 9 adults and elderly, aged 4 to 11 years children and 27-62 years adults. They presented a single skin lesion (8 cases) or multiple lesions (3 cases), localized on upper limbs and the face. Patients are from rural areas, working in animal farms, with the exception of 4 patients living in urban areas, which are part of two families (mothers, son and daughter) who went to the mountains to spend a weekend at sheepfold. We are mentioning that 3 cases were presented to the dermatologist being sent by the veterinarian with the presumption of correct diagnosis: *Note orf*.

Local dermatologic examination revealed erythematous nodular lesions, exudative, some covered by haematic crust with an inflammatory halo, located in the fingers of the hands (fingers I, II and V of the right hand in most cases), dorsal hands, forearms and face.







The 11-year-old patient presented inflammatory large inflammatory plaque in the onset, 4.5 cm diameter, which evolved into central necrosis, located at the middle phalanx of right medius. Local treatment with antiseptic solutions and antibiotic healing ointment, associated with systemic inflammatory drug (ibuprofen) resulted in a favorable evolution toward healing without the need for surgery. It is worth mentioning that the patient's mother and the other two infected people almost simultaneously (a 4 year old boy and his mother) had 1-1.5 cm diameter nodular lesions evolving asimptomatic. Medical history of the mother

and 4-year-old patient was insignificant, but the little girl 11 years old presented left intercostal herpes zoster one year ago.

In a female patient of 32 years, wife of a man working in animal husbandry, rural area, the evolution of orf was complicated by erythema multiforme with eritemato-papular exanthema, with characteristic target lesions, located on palms, fingers, dorsal hands, forearms and face.

Lymphangitis located on the back of the right hand and on the anterior surface of the right forearm was present as complication a case of orf with 2 nodular lesions in the back of the right hand, the I-st and II-nd metacarpal-phalangian joints for a man of 36 years, shepherd.

Secondary bacterial infection was present in 3 cases, men, adults, rural, working in animal husbandry. The culture of pus revealed the presence of *Staphylococcus aureus* in 2 cases and *Enterobacter spp*. in the third case.

Clinical examination of organs and systems was normal.

Routine laboratory examinations were normal, except for a red blood cell sedimentation rate of 30-40 mm/h in children. Viral serology for herpes, infectious mononucleosis virus, coxsackie virus, parvovirus, and bacterial tests for Chlamydia and Mycoplasma have been negative in all patients who were evaluated.

Electron microscopy of biopsy pieces demonstrated the presence of specific intracellular viral particles in 7 cases (for example, biopsies were made of the lesions of adult patients as parent and not for their children or wife).

Under general treatment with anti-inflammatory and local healing ointment with antibiotic nodular lesion lesions completely disappeared within 10-18 days. Local treatment with dermocorticoids was beneficial for erythema multiforme lesions and systemic antibiotics were given for bacterial superinfection and lymphangitis.

Discussions

Orf (also called: ecthyma contagiosum, contagious pustular dermatosis, infectious pustular dermatosis) is an endemic viral disease manifestations among sheep, goats, deer, reindeer, yak, Japanese serow (*Capricornis crispus*) and rare in humans. The etiologic agent is a double-stranded DNA virus, large (260x160 nm), Parapoxvirus genus of the family of Poxviridae. The virus is resistant to the external environment and it is viable even in winter on contaminated objects.

Orf cases are often reported in Europe and New Zealand, rarely in North America, only the Caucasian population, without differentiation by gender or age. Although Japanese serow can manifest orf, we did not found in the medical literature reported human cases of Japanese doctors. It is recorded a higher number of cases in the spring (when lambs are born) and periods of holidays when lambs and sheep are sacrificed (Easter).

Orf virus often affects animals in the first months of life, devoid of viral immunity. Contagious pustular dermatitis begins in the mouth, through vesiculo-bullous pustules, erythematous nodules, inflammatory, exudative, continuing with mouth ulceration, which heal spontaneously in about one month, causing permanent immunity to vorf virus. Ulcers can persist for more than 1 month, extending from perioral area to gingival, perinasal, nipple, genital and perianal. Frequent complication is bacterial infection. Orf virus transmission can be done directly from the sick to the healthy animal or indirectly (barn, trough, food, fence, knives, scissors lawn animals, doors etc). There are no known cases of transmission of the virus to cattle or other animal groups.

Transmission to humans is rare, being mentioned for the first time in 1932 by Brandenberg. Orf is manifesting among farmers, shepherds, veterinarians, shearers, butchers, slaughterhouse staff and cooks handling infected meat. The history is very important because even if we consider that the affected persons as farmers, we must take into account the temporary or accidental caring for animals (hobby or weekend visit to the farm for educational purposes), religious communities mutton consuming that are personal sacrificing animals.

The virus is transmitted to humans directly, during care, feeding of sick animals, handling carcasses, fragments of infected animal with bare hands or indirect (contaminated objects, food handling). Contamination is exceptional interpersonal, but explains manifestation of family occurred condition.

After an inoculation period of 5-6 days, there is a single lesion or a small number (up to 10) of lesions, with or without low-grade fever, malaise for 3-4 days. In the onset, there is an eritemato-violet papule, hard to the touch, which grows in diameter and evolves into an eritematous, edematous and prominent plaque or inflammatory nodules 2-3 cm in diameter, with a central loose bulla or pustule. After a few days occurs an ulceration covered with a thick haematic crust, surrounded by a purple-gray ring and an erythematous area. Erythematous nodule may become prominent or papillomatous, but it flattens in time, the crust is detached and slow healing occurs. Lymphangitis and lymphadenopathy of afferent lymph nodes may occur. The clinical aspect of orf in humans is often represented by a single nodular lesion that appears at the site of inoculation, with self-limiting evolution in 6-8 weeks. Rarely, in immunocompromised persons (chronic lymphocytic leukemia), multiple nodular lesions are present, almost simultaneously occurred, large (5 cm diameter), locally destructive or complicated with impressive pyogenic granuloma, severe evolving. Sometimes it can cause relapses.

The most common complication of orf is bacterial superinfection determined by *Staphylococcus spp.* (impetigo) and *Streptococcus spp.* (lymphangitis, cellulitis). There have been described in the literature cases of local skin necrosis or secondary skin macular, papular, vesicular rash accompanied, but they often appear to immunocompromised patients. Erythema multiforme is appearing classically 10-14 days after the onset of orf and lasts 7 days and not affect the mucous membranes. The first reporting of this associated diseases was in 1948 by Blakemore et al. Later, Johannensen et al. and other researchers have published a further 21 similar cases. Rarely, can occur pemphigoid-like rash usually self-limiting, with papulo-vesicles or bullae, almost 3 weeks after orf; it heals spontaneously within weeks or require immunosuppressive treatment. Murphy et al. published 5 cases of generalized bullous pemphigoid secondary to orf. It differs from classical bullous pemphigoid of the young age of occurrence of disease, presence of isolated deposit of C3 and linear deposit of IgG at dermo-epidermal junction and absence of antibodies anti- basal membrane into the blood.

Location of orf in humans is often on hands, forearms and less on the face. The most common location is on the back of the proximal and middle phalanx of the right index.

It was found that infection of pregnant women in the last trimester of pregnancy does not affect the fetus.

Orf virus, with a tubular structure, may be evidenced in negative stain electron microscopy.

The differential diagnosis should be done with milker's nodules, erisipeloid, sporotrichosis, acute febrile neutrophilic dermatosis, pyogenic granuloma, impetigo, herpes

simplex, anthrax, tularemia, benign skin tumors, tuberculous chancre, syphilitic chancre, atypical infection with *Mycobacterium spp.* etc.

Laboratory diagnosis can't be based on specific serological tests as serology can't distinguish orf virus by other parapoxviruses as paravaccinia.

Correct diagnosis is the microscopy of a fragment of crust or a piece of biopsy demonstrating the presence of ovoid and striae viral particles intracytoplasmic into the degenerative keratinocytes. The electron microscopy of exudate did not bring a benefit because only revealed the presence of ovoid virions without differentiating them from other parapoxviruses.

The orf virus can be grown on culture cells derived from sheep, but the growth of virus is slow and inconstant.

Groves et al. established that histopathological examination of incisional biopsy piece has significant diagnostic value: a marked pseudoepiteliomatous hyperplasia affecting keratinocytes in the upper third of epidermis, with vacuolar degeneration of keratinocytes, with intracytoplasmic vacuoles showing eosinophilic inclusions, in the initial stages. Occasionally intranuclear inclusions can be observed. In time, the invagination of epidermis into the dermis occurs as glove fingers. Keratinocyte disintegration is often present. Necrosis of the epidermis, with ulceration, is present in the center of nodular lesion. Papillary dermis is swollen and has a dense infiltrate, with central macrophage and histiocytes, with lymphocytes and plasma cells in the periphery, with very few neutrophils and eosinophils, which extends to the deep dermis. Large atypical lymphocytes are present into dermal inflammatory infiltrate, often expressing the antigen CD 30. In the upper dermis massive capillary proliferation and vasodilation are present, with hypertrophied endothelial cells and endothelial cell proliferation.

PCR on frozen fragments of lesion biopsy or tissue debridement or exudate can certainly identify orf virus. Real-time PCR is more sensitive than standard PCR technique. I thas been shown that orf virus is producing a homologue of VEGF (vascular endothelial growth factor) which recognizes certain VEGF receptors and thus were responsible for intense vascular proliferation in the dermis.

Orf treatment in humans is not specific antiviral and although it is a self-limiting disease, are useful: immobilizing finger, wet compresses with mild antiseptic to stop exudation (open bullae or pustules under antisepsis, then apply gentian violet, methylene blue or antibiotic and corticosteroid spray, as Oximed). Emollient cream with antibiotic and corticosteroid is useful in crust stage (Diprogenta, Fucicort, Fluocinolon N). Treatment is often only symptomatic (anti-inflammatory, analgesic, antipyretic), but most often can be administered prophylactic antibiotics to prevent bacterial superinfection, or curative antibiotics if bacterial infection is already installed up at presentation. Depending on the severity of the case, antibiotic treatment can only be local or may be associated with the systemic.

There are reports of series of orf cases successfully treated with idoxuridine 0.8% ointment, applied 3 times/ day.

Imiquimod topical treatment induced rapid regression of lesions in some studies, and cidofovir cream had promising results. It has been shown that injection of interferon-α have poor benefit.

Surgical treatment is unusually, but is addressed cases with exofitic lesions, which may be excised, curetted or shaved. If exudative nodular lesions are persistent are indicated:

curettage and electrocoagulation. There are cases successfully treated by cryotherapy with liquid nitrogen in order to faster healing. Radiation contact should be avoided and the decision of finger amputation is not needed in most cases.

Treatment is performed correctly only if the general practitioner is informed to active detecting of similar cases in the community, workplace or patient's family and also veterinarian in whose care are the source animals.

It is necessary to vaccinate lambs and kid goats from the farm yard and the patient operates.

Conclusion

Orf is a viral zoonosis with endemic evolution, rarely seen in dermatologic surgery, frequently manifesting in spring, the adult Caucasian population, without gender or age predisposition. It can be spread by direct contact with sick alive animals or indirectly through contaminated objects (barn, trough, food, fence, knives, scissors lawn animals, doors etc), slaughterhouse. Contamination from a human source is possible, but rare.

Evolution of orf is often with self-limiting lesions, with spontaneous healing in 6-8 weeks, but it can sometimes complicate or may have long-drawn evolution.

Orf can be a cause of erythema multiforme, lymphangitis, soft tissue necrosis of the fingers.

Healing of orf is often *retitutio ad integrum*, rarely with scar or amputation of the affected finger.

Medical Letter addressed to family doctor and veterinarian is necessary to be informed about the disease and to take measures to vaccinate young animals and for the maintenance of animal health care. The veterinarian is the one who sends the human patient to a dermatologist with the correct assumption.

The knowledge and recognition of orf by physicians is important and also collaboration of dermatologist and family doctor with veterinarian is essential for maintaining the health of the population.

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