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CUPRINS

MARKERS FOR TUMOR MARGIN ASSESSMENT THROUGH RAMAN SPECTROSCOPY IN COMPARATIVE ONCOLOGY

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Abstract

The occurrence of tumour diseases in both animals and humans is continuously increasing. Research in nanosciences and molecular biology has put lately an intense effort to identify the aetiology factors and seek for new ways of diagnostic and targeted therapies aimed at reducing mortality and increasing chances to healing. Extensive development of cancer tumours is frequently counteracted through surgery. Assessment of a clean surgical margin is vital and a precise and rapid diagnostic down to molecule level represents a technical challenge with important clinical implications. We present a new way of using surgery instruments and surface enhanced Raman spectroscopy for direct ex vivo (no freezing, no staining) and in vivo diagnostic of clean margins in mammary tumour surgery of pets (dogs and cats).Raman spectroscopy extracts chemical information with reported 100%sensitivity, 100% specificity and overall accuracy of 93% in identifying carcinomas. Our main result stays in identification of a set of molecular markers (carotenoids, lipids and intramolecular water) for Raman diagnostic in cat and dog mammary tumour surgery. Those markers have already been confirmed for human patients.

Key words: *markers, Raman spectroscopy, tumour margin*

Introduction

Nowadays, in the field of human cancer research, there is an increased interest in clinical development of a new model system that will determine the border between the clean margin and the positive margin of the cancerous tissue. (Lutful K. L., et al., 2015)

Comparative oncology is a field that examines the tumor development across species and it is characterized by collaboration of both human researchers and research veterinarians. (Schiffman J. D., et al., 2015)

The dogs are considered one of the best preclinical models of cancer, especially because they share the same environment, risk factors and disease characteristic with the human population. (Lutful K. L., et al., 2015) Cancer occur spontaneously in dogs and have a clinical presentation and pathophysiology that is similar to the ones in human cancers. (Schiffman J. D., et al., 2015)

Tumor margin assessment is an important part whenever surgical excision is performed. (Withrow S. J., et al., 2014) Obtaining clean margins in surgical interventions for different types of cancers is an essential factor in the treatment of cancer while conserving the healthy tissue. (Sebastian M., et al., 2015)

The advancements in the field of surgical oncology include the intraoperative assessment of surgical margins. A number of methods are currently used in medicine to evaluate the margins of tumors. The gold standard method in the margin evaluation is histopathology. The disadvantage is the lack of knowledge whether the tumor has been entirely or incompletely removed at time of surgery. Re-excision rates following histopathology evaluation of surgical margins in breast-conserving surgery are 25 to 35%. Other frequently used intraoperative techniques are frozen sections and imprint cytology, mainly because, compared to histopathology, the re-excision rates are 5 to 10%. (Liptak J., et al., 2013)

The research on margin assessment in human oncological surgeries focused on intraoperative techniques that include Raman spectroscopy, optical coherence tomography, radiofrequency spectroscopy, near-infrared fluorescence optical imaging. (Liptak J., et al., 2013)

Raman spectroscopy is based on inelastic scattering of monochromatic light on chemical bonds in a sample. The energy of monochromatic photons incident at a sample surface excites intra-molecular vibration modes whose frequencies are specific to the involved chemical bonds. The process develops with energy and momentum conservation i.e. a photon of lower energy is emitted (Raman photon) and a transition from one energy level to another occurs resulting in a frequency shift of the emitted photon.

These shifts are seen as individual bands in the Raman spectrum and their assessment would permit accurate analyses. Some particular advantages of Raman spectroscopy in precise diagnosis result from: *a*) sensitivity to many different functional groups, with access to C=C, S-S, CS bonds that are weak in the infrared (IR); *b*) highly selective fingerprint, since it can discriminate similar compounds; c) no sample preparation; d) compatibility with aqueous solutions; *e*) high spatial resolution that permits single cell level analysis. However, a drawback of the technique results from the weakness of the scattered signal and auto-fluorescence when applying Raman spectroscopy to biological samples such as body fluids, tissue, fat, etc.

An effective way to improve the weak signals is to use surface enhanced Raman scattering (SERS), where metal nanostructures could provide up to 10^{17} signal intensity amplification due to the resonant interaction of light with the surface plasmons excited at the surface of the structure. This not only increases the spectral resolution but also shortens significantly the duration of acquiring the spectra.

To draw Raman spectroscopy into the clinic for assessment of surgical margins ex vivo and in vivo would need one marker or several. This would make a good relative qualitative approach for clinical validation of the method and a step forward towards absolute quantitative diagnostic. (I. A. Birtoiu et al, Interface Focus 2016, Accepted).

In this paper, we propose SERS for margin assessment (I. A. Birtoiu et al., 2015; Micsa C. et al.,) through spectral markers assigned for malignant and respectively benign/healthy tissue.

Materials and methods

The study was performed on mammary tumors of dogs and cats which were surgical removed through mammectomy during general anesthesia. All cases were brought in for mammectomy in the Obstetrics and Gynecological Pathology Department of the Faculty of Veterinary Medicine of Bucharest. For the research we used 9 dogs and 3 cats of mixed breeds, all females, with ages between 3 to 15 years.

The patients were put under general anesthesia and were prepared for the surgical intervention. After the mastectomy, every patient received postoperative care and medication. They were given antibiotics and anti-inflammatories. Their body was strapped with a piece of cotton sheet for 10 days to prevent the collection of serous fluid. After two weeks their stitches were removed.

Direct *ex vivo* samples of normal (healthy) tissue, breast tumors, skin and pure fat from female dog and cat patients were investigated following regional mastectomy (removal of one mammary gland chain, the skin covering the breasts and the corresponding one side lymph nodes). The tumors were divided into two parts, from which one followed the common way of cytological and histopathology analyses and the second part was sampled for direct Raman

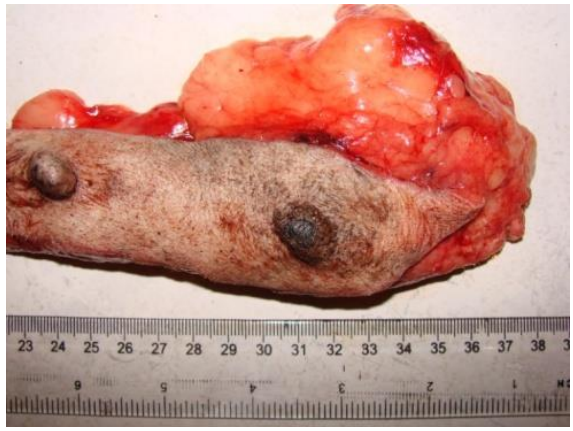
exploration. The most relevant cases and the comparison between cytology, histopathology and Raman diagnostics are shown in the table 1 and associated images.

The smears prepared for the cytological exam were obtained through skin scrape and touch imprint of the mammary tumors and then were then stained with the standard coloration May-Grűnwald Giemsa.

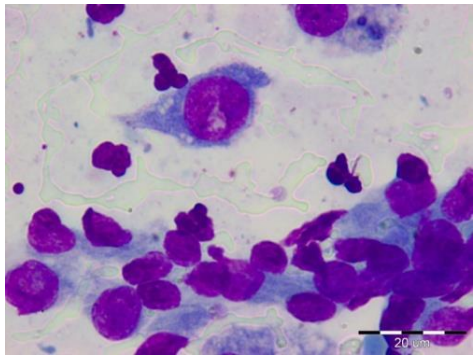
For the histopathological exam, fragments were collected from each mammary tumor and were included in one or several blocks. The probes were immersed in 10% neutral buffered formalin for 24 hours. After the fixation, the samples were embedded into paraffin blocks to support the tissue structure and to enable very thin sections to be cut and mounted onto microscope slides for analysis. The coloration of the slides was made manually using the Hematoxylin and Eosin stain.

Table 1

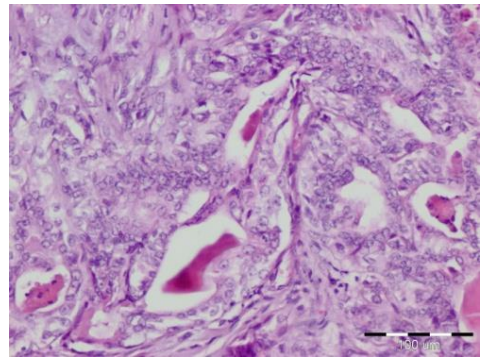
Case number	**Receiving date**	**Animal identification data**	**Cytological diagnosis**	**Histopathologic diagnosis**	**Raman result**
1	10/30/2014	Canine, German Shepard, female, 3 years, unspayed	Suspected benign tumoral hyperplasia	Secretory adenosis (lobular hyperplazia)	Benign
2	12/3/2014	Canine, Mixed breed, female, 15 years, unspayed	Suspected mammary carcinoma	Tubular and papillary mammary carcinoma	Malignant
3	12/3/2014	Canine, Cocker Spaniel, female, 11 years, unspayed	Suspected mammary adenocarcinoma	Tubular and papillary mammary carcinoma and lobular hyperplasia (adenosis)	Malignant
4	12/12/2014	Feline, European, female, 14 years, spayed	Suspected mammary carcinoma	Cribriform mammary carcinoma	Benign
5	12/16/2014	Feline, European, female, 15 years, spayed	Suspected mammary carcinoma	Tubular and papillary mammary carcinoma	Not investigated
6	12/19/2014	Canine, French Bulldog, female, 11 years, spayed	Suspected mammary carcinoma	Mixed type mammary carcinoma	
7	12/19/2014	Canine, Golden Retriever, female, 11 years, unspayed	Suspected mammary carcinoma	Tubular mammary carcinoma with lymphonodal metastasis	Malignant
8	1/13/2015	Canine, Boxer, female, 9,5 years, unspayed	Suspected mammary carcinoma	Mixed benign tumor	Malignant
9	1/21/2015	Feline, Norwegian Forest, female, 11 years, spayed	Suspected mammary adenocarcinoma	Simple cribriform mammary carcinoma	Benign
10	3/23/2015	Canine, Alsacian Shepherd, female, 10 years, unspayed	Suspected mammary carcinoma	Mixed mammary carcinoma	Malignant
11	11/20/2015	Canine, Mixed breed, female, 13 years, unspayed	Suspected mammary carcinoma	Simple tubular, papillary and solid mammary carcinoma	Malignant
12	4/14/2016	Canine, Mixed breed, female, 11 years, unspayed	Suspected mammary carcinoma and malignant myoepitelioma	Benign glandular hyperplasia - secretory adenosis with carcinomatous transformation, tubular type, with a tendency to become solid	Benign



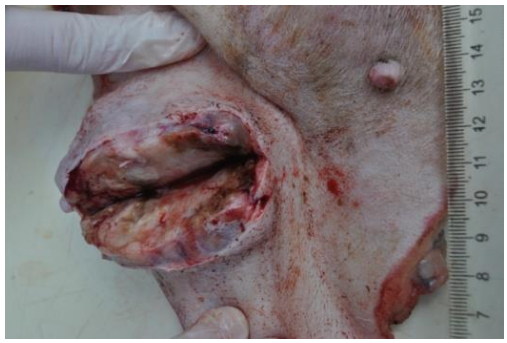
Case number 3 – Cytological aspect of mammary tumor – suspected mammary adenocarcinoma



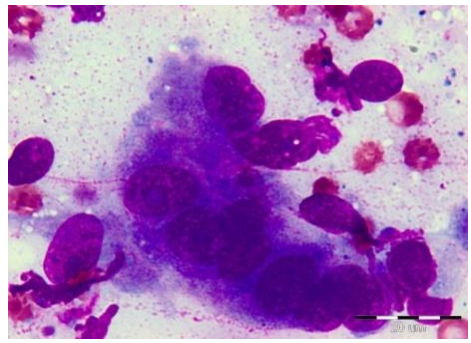
Case number 3 - Macroscopic aspect of mammary tumor



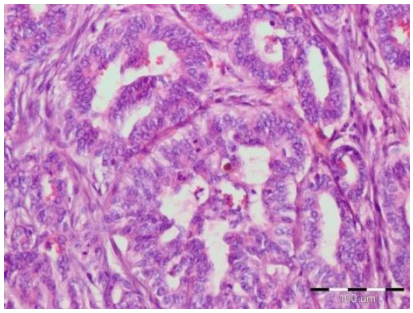
Case number 3 – Histopathological aspect – Tubular and papillary mammary carcinoma and lobular hyperplasia (adenosis)



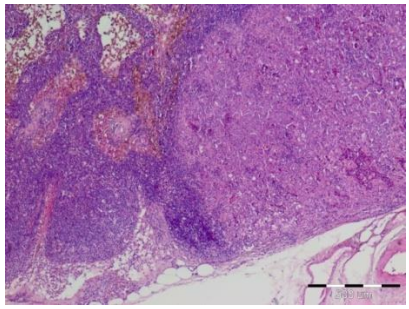
Case number 7 – Macroscopic aspect of mammary tumor



Case number 7 – Cytological aspect – Suspected mammary carcinoma



Case number 7 – Histopathological aspect – Tubular mammary carcinoma



Case number 7 – Histopathological aspect – Lymphonodal metastasis

Raman measurements

The Raman spectra were acquired using a LABRAM HR 800 (Horiba JobinYvon) micro-Raman spectrometer, in the backscattering geometry with aλ = 632 nm HeNe laser for excitation source. Later developments have led to a portable Raman instrument based on fibre optics (Fig 1a) intended to be used in the operation theatre.

The chosen wavelength permits a reasonable signal intensity, smaller energetic impact with the samples and a spectral range extending between 100 cm^{-1} to 4000 cm^{-1} to include all lines above 2200 cm^{-1} that cover all vibrations of biological interest. This large spectral window would not be available with an IR excitation source. The study of the vibrational properties of water confined in the non-cancerous and cancerous human breast tissue in comparison with the properties of water confined at interfaces for DNA and phospholipids shows that the normal tissue from a negative margin contains a significantly higher number of hydrophobic adipose cells than the cancerous tissue, which is mainly composed of hydrophilic proteins

Water confined in the cancerous tissue exhibits a single band **at 3311 cm^{-1}**. A sensitivity of 83% for lipid markers (**2800–3000 cm^{-1}**) and 69% for carotenoids (**1158, 1520 cm^{-1}**) have been found in human assays. The comparison between the micro-Raman spectra of normal and malignant tissues shows strong signals from carotenoids (**1158 cm^{-1} and 1520 cm^{-1} and at 1161 cm^{-1} and 1527 cm^{-1}**) for the normal tissue, which are absent in the spectrum of cancerous tissue where peaks assigned to proteins are dominant.

The schematics of the portable Raman spectrometer and the Raman spectra of some of the samples mentioned in the table 1 are shown in figure 3 (a) and b) respectively).

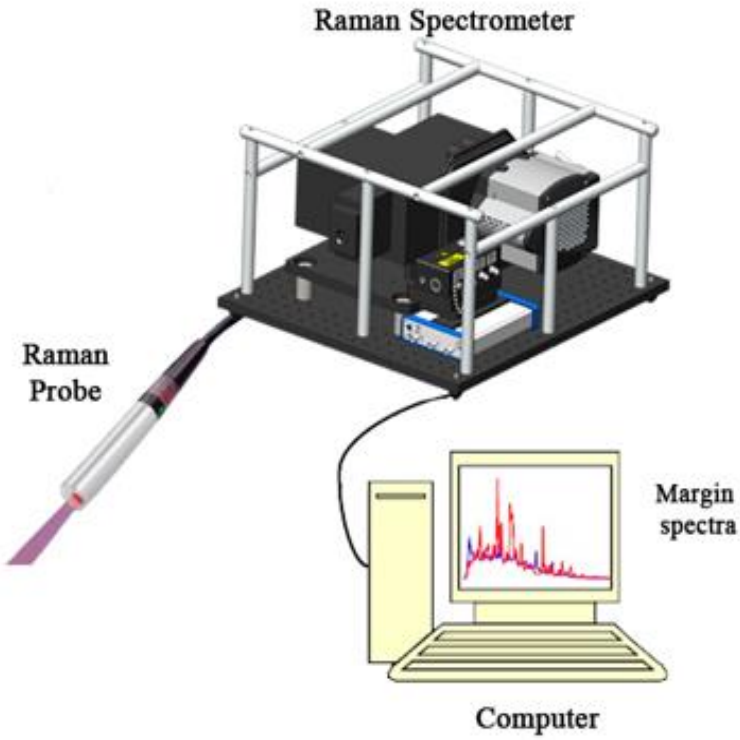


Figure 1. a) Schematics of the portable Raman spectrometer for real time margin assessment

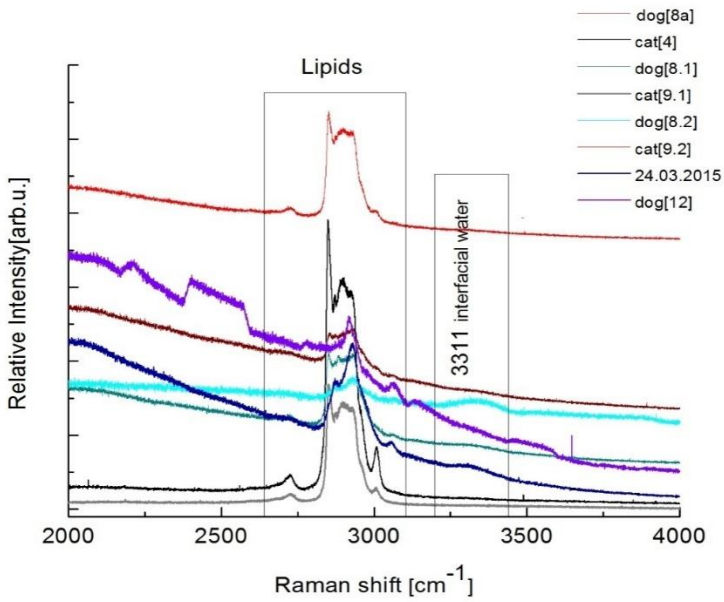


Figure 1. b) Raman spectra of the samples 4, 8, 9 and 12 in the table 1. The peak at 3311cm^{-1} is noticed in samples 10 (24 03 15) and sample 8.

Results and discussion

We have mainly exploited the high frequency region (2000 to 4000 cm^{-1}) in order to show evidence of interfacial water (3311 cm^{-1}) that is found exclusively in the cancerous tissue. The frequency range from 100 to 2000 cm^{-1} (not shown here) presents the bands of carotenoids at 1158 and 1527 cm^{-1} for the samples denoted "benign" in table 1.

The absence of the 3311cm^{-1} peak from a tumor margin can signify "negative margin". It is much easier at this stage to use a single marker instead of two or four of them to make a "yes/no" decision concerning margin assessment.

Although Raman diagnostic and histopathology diagnostic do not match at 100% from table 1 the spectra of the margins have been always free of the 3311cm^{-1} peak.

Conclusions

The potential of Raman spectroscopy to assessing mammary surgical margins in dog and cat oncologic surgery has been investigated.

The direct ex vivo measurements (no sample preparation) have shown that the 3311cm^{-1} peak corresponding to interfacial water in cancerous tissue can be used as a marker for a rapid decision on the margins status.

The Raman band of interfacial water has been first found in human cancerous breast tissue in vitro. Its presence in the dog and cat mammary tumors adds to the field of comparative oncology.

Further work will be devoted to translation of Raman portable instrument in the veterinary operating theatre.

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RESEARCHES REGARDING THE CORRELATION BETWEEN RENAL PARAMETERS AND THE EVOLUTION OF ELECTROLYTES IN RENAL FAILURE IN DOGS

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Abstract

Renal failure is a medical emergency defined as an acute syndrome of partial or total rapid loss and potentially reversible of the renal excretion function, typically on a healthy renal parenchymal and rarely on an old nephropathy untreated on time that can lead to chronic renal failure, or be incompatible with life. The cases studied are canine patients of different ages, belonging to different races and sharing acute or chronic renal insufficiency of different etiology. Determination of renal parameters was performed in all cases and their progress was closely correlated with the electrolyte and sanuguine gas parameters. The purpose of this research is to determine, based on the cases under consideration, the AnGap influence in establishing a vital prognosis and a therapeutic protocol. Determination of blood gases and electrolytes is an important component in determining the degree of dehydration, electrolyte imbalance and the degree of kidney damage. The difference between electrolytes and pH value are important indexes and provide extremely useful data to guide the therapeutic act. The following study was performed in the Faculty of Veterinary Medicine's Clinic and it was based on case studies spontaneously presented in the Clinic. This activity was conducted during 16 months (March 2015 - June 2016). During this period, 30 patients who presented clinical signs of renal impairment and were confirmed by biochemical analysis were introduced into the study. The studied cases are dogs of different ages, belonging to different races and sharing acute or chronic renal impairment of different etiology. Determination of renal parameters was performed in all cases and their progress was closely correlated with the electrolyte and sanguine gas parameters.

Key words: *dog, renal failure, electrolyte balance, AnionGap*

Introduction

One of the most important functions of the kidneys is to maintain the acid-base balance and to ensure a normal cellular function necessary to maintain blood pH within narrow limits around 7.4. (1)

Maintenance of constant pH involves the optimum operation of various intra- and extracellular buffer systems, of the respiratory and excretory system. The first two have a role in the rapid changes in pH, while the kidney is responsible for the long-term homeostasis. (2)

Disruptions of fluid and electrolyte balance are often emergencies and therapeutic measures depend on the accurate understanding of the pathophysiology of these disturbances. Fluid and electrolyte disorders may be of interest: mostly water, predominantly various electrolytes, water and electrolytes alike. Electrolytes are represented by ions of the main elements: Sodium (Na^+), Chlorine (Cl^-), Hydrogen (H^+), Bicarbonate ($HCO3^-$), Potassium (K^+), Sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), calcium (Ca^{2+}) , Magnesium (Mg^{2+}). In this matter, it is known that the action of magnesium, calcium, iron and zinc ions is as activators of enzymes, calcium's role in blood clotting and neuro-muscular irritability, the importance of calcium/phosphorus ratio in the process of ossification, the main role of sodium and potassium in osmotic control of water metabolism, regulation of acid-base balance. (1)

The samples were submitted for laboratory investigations for electrolytes, blood gases, acid-base balance, AnGap, tCO_2 and bicarbonate, and played an important role in emergency medicine and in cases of severe hydroelectrolytic deprivation, providing essential information for clinical diagnosis. The laboratory parameters shown above are pre-programmed with limits and benchmarks for veterinary medicine and species-specific. (1)

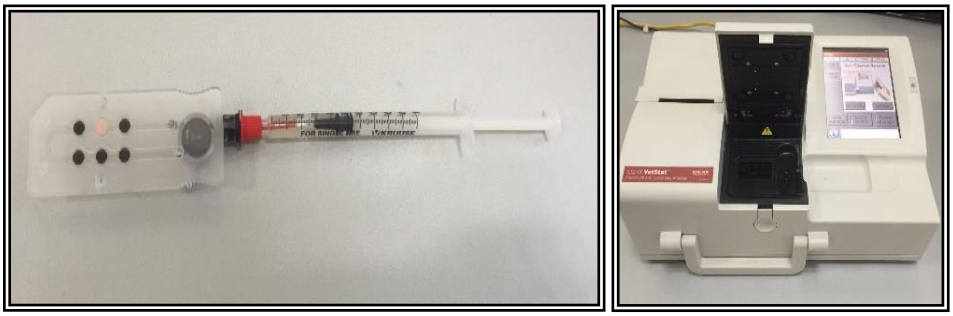
Materials and methods

The study was conducted on 30 canine patients of different ages, belonging to different races and presenting acute or chronic renal failure of different etiology. Determination of renal parameters was performed in all cases and their progress was closely correlated with the electrolyte and blood gas balances.

The principle of the method is based on the quantitative analysis of the presence of molecules of the gas and electrolyte at a point in the venous blood circulating through interaction with fluorescent sensor molecules that give to the final results needed for balancing the canine patient.

The analysis offer fast results of the following parameters: Sodium, Potassium, Chlorine, Hydrogen ion concentration (pH), carbon dioxide partial pressure (PCO_2), total carbon dioxide (tCO_2), bicarbonate (HCO_3), AnionGap.

For the analysis of blood gases and electrolytes the blood is sampled in a 1 ml vial with green cap (Li-heparin anticoagulant). The sample is then analyzed in a very short time (less than 30 min for blood gases), using the VetStat Idexx machine. Urea and creatinine were analyzed using the Idexx Vet Lab Station for blood biochemistry.



Picture 1. Disposable box for electrolytes determination - left, VetStat Idexx for blood gases and electrolytes determination (orig.)

Picture 2. Idexx Vet Lab Station - blood biochemistry analyzer (orig.)

Results and discussions

All data were summarized in tables and analyzed in the context of clinical patient dynamics. The tables were analyzed and the data obtained was introduced in graphs and subsequently analyzed.

Table 1.

Case dynamics and the relationship between AnGap and renal values

	BUN	CREA	pH	AnGap	HCO_3	PCO_2
Case 1	+	+	-	+	-	-
	+	+	-	+	-	-
Case 2	+	+	-	+	-	Normal
Case 3	+	+	Normal	Normal	Normal	Normal
Case 4	+	+	-	+	-	-
Case 5	+	+	Normal	+	-	-
Case 6	+	+	-	+	-	-
	+	+	-	+	-	-
Case 7	+	+	-	+	-	-
	+	+	Normal	Normal	-	Normal
	+	+	Normal	+	Normal	Normal
	+	+	Normal	Normal	-	Normal
	+	+	Normal	+	-	Normal
	Normal	+	+	Normal	Normal	Normal
	+	+	-	+	-	-
	+	+	Normal	Normal	Normal	Normal
Case 8	+	+	+	+	-	-
Case 9	+	+	Normal	+	-	-
Case 10	+	+	-	+	-	Normal
Case 11	+	+	-	+	-	Normal
	+	+	+	Normal	Normal	Normal
	+	+	+	Normal	Normal	Normal
	+	+	+	Normal	+	Normal
	+	+	+	+	Normal	Normal
	Normal	Normal	+	Normal	+	Normal
	+	+	+	Normal	Normal	Normal
	Normal	Normal	+	Normal	Normal	Normal
	+	+	+	Normal	Normal	Normal
Case 12	+	+	Normal	+	-	-
Case 13	+	+	+	+	-	-
Case 14	+	+	-	+	-	Normal
Case 15	+	+	Normal	+	-	-
Case 16	+	+	-	+	-	Normal
Case 17	+	+	Normal	+	-	-
	+	+	+	+	Normal	-
	+	+	+	Normal	+	Normal
	+	+	+	Normal	Normal	-
	Normal	Normal	+	Normal	Normal	Normal
	Normal	Normal	+	Normal	Normal	Normal
	+	Normal	+	Normal	Normal	Normal
	+	+	Normal	Normal	-	Normal
	+	+	+	+	Normal	Normal

Case 18	+	+	-	+	-	-
	+	+	-	+	-	-
	+	+	-	+	-	-
Case 19	+	+	-	+	-	-
Case 20	+	+	**Normal**	**Normal**	-	-
Case 21	+	+	-	+	-	-
Case 22	+	+	**Normal**	+	-	-
Case 23	+	+	**Normal**	**Normal**	**Normal**	**Normal**
Case 24	+	+	**Normal**	+	-	-
Case 25	+	+	-	+	-	-
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	-	+	-	**Normal**
Case 26	+	+	-	+	-	-
	+	+	-	+	-	-
	+	+	**Normal**	**Normal**	**Normal**	**Normal**
Case 27	+	+	-	+	-	**Normal**
	+	+	-	+	-	**Normal**
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	**Normal**	**Normal**	**Normal**	**Normal**
Case 28	+	+	**Normal**	+	-	-
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	**Normal**	**Normal**	**Normal**	**Normal**
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	+	**Normal**	**Normal**	**Normal**
	+	+	+	**Normal**	**Normal**	**Normal**
	Normal	+	+	**Normal**	**Normal**	**Normal**
Case 29	+	+	-	+	-	-
Case 30	+	+	**Normal**	+	-	-

Analyzing the studied cases reveals that the 30 cases generated 71 complete episodes of analysis, diagnosis and treatment.

From the 71 tests, AnGap was determined normal in 31 cases. Direct association of AnGap values with urea, creatinine, pH, bicarbonate and venous carbon dioxide revealed interesting data that corresponds with the actual literature, extremely poor in specific data related to the change AnGap domestic canidae with renal failure.

In 64 cases AnGap was higher than normal associated with high urea and creatinine or when one of these values have been higher than normal. This represents a percentage of 90.14% of the studied cases, which confirms that AnGap is an extremely useful index in evaluating and forecasting the progression of renal failure in dogs.

Out of the 71 tests, in 50 the pH was modified (metabolic acidosis or alkalosis). From the 50 cases, in 29 cases AnGap was higher and in 21 cases AnGap was normal. This demonstrates that the change in blood pH does not influence the variation of AnGap. The correlation between the two values is poor statistically and predictive.

Out of the 71 tests, 26 showed both HCO_3 and pCO_2 within normal limits. In 23 (88.46%) cases AnGap was normal, which demonstrates a clear and direct correlation with the

literature that AnGap is directly influenced by venous blood gas values. Only in 11.54% of cases, AnGap was had higher values than normal.

Certainly, the determination and analysis of the ionogram and the venous blood gases is the right and impetuous way in the diagnosis and treatment of canine kidney diseases.

Once blood gases and electrolytes balance is adjusted, that leads to the adjustment of umoral balance that leads to an improvement of clinical signs of the patient.

Conclusions

The results of the present study lead to several important conclusions.

AnGap is an extremely useful index in evaluating and forecasting the progression of renal failure in dogs, given that in 64 cases, AnGap was higher than normal, when urea and creatinine or one of these values have been higher than normal.

Changes in blood pH does not influence the variation of AnGap. The correlation between the two values is statistically and predictive weak.

AnGap is directly influenced by venous blood gas values.

Determination of ionogram and venous blood gases is the right and impetuous way for the diagnosis and treatment of canine kidney diseases.

Once blood gases and electrolytes balance is adjusted, that leads to the adjustment of umoral balance that leads to an improvement of clinical signs of the patient.

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BCL2 AND BAX GENE EXPRESSION IN CUMULUS-OOCYTES COMPLEXES IN COW

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Abstract

In vitro maturation (IVM) is the first and the most important step for IVF technique because the success of in vitro embryos production highly depends on oocyte quality. In the present study it was aimed to identify the optimal time of maturation of cattle oocytes, in which IVF techniques are very important and frequently applied. One of the indicators of IVF failure is the apoptosis, occurring in the participating cells on the whole cycle of development. Therefore is essential to identify highly accurate the apoptotic moment. In this respect, the expression of two genes: Bax and Bcl2 was assessed in three stages of maturation of oocytes: time 0, 24h and 48h of cultivation on maturation culture media. These genes have been previously shown to play an essential role in apoptotic process of the cells. The genes expression was evaluated by Quantitative Revers-Transcription PCR, using SYBR Green reagents, having as target the total ARN isolated from the complex oocytes - cumulus cells. The value of genes expression was normalized using as control the Actin gene biomarker. Obtained data were interpreted using $2^{\Delta\Delta}C(T)$ method and the statistical analysis was performed using ANOVA algorithms. In the determinations, it was found that the expression of the two : Bax and Bcl2 genes are somewhat antagonist, so that at 24 hours of maturation time are both over-expressed comparing to the 0 moment. By comparison, Bcl2 expression is low, which indicates that the apoptotic process has not started and that the stress of in vitro cultivation has not reached the maximum level. Bax gene expression indicates the existence of high stress factors, but not the entry of a cell into apoptosis process. Therefore, it can be considered that the optimum time interval for maturation in culture media is 24h. At 48h the Bax gene overexpression compared to the Bcl2 gene expression indicates the entry of cells into apoptotic process, knowing that a very high level of the Bax gene may have an inhibitory effect on Bcl2 gene experssion.

***Key words** : polimorphism screening, variabile number of tandem repeats*

In vitro maturation (IVM) is the process by which immature oocytes are harvested and subject to artificial maturation conditions in the laboratory before being fertilized. Is a breeding technique that generates the mature oocytes capable of supporting embryonic development before and after implantation. Harvesting oocytes can be done on living animal, transvaginally by OPU technique (Ovum Pick-up) or post mortem after slaughter, by aspiration of ovarian follicles. IVM involves the removal of cumulus-oocyte complexes artificially (COC) of antral follicles and their cultivation in standard essential conditions for 24-28 hours (depending on species) to reach metaphase II (MII). (Chankitisakul et al., 2013, Coyral-Castela et al., 2012, Ferre et al.,2016, Gilchrist and Thompson, 2007).

Proper maturation of oocytes is necessary for them to develop their competences necessary in embryonic development. During maturation in vitro, the release of the first polar globe, in cows, corresponds with metaphase stage II and is reached at 18-24 hours after initiation of oocyte cultivation. Sperm can fertilize the ovum even after 16 hours of cultivation, in metaphase II, but the best is that oocyte meiosis is completed. Even if oocyte releases the polar globe after 16 hours of cultivation, it needs a longer period (24 hours) to accumulate the necessary competence for embryonic development.(Ward et al., 2002).

Gene expression is the process by which the gene information is transcribed to synthesize a functional gene product. Quantifying the expression level of a gene in a cell, tissue or organism, provides the necessary information to better understand the functioning of living organisms. The most commonly used method for quantifying gene expression is PCR (Polymerase Chain Reaction) which investigates changes, decreases or increases, of gene

expression or of a set of genes by measuring the amount of the resulting product (Mircu et al., 2015).

Apoptosis is a genetically controlled act, which can be initiated in two major ways, the dead receptor and mitochondrial pathway. The members of the cell protein family Bcl-2 (B-cell leukaemia / lymphoma-2) participate in the regulation pathway of mitochondrial apoptosis and represents one of the most relevant class of regulators of apoptosis by acting in the effector stage of the of apoptosis (Reed and Hematol, 1997).

Bcl-2 gene family comprises around 20 homologues regulators, pro and anti-apoptotic, of programmed cell death. They either disturb or preserve the integrity of mitochondria, thus induce or prevent the release of apoptotic factors such as *cytochrome c*. (Chakravarthi et al., 2015, Kirikin et al., 2013, Sanchez and Smitz, 2012).

The protein Bcl-2 prevents apoptosis and maintains the survival of cell by influencing the release of *cytochrome C* from mitochondria (Yang et al., 1997). BAX (also known as BCL-2-like protein 4), identified in co-immunoprecipitation with Bcl-2 protein, can suppress the Bcl-2 ability to block apoptosis (Jurgensmeier et al., 1998). Cell vulnerability to apoptotic stimulus was determined by the relative ratio between pro- and anti-apoptotic members of the Bcl-2 family (Chao and Korsmeyer, 1998).

Intrinsic mechanism of apoptosis is present in ovarian granulosa cells, and the ratio of Bcl-2 / BAX appears to play an important role in their apoptosis (Senbon et al., 2003, Tilly, 1996).

The evolution of the follicle, regarding the atresia, degeneration or development until ovulation is determined by complex interactions between the members of the Bcl-2 family of proteins. Granulosa cell survival promoted by the gonadotropic hormones is correlated with a reduction in the level of expression of the Bax gene, with no observed changes in the levels of Bcl-2 and Bcl-X_L. High levels of BAX are correlated with granular cell death and follicular atresia. BAX encoding gene expression is found in germ cells and an increased level is correlated with apoptosis of fetal germ cells, but without any change in the level of Bcl-2 (Kim and Tilly, 2004). It was observed that in the absence of BAX protein, granulosa cells are not able to enter into apoptosis, leading to the extension of their reproductive life in mice that were genetically engineered with this deficiency (Kim and Tilly, 2004, Sanchez and Smitz, 2012). Bcl-2 gene expression in the mouse oocyte both during fetal development and in postnatal life and suppress apoptosis indicating that Bcl-2 can promote the survival of germinal cells (Kim and Tilly, 2004, Kirikin et al., 2013).

In cattle, the wave of gonadotropin hormones in granulosa cells induced progesterone receptor expression, developing their resistance to apoptosis (Vintilă,2005). This relationship was also found to cumulus cell complex of bovine origin, in whom treatment for 24 hours with progesterone ratio decreased Bcl-2 gene transcription (Marek et al., 2014, Goovaerts et al., 2011).

The aim of this study was to identify the optimal time of maturation of oocytes collected from cattle by assessment and quantification of Bcl2/ BAX genes expression biomarkers which are recommended in the literature. The genes included in this study indicate the maturation and fertilization in vitro preparation, their expression is correlated with the time of oocyte entrance into the cell apoptosis process.

Material and Methods

The ovaries were obtained from Agrocompany slaughterhouse, Nojag, Romania, from where were transported within one hour in a saline solution supplemented with antibiotics (NaCl 0,9% şi streptomicyn) at 35-37º C. Oocytes were harvested through puncturing the

ovaries with 18G needles, the follicular liquid being aspired into the 5 ml syringe and then introduced in sterile 50 ml tubes containing PBS. After 10 minutes the sediment was removed using sterile pipettes and transferred in Petri dishes containing PBS, for the two cycles of washing. In this manner 75 cow oocytes were obtained.

Oocytes were cultivated on TCM maturation media. For this, oocytes are placed in a Petri dish containing and 10ml TCM and 1ml ECS, this representing the first TCM wash. The second wash was carried in the same conditions and finally, the oocytes are placed into 400 µl TCM media added with 15µl of FSH. Mineral oil is poured drop wise in order to prevent evaporation while placed at incubation. The incubation conditions are as follows: temperature of 38.5°C, with 5% CO_2, for equilibration, for 48 hours.

Biological samples consisted of oocytes harvested before maturation (the 0 moment), after 24 hours and 48 hours of maturation.

The oocytes along with cumulus cells were transferred in another Ependorf tube and centrifugated for 5 minutes at 200 x g (1060 rpm). The sediment was recovered, suspended in PBS and again centrifugated using same parameters. The supernatant was removed and the samples were deeped in liquid nitrogen for 1 minute and after that stored at -80º C till total RNA isolation. For each harvesting moment six biological samples were used according to Table 1.

Table 1

Experimental variants used for gene expression quantification

Bovine oocytes/ ,,0'' moment (**D**)	Bovine oocytes/ ,,24 h'' moment (**E**)	Bovine oocytes/ ,,48 h'' moment (**F**)
D1	E1	F1
D2	E2	F2
D3	E3	F3
D4	E4	F4
D5	E5	F5
D6	E6	F6

Prior to proceding with RNA isolation the cell samples were washed with PBS buffer and sedimented by centrifugation at 3000 x g for 5 minutes. For each sample aproximative 1,5x 103 cells were harvested, this corresponding to a 50 mg quantity as requiered by the protocol. Total ARN was isolated and from sedimented cells using *SV Total RNA Isolation System* (*Promega, US*) commercial kit according to manufacturer's protocol.
Quantity and quality of extracted RNA was assessed by measurements with *NanoDrop 8000* spectrophotometer (*Thermo Scientific*).
From isolated RNA, the cDNA was synthesized using *Hight-capacity cDNA Reverse Transcription* kit (Applied Biosystems) following the producer indications and oligo dT(8) primer, also provided with the kit.

Obtained cDNA was used as template in qPCR reactions using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), according to provided protocol with a *Stratagene Mx3000P* (*Agilent*) real time PCR equipment. The primers sequences (Table 2) used in this study were obtained from the reference literature and were synthesized by *Eurogentec* (Belgium).

Table 2

Sequences of primers used in this study

Expression marker	**Primer sense 5'-3'**	**Primer antisense 3'-5'**
BAX	TCTCCCCGAGAGGTCTTTTT	TGATGGTCCTGATCAACTCG
BCL2	ATGTGTGTGGAGAGCGTCAA	CTAGGGCCATACAGCTCCAC
β-actin	GAGCGGGAAATCGTCCGTGAC	GTGTTGGCGTAGAGGTCCTTGC

Each sample was analyzed in duplicate. For normalization of gene expression in terms of number of copies β - Actin gene was used. For each primer a sample without DNA template considered as negative control was run. For the relative quantification the Δ (ΔCt) method was used (Livak and Schmittgen, 2001). For all of the samples the number of cycle's threshold (Ct) were determined. For relative quantification the Δ (ΔCt) method was used. According to this method the R (the relative ratio between the control and stressed variant) is calculated with the following formula: $R = 2^{-\Delta\Delta Ct}$. The obtained data were interpreted with ANOVA software (Table 3 and 4).

Results and discussions

Reproductive biotechnologies are widely used in animal breeding. Methods like artificial insemination, super ovulation and embryo transfer are allowing the genetic potential of parental forms to be harnessed effectively. In vitro fertilization permits the use of biological material derived from valuable parental forms, but however little used because the survival rate of embryos obtained is very small. The main cause of low survival rate appears to be cell apoptosis induced by the stress of *in vitro* cultivation. In this frame, quantification of apoptosis gene expression in different moment of oocytes maturation can be used for the improving of IVF protocols.

Total RNA was successfully isolated from all biological samples, the quantity and quality being considered optimum for the following qPCR experiments.

The Real-Time qPCR results were analyzed and presented in graphical form (Figure 1 and 2).

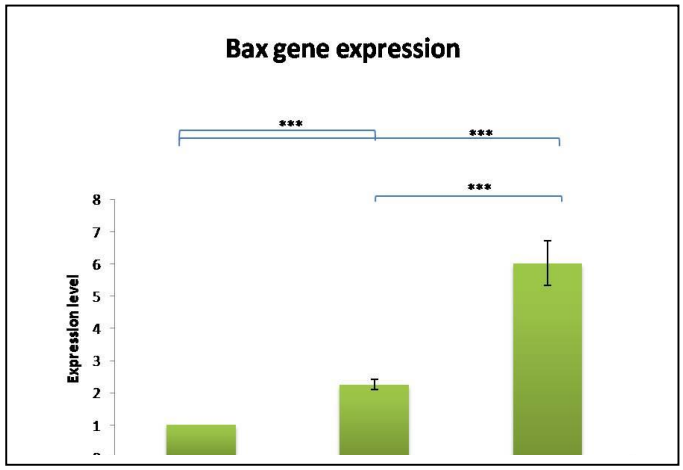

Fig. 1. Expression levels of BAX gene scored in three time intervals.

Gene expression BAX in the case of in vitro matured oocytes with cumulus cells increases proportional with the passage of time from the moment 0 of sowing in culture medium, up to 48 hours, thus showing that the mechanisms of cell protection against stress factors are overwhelmed, heading toward cells apoptosis, a phenomenon closely related to survival of cells (Kirikin et al., 2013). Increasing the level of expression of these genes is very high statistically significant, progressive in all times of observation of cells evolution.

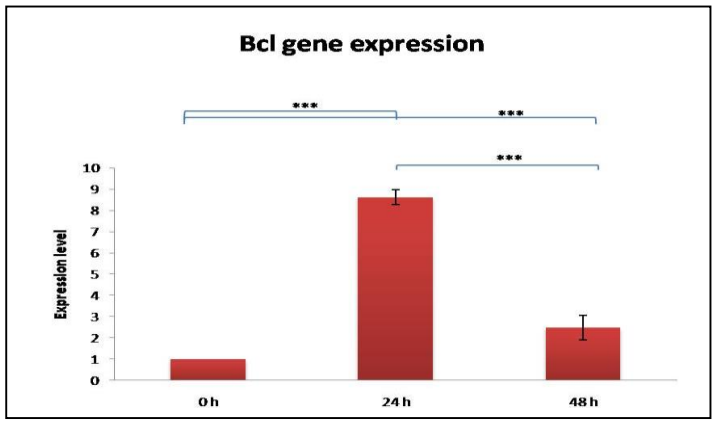

Fig. 2. Expression levels of Bcl 2 gene scored in three time intervals.

Gene Bcl2 is over expressed at 24 h from inoculation, proofing that cells subjected to stress factors, are attempting to adapt to new conditions. Decreasing the expression of this gene at 48 h correlated to enhanced BAX gene expression indicates exceeding the compensatory mechanisms of cell. Also indicates that these cells entered earlier than 48 h into apoptosis, correlated with decreasing viability time related.

The same pattern was observed when the culture medium is added with cysteine, except that this addition results in a period of viability of the oocyte over 48 hours by an almost double expression compared to cells of the cumulus cultured in medium culture without the addition of cysteine (Mircu et al, 2015). Differences in the expression of this gene between all three moments of examination is very high statistically significant.

Gene expression ratio of BAX / Bcl2 is subunitary after the passage of 24 hours of cell culture indicates their ability to adapt to their new environment and preserving the sustainability of these cells at this studied interval. At 48 hours after the passage this ratio is reversed becoming supra unitary, being an indicator of apoptotic processes through which cells lose their viability and thus the ability to be used for in vitro fertilization.

Conclusions

Gene expressions in Bcl2 and BAX evolve somehow antagonistic, so that in 24 hours of maturation both have increased levels of expression compared to moment 0.

At 48 hours the over expression of the Bax gene as compared to the Bcl2 gene expression indicates the entry of cells into apoptotic process.

According to obtained data the apoptotic process begins after 24 hours in cattle. It was found that after 48 hours of cultivation the oocytes are found in the apoptotic process.

Given the correlations between the experimental data and the applicative model it can be stated that identifying the moment of entry into apoptosis of oocytes is indicated accurately by genes BAX and Bcl2. Moreover, these genes evaluated together can indicate the homeostatic status of oocyte.

Aknowlegdements

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PRONUCLEI FORMATION SUBSEQUENT TO INTRACYTOPLASMIC SPERM INJECTION IN BOVINE

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Abstract

ICSI represents the top of the range of the assembly protocols of assisted reproduction which presumes the existence of sophisticated equipments, prior acquired routine in preparing the oocytes and sperm and a very good organization and synchronization of the working time. The aim of the present paper is represented by the assembly ICSI technique in the assisted reproduction laboratory within CLC-HC Timisoara . 70% of the oocytes were considered able for ICSI and out of this number 12 (17%) were destroyed meanwhile micromanipulation; the remaining were fertilized using sperm treated with PVP (G1), TritonX (G2) or untreated sperm (control). Taking into account the outcome results the superiority of the method that prepares the sperm with Triton X, even though the fertilization percentage (35%) is clearly inferior toward the ones reported (60-80% by Sekhavati et al., 2012). Using PVP to prepare the sperm has generated a lower percentage of success (30%) besides a overwhelming proportion of oocytes with one pronucleus (1PN-80%) or unfertilized (NE-20%). Previous reports emphasize the fact that the volume of PVP which gets in the oocyte cytoplasm consecutively injecting the sperm can have a harmful effect on the zygote. The efficiency of Triton X to remove the acrosome, in this way dislodging a consistent enzymatic volume and allowing to decondense the male pronucleus it is demonstrated by the 7 fertilized oocytes (35%) by injecting the sperm treated previous with this solution. Untreated sperm it has generated the oocytes fertilization only in proportion of 11% whereas the male pronucleus does not cover in an useful time and requested proportion the transformations needed to support the fecundation. The culture media used for gamete and zygote preparation had generated lower results toward the reports in the literature. The modest outcomes can be explained both through the culture media composition and through the long execution of ICSI. The presence of both pronuclei in some of the oocytes subdued to ICSI (between 30 and 35% depending on the substance used to treat the sperm), proves our capacity to assemble ICSI technique in the assisted reproduction laboratory within CLC-HC.

Key words*: ICSI, bovine, PVP, TritonX*

Assisted reproduction has massive benefited of the appearance and using the intracytoplasmic sperm injection (ICSI), followed by the birth of many products. Despite the success, there is still pondering the likelihood that ICSI be responsible for the increased infertility in males, hetero- and autosomal chromosome aberrations and mental, physical or reproductive anomalies registered in the offspring that have resulted from the use of this technique. Currently, due to the progress recorded in the use of ICSI, using immature sperm or those with abnormal morphology it is likely to have increased the risks associated with the use of this technique.

Cattle have represented a promise due to the technique well established, but the results were not as expected, with a limited success or even getting a single descendant (Goto et al., 1990), although the published results of Wei and Fukuy (2000) proofed that the oocytes of cattle, consecutive ICSI, are activated and undergo normal fertilization, comparable with IVF, but the division and the blastocyst formation are modest. Unlike in human, in cattle is necessary to apply an activating stimulus of electrical or chemical nature subsequent ICSI, in order to ensure the resumption of meiosis (ionophore calcium A23187 or ethanol).

Keskintepe and Brackett (2000) have enabled oocyte at 30 minutes after injecting the spermatozoon by incubating in a medium containing 50 µM of the ionophore of calcium (A23187) for 5 minutes; the bull sperm was capacitated before injection by incubating it for 1 hour in medium containing heparin. Thus were obtained the rates of division of the blastocyst of 52.4% and 24.4% with reference to the development of the blastocyst.

Chung et al. (2000) have analyzed a method of activation that mimic the oscillations of calcium observed during natural fertilization of cow: the oocytes were activated by three consecutive incubations of 5 minutes each with ionomycin, the periods of incubation being separated by breaks of 30 minutes. Their results have confirmed the observations of those who claimed that only the technique of ICSI itself is not sufficient for the activation of oocytes. They called the partial activation that they have identified as metaphase III (a abnormal stage in which the chromosomes remain condensed after telophase II due to insufficient activation of the ooplasm). They also assumed that such a partial activation could sporadically ensure sufficient cytoplasm factors to initiate the formation of female pronucleus but not enough to process the very stable spermatozoon of bull (they have a very tightly packed core because it contains protamins of type I that are the most cross-linked of all protamins). They assumed in equal measure that the partial activation could be occurring due to the membrane of the spermatozoon that it remains intact. Subsequent investigations have shown that the use of immobilized sperm has led to the improvement of the activation degree of the oocyte as well as the percentage of fertilization.

The successful use of ICSI in cattle is mainly limited due to the reduced capacity of bull sperm to decondense after ICSI. In the last stages of sperm maturation, the nucleus powerful condenses by replacing the histones with protamins and stabilizes by forming numerous disulfide links between protamine. Reduced glutathione (GSH), the endogenous reducer of disulfide plays a key role in the development of the germinal vesicle in the stage of metaphase II as well as in ensuring the protection of the cell against oxidative stress. GSH plays an important role in spermatozoon decondensation and formation of the male pronucleus by reducing the S-S ties to S-H groups in the chromatin structure of the spermatozoon during fertilization and the degree of decondensation of the SPE nucleus at the time of ICSI affect its success (Sekhavati et al., 2012). ICSI avoids the competition that occurs between the sperms in the female reproductive tract as well as the interaction of sperm-oocyte at the levels of zona pellucida and oolema and through these features proves its effectiveness when the number and the quality of the SPE are inadequate for the completion of IVF. In cattle, the success rate of ICSI is restricted by the low proportion of head decondensation of the sperm consecutive ICSI. Activating the oocytes with additional stimuli during ICSI increases the free fraction of cytosolic calcium that will determine the destruction of the cytostatic factor as well as the degradation of the maturation promoting factor (MPF) essential for the resumption of the second meiosis as well as for the formation of the two pronuclei. The activation of the oocyte can be induced by different physical or chemical factors, including electric throb, ionomycin, calcium ionophore, 6-dimetylaminopurine and ethanol, obtaining different results depending on the species.

Consecutive ICSI, the intact acrosome as well as the attendant perinuclear sheath must be removed by the cytoplasm of the oocyte. These events generate extension of the remodeling process of the spermatozoon head as well as the creation of an asymmetry in the chromatin decondensation. Due to the persistence of the perinuclear sheath at the base of the acrosome, the access to the apical part of the spermatozoon chromatin as well as its remodeling are affected creating a situation in which the rear portion of the spermatozoon chromatin is decondensated when the portion of the apical remains unaffected. This postponement of the partial decondensation of chromatin generates a delay in the process of nuclear remodeling including the recruitment of the constituents of nuclear pore as well as delaying the replication of DNA and the first cell cycle after fertilization. From these considerations, the removal of the acrosome or at least its deterioration would lead to superior results of ICSI (Varghese et al., 2005).

The use of Percoll to select useful sperm presents two major advantages: first, the mobile sperms are effectively isolated and secondly, the mobile sperm will be easily selected from the seminal plasma, somatic cells, and dead sperms or immobile. In spite of the indisputable efficiency in the sperm selection, the Percoll method can lead to the calcium coupling and in this way the fertilization interferes.

The failure of ICSI in cattle seems to be due to the incomplete activation of the oocyte generated by the late disintegration of the plasmatic membrane of the spermatozoon inside the oocyte as well as the incorporation of the acrosome into the oocyte which contains a wide spectrum of hydrolysing enzymes. Between the attempts to improve these shortcomings it is stated the individual spermatozoon dismemberment shortly before the injection with the help of lysolecithin- a hydrolysis product generated from membrane phospholipids.

The radical difference between fertilization and ICSI lies in the fact that in the latter, both the plasma membrane and the acrosome are introduced into the oocyte, the contents of the acrosome may exert a harmful effect on the oocyte (Morozumi et al., 2006). Another notable difference between fertilization and ICSI is represented by the fact that the repeated transient elevations of intracellular Ca^{2+} from the oocyte (Ca^{2+} oscillations) - the key signal of the oocyte activation - begins much later in the oocytes subjected to ICSI. The oocyte activation, as evidenced by the completion of meiosis, appeared much earlier in the cases in which the spermatozoon was released from the plasma membrane prior to ICSI.

The quick disintegration of the spermatozoon membrane is important because the activation of the oocyte depends on the oocyte activation factor induced by the spermatozoon (SOAF – sperm-borne oocyte activating factor). In mammals, the SOAF is represented by phospholipase C, localized both in the perinuclear sheath of the acrosomial region (TPRA) and under the plasma membrane of the equatorial segment of the acrosome (MPSEA). Injecting a whole SPE allows the exposure of the SOAF to the oocyte cytoplasm just after the plasma membrane of the two regions disintegrates. Degradation may occur more quickly in some eggs than in others. If the plasmatic membrane of the spermatozoon is removed before ICSI, the SOAF will leak out into the environment and it will be lost. The SOAF coupled to the perinuclear sheath (TPRA) will remain and will be exposed to the ooplasma only after the injection that triggers the Ca^{2+} oscillations. The phenomenon is almost similar with natural fertilization, when SOAF comes into contact with the ooplasma, consecutive to the membranes merger of the spermatozoon and the oocyte.

Another reason for enhancing the embryo development consecutively using chemically treated sperms could be also the fact that the acrosome could remain on the outside. TritonX removes both the membrane of the spermatozoon and the acrosome which contains many enzymes (hydrolases) with a potential negative effect on zygote.

The curtailment of the interval elapsed between the destruction of the membrane of the spermatozoon and the time of ICSI is essential. As this interval is longer, the greater is the likelihood of damaging the spermatozoon nucleus. Endogenous nucleases, which are activated by Ca^{2+}, can cleave the sperm DNA when the core of the spermatozoon is directly exposed to the artificial environment.

The fusion of the sperm and oocyte increase the intracellular concentration of the free Ca^{2+} and stop the histonH1-kinase activity. This determines the production of IP_3 (inositol 1, 4, 5-tri phosphate) at the receptor level via protein G as well as the production of tyrosine-kinase. Through a specific receiver, IP_3 stimulates the type 1 receptor IP_3. This generates an increase of free Ca^{2+} through its exit from the endoplasmic reticulum. The Ca^{2+} ionophore is one of the most used artificial factors for the oocyte activation and balancing the maturation supporting factor. Ethanol stimulates the activation of metaphase II of oocytes and leads to the

IP_3 stimulation from the plasma membrane in the late embryo stages. The combined treatment of oocyte activation consists in phosphorylation or inhibitors of protein synthesis (Korkmaz et al., 2013).

The intracytoplasmic sperm injection technique (ICSI) represents the top of the range of the assembly protocols of assisted reproduction and some of the stages that form it represent the initial steps for cloning, generating and using of stem cells or induced pluripotent cells.

The execution of this technique presumes besides the existence of sophisticated equipment and a prior acquired routine in preparing the oocytes and sperms, and a very good organization and synchronization of the working time. Considering this aspects, by going through all the necessary steps, the aim of the present paper work was represented by the assembly of ICSI technique in the assisted reproduction laboratory within CLC-HC Timisoara and also by the evaluation of the obtained results reflected by attesting the fecundation, phenomenon mirrored by the presence of both pronucleus (female and male).

Materials and methods

Oocytes were obtained from slaughtered cows ovaries. The ovaries were transported in 0.9% NaCl solution within two hours to the CLCHC laboratory of assisted reproduction. Oocytes were harvested by suction (Simona Marc-Zarcula et al, 2015) and only first quality oocytes were cultivated, according to the protocol described by Simona Marc-Zarcula et al, 2014. Since the cultivation time for cattle may be between 22 and 24 hours, after 22 hours from the time of placing in the incubator, the oocytes were subjected to examination for the presence of the first polar body. Oocytes were denuded by vortexing for 30 seconds in TCM199 medium containing 0.1% hyaluronidase (Sigma) and washed in two steps of PBS. Oocytes suitable for the next stages of the ICSI technique were distributed to group 1 (G1) and injected with sperm treated with PVP, Group 2 (G2) - injected with sperm treated with TritonX and the control group (M), in which the injected sperm was not subjected to any treatment affecting their movements or cell membrane.

After thawing, the sperm was evaluated from the point of view of motility on a scale from 0 to 5 and was used only if it was at least 4. The defrosted sperm was selected with mini-Percoll gradients (90% and 45% - Mircu et al, 2015) and resuspended in Sperm-TALP medium. Afterwards they were treated with PVP, TritonX or used directly. PVP is a polymer used in ICSI procedure to increase the viscosity of the sperm solution, facilitating sperm handling and immobilization. For ICSI, the sperm is first suspended in a medium containing PVP and a single sperm is selected and injected into the oocyte, along with a small amount of agent. PVP (10%) (40,000 IU) was prepared by dissolving PVP in culture medium (MSOF active solution). The solution was aliquot and store in refrigerator for up to 2 weeks. Triton X - 50 µl of the sperm suspension was mixed with an equal volume of TCM. The mixture was vortexed 0^0C for 1 minute. The mixture was vortexed at 0^0C for 1 minute. Sperm is washed in TCM by centrifugation (2000x G for 3 minutes) before ICSI. According to literature, the time between preparation of sperm and injection into the oocyte should not exceed 30-45 minutes (Nakai et al., 2011). All steps were performed at the NARISHIGE micromanipulator Axiovert 40 CFL, equiped with a NIKON inverted phase contrast microscope. After removal of cumulus cells, oocytes were put in drops containing 5 µl of IVF-TALP (in vitro fertilization-Tyrode's albumin lactate pyruvate). The sperm was transferred into 10µl culture medium (Sp-TALP) containing 10µl / ml heparin. Before the injection of SPE, 4% PVP was used to slow them down. Each SPE for injection was selected from those who placed into 10µl drop of PVP acceded there head to the bottom of Petri plate. The aspiration needle was used to pierce the oolemma, forming a access to the clear area of the ooplasm, and then was sucked into a small volume

pipette ooplasm, , and then was sucked a small volume of ooplasm into the pipette. SPE and ooplasm sucked were then injected into oocyte with a minimum volume of PVP. ICSI was performed under a microscope, magnification of x200, in drops of 30µl of medium TCM199 + BSA 3 mg / ml covered by the silicone oil, the culture plate 60x15 mm (Falcon, Fisher Scientific) and maintained at 37 ° C in the plate heating microscope. Two drops of TCM199 medium + BSA were positioned on the fertilization micro-plate in witch were introduced five oocytes and were covered with a drop of mineral oil. On the same micro-plate were positioned two drops SOFplus PVP medium or TritonX from which was extracted one sperm. Each SPE for injection was selected from those who placed into 10µl drop of PVP acceded there head to the bottom of Petri plate. The aspiration needle was used to pierce the oolemma, forming access to the clear area of the ooplasm, and then was sucked into a small volume pipette ooplasm, and then was sucked a small volume of ooplasm into the pipette. SPE and ooplasm sucked were then injected into oocyte with a minimum volume of PVP. The oocyte was fixed in micromanipulator with polar body at 6 o'clock and sperm was injected perpendicular (about 3 o'clock) in the perivitelin space after the oocyte membrane cell was dotted and a small amount of cytoplasm drained. After ICSI, the oocytes were transferred to the culture medium represented by TCM199 + BSA 3 mg / ml covered by the silicone oil, the culture plate 60x15 mm (Falcon, Fisher Scientific) and placed in an incubator at 37 ° C, humidity 100% and 5% CO2. 18 hours after sperm injection, oocytes were examined for the presence of pronucleus (Sekhavati et al., 2012). The existence of both pronuclei attests the full completion of fertilization process. Depending on the aspects observed under a microscope, the oocytes were classified into the following categories: 2N (presence of both pronuclei and absence of sperm), 1N (there presence of female pronucleus and the sperm head) and NE (oocytes inactivated / unfertilized inside which no pronucleus was observed). Garcia-Mengual et al. (2015) attests activation of the oocyte through its ability to resume meiosis without the presence of a visible metaphasic plate and having at least one pronucleus.

Results and discussions

The results achieved by the ICSI technique are presented in Table 1 and Chart 1.

Table 1.

The results obtained by the treatment of sperm

Group	Oocytes x ICSI	2PN		1PN		NE	
		n	%	n	%	n	%
G1	20	6	30	10	50	4	20
G2	20	7	35	8	40	5	25
G3	18	2	11,11	3	16,6	13	72,44

During the passage through the epididymis the mammals SPE is highly resistant to chemical and physical disintegration due to conversion of protamines sulfhydryl group in disulfide groups. SPE cattle nucleus possesses particularly strong disulfide bonds, which could explain the presence of stable chromatin, which could prevent decondensing of the nucleus. In ICSI procedure, puncture of the oolemma or aspiration of cytoplasm with micropipette can contribute to oocyte activation. Centrifuging clarifies ooplasm by polarizing lipids, which are recognized in the mask of cellular organelles in most ungulates. Tatham et al. (1996) states that the centrifugation of oocytes (15800xg for 10 minutes) prior to the ICSI procedure contributes

to better visualization of the injected SPE, while Wall and Hawk (1988) believe that the centrifuging does not influence the further development as the polarization of the ooplasm is only temporary. Rho et al. (1998) observed redistribution of lipids in the ooplasm 10 hours after centrifugation. After removal of the cumulus cells, about 70% of the in vitro matured oocytes had GP visible and a dense and uniform ooplasm making them thereby suitable for ICSI (Rho and col., 1998). Of the 761 oocytes injected, 57 (8%) were destroyed in the process of micromanipulation and degenerated within 3-24 hours. Other 32 (or 4%) expelled the SPE into perivitelin space.

The amplitude and the increased concentrations of Ca2 + was believed to play an important role in the events resulting in activation of the oocyte, including regulation of maternal mRNA stored in the early zygote, which could affect gene expression in the late stages of embryonic development and the cellular composition of the blastocyst (Nakai et al., 2011). In pigs, formation rate of pronucleus was lower when using sperm treated with Triton X compared to those resulting consecutively using sperm intact (Nakai et al., 2011).

Concentration and activity of phospholipase C in SPE may be slower due to the treatment of SPE by processes commonly used prior to ICSI in rent animal species. It seems that the procedures used to decondensing the head of SPE before ICSI in pig also causes the loss of phospholipase C, resulting in a weaker signal oocyte activation. Korkmaz et al's study (2013) demonstrated that transferring oocytes after ICSI in culture medium containing 3 mg / ml BSA and CR1aa and then (on the 5th day) in freshly prepared culture medium and supplemented with 5% calf serum affected positively embryonic development. The same study revealed that the best ICSI results were achieved consecutively ICSI using an ionophore calcium in combination with 6-DMAP and high quality oocyte.

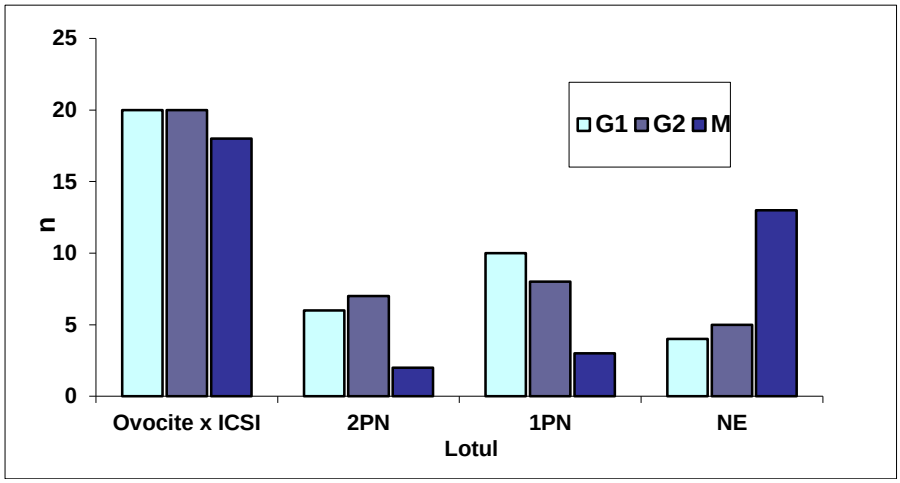

Chart 1. Number of pronuclei variation related to spermatozoa treatment

Out of the 70 oocytes considered fit for ICSI 12 (17%) were destroyed during micromanipulation, a proportion which can vary from case to case depending on both the biological material used and skills of the performer.From the data presented in Table 2 result the superiority of the method which appeals to preparation of sperm with Triton X, even if the percentage of fertilization (35%) is significantly lower than those reported in the literature (60-80% Sekhavati et al., 2012). Chart 1 captures the dynamics of unfertilized oocyte whose percentage rate clearly increases in the situation in which the injected sperm was not subject to any treatment.

Using PVP to prepare sperm generated besides a lower success rate (30%) and an overwhelming proportion of essential active oocytes (1 PN – 80%) or completely inactive (NE – 20%). Specialized studies show that PVP volume reaching oocyte cytoplasm consecutively sperm injection can be harmful for the zygote. The 7 fertilized oocytes (35%) by injecting sperm prepared with Triton X can demonstrate its efficacy knowing that Triton X has the ability to remove the acrosome, deploys in this way a consistent volume of enzyme and allowing decondensing the male pronucleus. Untreated sperm generated only 11% oocyte fertilization – a value highlighted by the literature that the male pronucleus does not make the necessary transformations in useful time to produce and sustain fertilization. ICSI has exceeded the time required to achieve the allowed 30 minutes for contact with sperm with PVP and Triton X in witch in both cases the values close to 60 minutes (59 PVP and Triton X-56). Unlike data reported consecutively human studies, bovine oocytes are not sufficiently activated only by puncture the oolema or aspirating the cytoplasm (Rho et al., 1998). Data presented by Morozumi and Yanagimachi (2005) reveal that when invariably a certain amount of PVP or Triton X reaches the oocyte, injection did not affect the oocyte whether ICSI was removed before the acrosome.

Conclusions

Culture medium and preparation of the zygote generated inferior results those presented in the literature. The modest results could be explained both by composite of the culture medium and long service and execution of ICSI. Both pronuclei in some of oocytes subjected to ICSI (between 30 and 35% depending to the substance used to treat sperm), demonstrates our ability to assemble ICSI assisted reproductive in the CLC-HC laboratory.

Aknowlegdements

The present research was carried in the Laboratory of Assisted Reproduction from the Horia Cernescu Research Unit established through POSCCE SMIS 2669 project.

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COMPARATIVE BOAR AND BULL SEMEN EVALUATION AFTER PERCOLL TREATMENT

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Abstract
There are numerous methods used in IVF both in bovine and pig for sperm separation and Percoll gradient is one of them. After using Percoll gradient spermatic parameters were as following: motility increased to 78.33% in boar samples and to 75% in bull samples; the number of live spermatozoa increased with 42.91%, 31.46% and 21.88% in boar sample, respectively with 19.37%, 9.34% and 5.85% in bull samples; normal spermatozoa increase to 98.52-98.92% in boar and to 84.22-93.97% in bull samples. Acrosome integrity, another parameter studied, indicate that in bull sample intact acrosome was before 83.97%, 87.58% and 88.1%, respectively 91.39%, 93.11% and 92.56% after using Percoll gradient, similar increase was in boar sample to 95.49%-98.42%. Therefore, Percoll is an easier and fast way to select viable and normal spermatozoa for IVF techniques
Keywords: *Percoll gradient, spermatic parameters, IVF*

In assisted reproduction techniques (ART) , used for research and practical purposes in animal field, both gametes quality have a great importance. Sperm separation methods have an important role in ART. These methods suppose selection of motile spermatozoa from nonmotile, removing seminal plasma, cryoprotective agents other debris and materials, and also to initiate the capacitation of the sperm.

In bovine ART there are used some methods for sperm separation such as Percoll, BoviPure and swim-up. Percoll and BoviPure are based on density gradient centrifugation and swim-up is based on motility spermatozoa selection.

Percoll is one commercial medium for the density-gradient centrifugation of cells, organelles, viruses and other subcellular particles. Percoll is especially useful as a first step to enrich for cell populations before attempting finer resolution or extraction of nucleic acids. Since its introduction in 1977 it has become the choice of researchers worldwide. Percoll is composed of colloidal silica particles (15-30 nm in diameter) coated with nondialysable polyvinylpirolidone (PVP). BoviPure is an iso-osmotic salt solution containing colloidal silica particles coated with silane. (11).

There are numerous studies both in bovine and swine reproduction regarding the effects of gradient Percoll on sperm preparation for IVF. In pigs, Noguchi et al. (2013) obtained significantly higher percentages of motile sperm, sperm with intact plasma and acrosome membranes, rates of penetration, cleavage and blastocyst formation after Percoll separation than simple centrifugation (8). In bovine, Samardzija et al. (2006) didn't find significant differences regarding sperm evaluation parameters between Percoll and BoviPure, although cleavage and blastocysts rate were significantly higher for the BoviPure group, the number of hatched blastocysts did not differ (11).

The purpose of this research was to evaluate the effect of Percoll gradient on some spermatic parameters (motility, viability, morphology, acrosome integrity) both in bull and boar.

Materials and methods

We used various solutions, either purchased or prepared in our laboratory. As a base for cell culture we have chosen Earl`s solution (1943) prepared in our laboratory following the

original receipt (for 100 ml solution we used Sigma products: 0.0265 g CaCl2x2H2O, 0.02 g MgSO4x7H2O, 0.04 g KCl, 0.68 g NaCl, 0.01586 NaH2PO4x2H2O, 0.1 g C6H12O6, 0.0011 Phenol Red and 0.22 g NaHCO3). All ingredients were solubilized in ultrapure water (Millipore) and subsequently sterilized by filtering with 22 μm filters (Millex GS Filter Unit) and kept at 4ºC until using. Initially a Earl 10X solution was prepared and for obtaining Earl 1X, 10 ml of Earl 10X were mixed with 90 ml ultrapure water and 0.22 g of NaHCO3.

Percoll stock solution was obtained by mixing 9 ml of Percoll with 1 ml Earl 10X. In order to avoid precipitation, the solutions were mixed through a continuous and very gentle stirring. There were necessary multiple attempts in order to obtain a perfect crystal clear solution. For gradient concentration centrifugation, were also prepared Percoll 90% and 45% by dilution with Earl 1X.

Bull semen sample consisted in three straws from bulls: Maradona, Heynckers and Bonaqua. The straws were thawed at 37ºC for one minute and their content was subsequently analyzed.

Boar semen sample consisted in three probes: Pietrain breed, ID boar 82000, Marele Alb, ID boar 88177 and Duroc breed, ID boar 689/33.

Mobility of spermatozoa was examined with a decimal system (Bara, 2012). Its viability was estimated following the eosine-negrosine (Vital Screen) staining. Morphological features of the semen were estimated subsequently to Spermac staining, using a 40X objective (Leica M3350).

There is no unanimously accepted Percoll protocol for use in veterinary assisted reproduction. The method principle consists of semen centrifugation in a concentration gradient followed by precipitate sampling and semen concentration, morphology, viability and motility assessment. 200 μl of bull semen, respectively 2000 μl of boar semen were slowly introduced in the centrifuge tube already containing Percoll 90% - 45% previously warmed at 37ºC for four hours. The mixture was centrifuged (Hettich 350R) at 600g for 20 minutes. The supernatant was removed and 2 ml of EarlX1 solution was added, than the mixture was centrifuged another 20 minutes at 200g. Again, the supernatant was removed and 700 μl of Earl 1X were added, followed by semen assessment.

Results and discussions

After using Percoll gradient occurs an increase in the number of motile spermatozoa for all samples examined (figure 1). When analyzing boar semen, the highest values were obtained from Large White boar (boar ID 88177), mobility has increased by 20%, and in Duroc breed (ID boar 689/33) with 35%. For the first sample, Pietrain breed (boar ID 82000) the mobility increased by 5% after using Percoll gradient. In average motility increased with 20% in boar samples. Also in bull sperm analyze, motility increased after using Percoll gradient with 5-10%.

In average motility increased to 78.33% in boar samples and to 75% in bull samples. A good boar semen should have during examination at least 70% motility. Due to fact that pig oocytes maturation time is around 48h, boar samples were examined after Percoll using also at 48h, and the motility was still high at 73.33% in average.

The data obtained show the importance of using Percoll gradient in the selection of mobile sperm for later use in assisted reproductive techniques such as in vitro fertilization and intracytoplasmic sperm injection (ICSI).

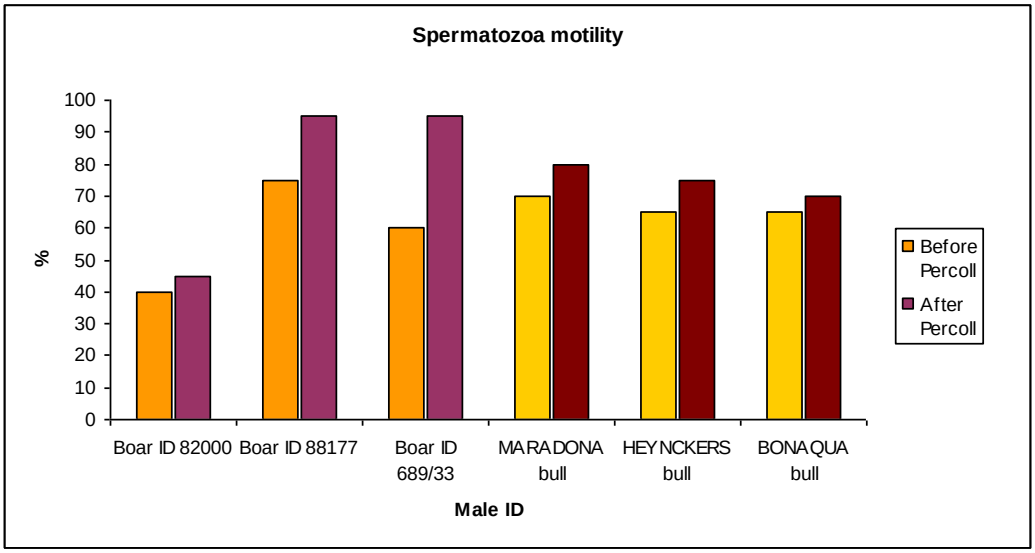

Figure 1. Boar and bull spermatozoa's motility before and after using Percoll gradient

Similar results on boar semen analysis were obtained by Grant et al. (1994), so the use of centrifugation the gradient discontinuous Percoll in the preparation of sperm boar for *in vitro* fertilization resulted in obtaining sperm with mobility and movements characteristic significantly higher ($p < 0.0001$) compared to the group prepared by simply washing. In vitro matured oocytes fertilized with sperm selected by gradient Percoll had cleavage rates significantly higher ($p < 0.0001$), although electronomicroscopic investigations did not reveal ultra-structural differences between groups. Similar results were reported also by Ding et al. (2000)(2).

Studies in pig IVF have shown that almost every studied parameter of boar semen in significantly different between penetration successes and failures, but most of them are interrelated, which emphasize the complexity of sperm functions and the difficulties in assessing boar fertilizing ability .

IVF systems can be used successfully for evaluating the fertilizing capacity and are more accurate than other methods.(3)

From figure 2 it can be seen a positive correlation between motility and viability, thus the number of live sperm after using Percoll gradient has increased for all samples, with 42.91%, 31.46% and 21.88% in boar sample, respectively with 19.37%, 9.34% and 5.85% in bull samples. Similar results analyzing bull semen were obtained by Mircu et al. (2015), Percoll raised the percentage of viable sperm to 40-56%.

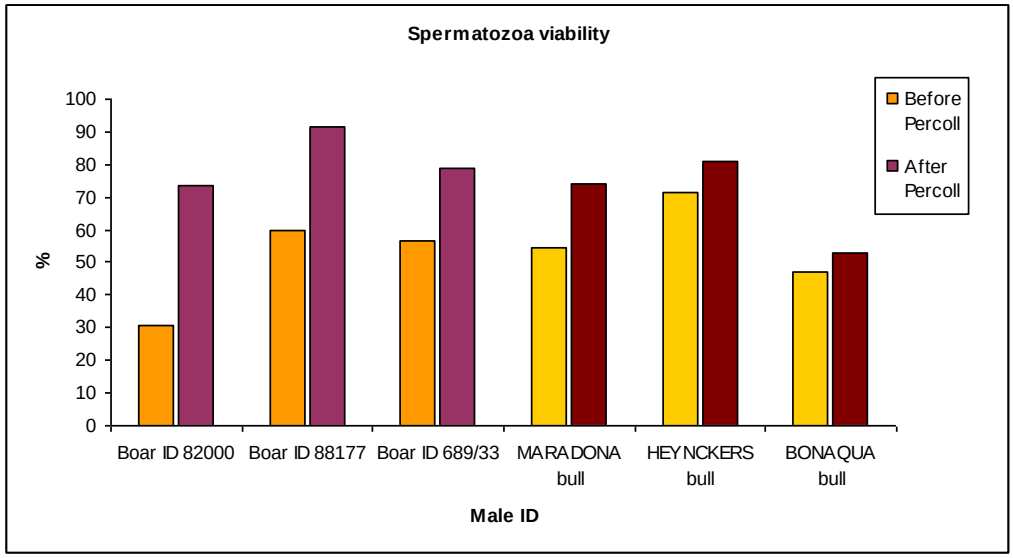

Figure 2. Boar and bull spermatozoa's viability before and after using Percoll gradient

Comparing Percoll gradient with Swim-up, another method used for viable sperm selection in bovine ART researchers obtained a large number of viable spermatozoa with intact acrosome subsequent to Percoll use. The difference of percentage for viable spermatozoa could be explained through centrifuge force action which can affect motility and sperm membrane integrity (Verberckmoes et al., 2000) and also by Swim-up method principle which relies on spermatozoa movements (7, 12). Percoll gradient selects sperm based on their density and is not a physiological mean of separating viable spermatozoa. In cattle more sperm were recovered with Percoll gradient than swim-up, however penetration rate was higher with swim-up separated sperm (Parrish et al., 1995). Similar results were obtain in buffalo (*Bubalus bubalis*) sperm research by Mehmood et al. (2008). They obtained significantly higher motility and greater IVF rate (cleavage rate and cleavage index) with swim-up method, although Percoll gradient separated greater number (6).

Regarding morphology in boar samples, the normal spermatozoa ranged between 34.29-94.05%, while anomalies vary between 5.45-65.71%., and increase to 98.52-98.92%, except sample ID 82000 were the number was lower after Percoll gradient. It can be seen, in figure 3 that the use of Percoll gradient does not increase significantly the number of normal spermatozoa. In bull samples normal spermatozoa increase to 84.22-93.97%.

Similar results in boar were obtained Matas et al. (2011). Thus the use of discontinuous Percoll gradient centrifugation 45/90 increased the sperm with normal morphology to values over 95% compared to 82% as was recorded in the control group. This decrease of sperm with anomaly is given by the spermatozoa with cytoplasmic drop (immature) (low density) and those with defective tail. Having a lower density, they remain in 45 gradient, thus being eliminated from the sample (5).

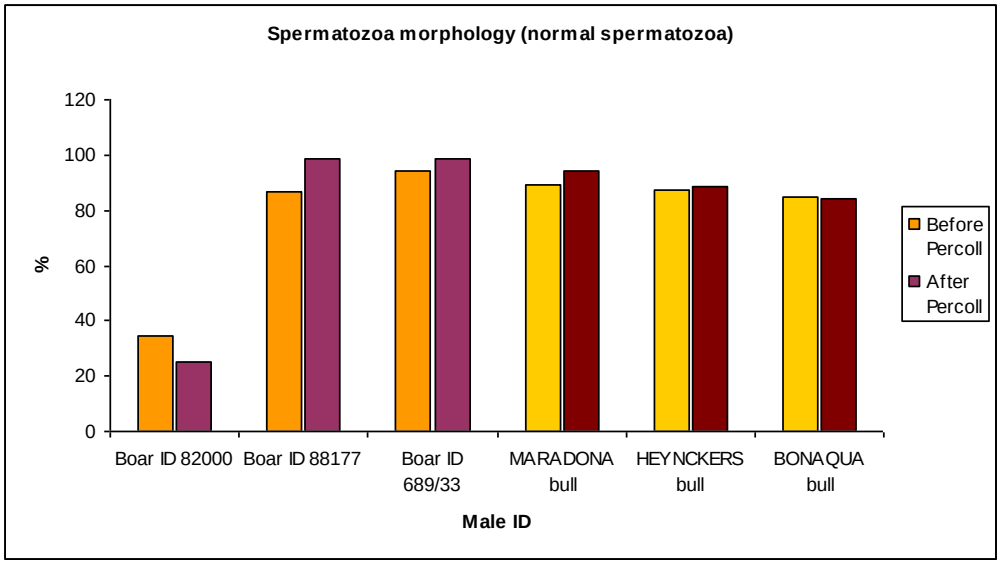

Figure 3. Boar and bull spermatozoa's morphology before and after using Percoll gradient (spermatozoa with normal aspect)

An intact acrosome is required for oocyte fertilization, so his integrity is vital to achieve optimum fertilization. If the percentage of acrosome-intact sperm is low, then fertility may be compromised. Acrosome reaction is seen as multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of sperm head, extensive formation of hybrid membrane vesicles and exposure of inner acrosomal membrane and acrosomal contents (11,14).

Studies indicate that acrosome integrity assessment provides a better characterization of sperm even to mobility. It is considered a good ejaculate if acrosome integrity is more than 51%. The same principles applied to mobility and morphology is valid for acrosome integrity, so if semen is stored for a period of time, this parameter decreases (10).

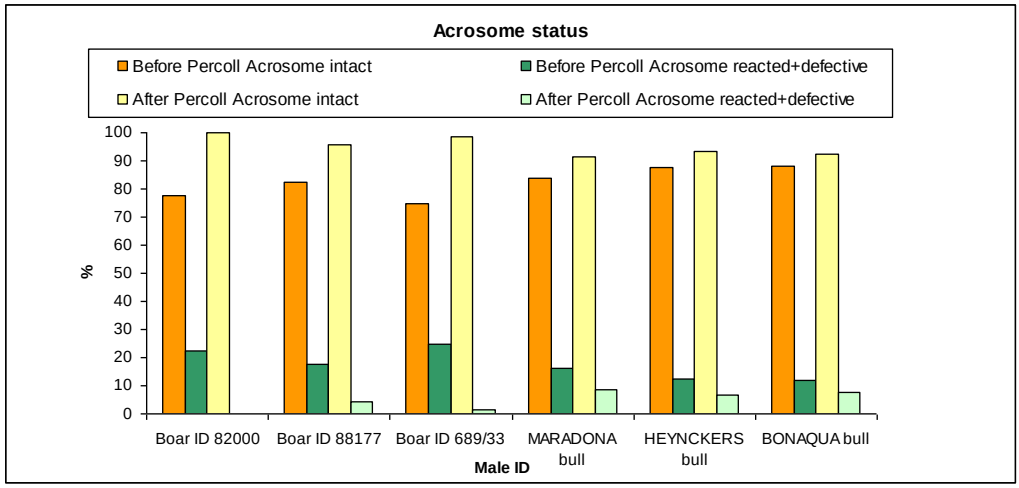

Figure 4. Boar and bull spermatozoa's acrosome analysis before and after using Percoll gradient

The results obtained by Noguchi et al., (2013) points out that the head of the sperm membrane integrity after using Percoll is more important in the development of the embryo *in vitro* than mobility. Also the percentage of motile spermatozoa and acrosome intact after using Percoll gradient was significantly higher than those obtained simply by centrifugation; and fertilization rate after IVF blastocyst formation were significantly enhanced separation on Percoll gradient and were positively influenced by intact membranes of the sperm head (8).

It can be seen in Figure 4 a reduction in the number of spermatozoa with reacted and defective acrosome, which indicates that by using Percoll gradient 45/90 we obtain a higher percentage of spermatozoa with intact acrosome, spermatozoa that can achieve fertilization. The same modification are observed in bull sample, so before Percoll spermatozoa with intact acrosome was 83.97%, 87.58% and 88.1%, respectively 91.39%, 93.11% and 92.56% after using Percoll gradient.

Conclusions

Spermatic parameters (motility, viability, morphology, acrosome integrity) analyzed in bull and boar semen samples after using Percoll gradient were higher than before.

Percoll is an easier and faster way to select viable and normal spermatozoa for IVF techniques.

Percoll gradient along IVF systems in bovine and pig reproduction can be used successfully for accurate evaluation of the sperm fertilizing capacity.

Aknowlegdements

The present research was carried in the Laboratory of Assisted Reproduction from the Horia Cernescu Research Unit established through POSCCE SMIS 2669 project.

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VITRIFICATION OF GERMINAL VESICLE AND METAPHASE II SWINE OOCYTES

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Abstract

In vitro maturation of oocytes is a technique that implies extended cultivation of immature oocytes, recovered by aspiring the cumulus-oocytes complexes from follicles, their maturation under adequate conditions for 48 hours, until they reach the metaphase II stage, so they can be in vitro fertilized later on. Oocytes, once recovered from the follicles, remain in the germinal vesicle stage, representing the diplotene of the first meiotic division at 0 hours of maturation. After maturation, swine oocytes evolve, passing through several stages, from germinal vesicle breakdown, to the first meiotic division and then achieving the metaphase II stage. Vitrification is one of the most interesting cryopreservation techniques, more and more used in the past few years, due to the advantages this technique has, compared to freezing. Vitrification prevents formation of ice crystals during the cooling process; the liquid gets a glassy solid structure, that stops molecular mobility, without any structural reorganization of the liquid that contains the oocytes. Freezing is a slow process, that can take hours until completed and it is very difficult to establish a balance between the agents that produce osmotic injury, due to ice crystals formation and due to osmotic stress. Assisted reproduction techniques rely also on using gametes stored at very low temperatures, in order to enable unlimited access to them. Whereas vitrification can be performed in different stages of gamete and zygote development, in this research we wanted to see the effectiveness of applying this process at the beginning or during gamete processing. Cultivation of oocytes to maturation and their potential use in assisted reproduction techniques, enabled the oocyte development to metaphase II for 52,63% of those belonging to Group 1 (vitrification / cultivation) and for 78,46% of the oocytes belonging to Group 2 (maturation / vitrification / cultivation). Obviously, cultivation of oocytes before vitrification brings more advantages for the meiotic resumption. Regardless the time of vitrification induction, after warming, the oocytes resumed their meiotic process. By using both protocols, we gathered satisfying rates (over 50%) of oocytes ready to be used in assisted reproduction techniques.

Key words*: vitrification, maturation, swine oocytes*

As long as assisted reproductive techniques represents a long chain of different steps, sometimes the lack of gametes could impede on their fulfillment. This is a strong reason for obtaining and preserving gametes in order to use them at request (Gardner et al., 2009). Vitrification is a simple and efficient gamete cryopreservation technique. Storing gametes at very low temperatures, in order to enable unlimited access thereof, can be a technique basic to assisted reproduction (Pankaj and Shashi, 2010). Vitrification can be performed in different stages of gamete and zygote development (Saragusty and Arav, 2011); scope of present research is limited to exploring effectiveness of such process as performed at the very onset, or during gamete processing.

Materials and methods

Ovaries used for present research were obtained from a sow slaughterhouse from Timișoara. COC complexes were collected by follicular puncture from 16 ovaries, from 1 to 5 mm follicles. We used a 18 gauge needle, attached to a 5 ml syringe, rubber piston type in order to prevent damage during emptying the syringe. After puncture, COC follicular fluid was aspirated. Every time the syringe was filled, it was drained into a 50 ml Falcon tube, placed in water bath at 37°C. After puncture, the Falcon tube was left for 15 minutes, for sedimentation of oocytes. The next step was the aspiration of the COC, by a pipette, from the bottom of the tube. This step was repeated 3 to 4 times, every time the pipette being emptied onto a 90 mm

Petri dish which contained enriched PBS medium. Such multiple pipette aspirations were performed to make sure that all of the oocytes (with cumulus cells associated) were collected. Next, COC were successively set onto two 60 mm Petri dishes, into TCM 199 medium, previously filtered and left for 3 to 4 hours in incubator, for equilibration. Thus, COCs were washed and placed onto Petri dishes. After such steps, COC were prepared for maturation, classified as Group 2. Part of the oocytes, after 2 successive washes in 60 mm Petri dishes with TCM medium, were directly denudated, without being maturated. After denudation they were vitrified in their germinal vesicle stage of nucleus, classified as Group 1.

After the final wash, the oocytes were classified into three categories, based on quality thereof (Marc Zarcula et al. 2014). Oocytes classified into such three quality categories, as based on morphological aspects, were then introduced to maturation into TCM 199 medium, supplemented with 10% ECS and FSH (Marc Zarcula et al. 2014). For such step, 3 Petri dishes were prepared, for the 3 categories of oocytes, each containing 50ml TCM 199, with extra 5ml ECS and FSH placed onto of 35mm Petri dish. Then the maturation medium was covered by mineral oil, in order to prevent oxygen acting on the oocytes. The plate was prepared 4 hours before the arrival of the ovaries in the laboratory, and placed in the incubator. The Petri dishes were placed in the incubator for 48 hours at 38.5˚C, in a 5% CO_2 solution, at maxim humidity. After 48 hours, the oocytes were examined by heated plate magnifying glass, to notice the expansion degree of the cumulus cells, the first of the aspects indicating the stage of maturation. By microscope examination, we also revealed the first polar body.

Oocytes in Group 1, which were to be vitrified at 0 time in germinal vesicle stage, were first denudated. Oocytes belonging to Group 2, were collected from the maturation dish by a BIOHIT 10-100µl pipette, after 48 maturation hours. Oocytes in both groups were set into two Ranque-Hilsch vortex tubes, each containing 1ml hyaluronidase 0,5%. After placing the oocytes, both tubes were vortexed for 35 seconds. Such time delay was found to be the optimal for denudation, for the complete removal of the cumulus cells and for avoiding mechanical damage of oocytes

After denudation, oocytes were washed successively in three Petri dishes, containing TCM 199 medium, for the complete removal of leftover cumulus cells. After washing, the oocytes, both group 1 and group 2 were placed each onto a 90mm Petri dish, containing a mix of cryoprotective substances, i.e. 124ml 45% ethylene glycol and 40ml 0.5% sucrose.

Exposure of the oocytes to the mix of cryoprotective substances is a necessary step previous to vitrification. The aspiration of oocytes and cryoprotective substances in the vitrification straws was performed by connecting the straws to the insulin syringe fitted with suction hose. Aspiration is a basic step, as straws will also contain air bubbles, to prevent straw breakage. Regardless of development stage of oocytes under vitrification, such oocytes must be aspirated within the straw.The next step was closing the straws, by melting and sealing, by a MRS1DUAL V2 device. After sealing, the straws were marked by inscription of the time at which they were made for each lot, next immersed into a liquid nitrogen tray, for 1 minute. Finally, straws were lowered into a liquid nitrogen container, to be therein stored, for 5 days. For both oocytes groups, we introduced two oocytes in every straw, to limit possible loss by chance breakage of straws, after warming.

After 120 hours storage in a liquid nitrogen container, the straws in both groups were thawed into warm water, at 37 °C, for 1 minute. The straws were unsealed by cutting the sealed end. The contents of the straws was poured into a 60mm Petri dish, by the insulin syringe used for aspiration, yet at present time discharging the contents into droplets. Next, the droplets were examined by magnifying glass, oocytes to be thus recovered from the cryoprotective liquid and placed onto a Petri dish of 60mm, into TCM 199 medium, for washing.

Oocytes in both group 1 and in group 2, recovered after the opening of the blades, were further washed, in preparation for cultivation. Two 35 mm Petri plates, one for each group of oocytes, were used. The droplets were supplemented by 10 ml FSH and ECS, such preparation being carried out 4 hours prior to usage. Group 1 oocytes collected from the washing medium were placed onto a Petri plate marked *Group 1,* and group 2 oocytes collected from the washing medium were set onto a plate marked *Group 2*. The two Petri dishes were placed into an incubator for 48 hours at 38.5˚C, in 5% CO_2, at maximal humidity. This procedure was testing viability of the two type post-vitrification oocytes. 24 hours later, we examined the oocytes by magnifying glass inspection, concluding that oocytes resumed their activity stopped before vitrification time.

Results and discussion

As concluding the experiment, we recorded results as further detailed.

From a total of 16 sow ovaries, we collected 107 oocyte cumulus complexes. Thus, a 6.68 COC per ovary, the mean value was obtained. Following the follicle puncture, aspiration and subsequent analysis by magnifying glass, COC were distributed as further detailed: Group 1: 42 oocytes for vitrification at 0 time germinal vesicle stage; Group 2: 65 oocyte for vitrification after 48 hours, after reaching metaphase stage II. In the case of group 1 we denudated and vitrified 42 oocytes. In the case of group 2, we denudated and vitrified 65 oocytes post maturation thereof. Analyzing data from Table 1, in terms of thawing after vitrification, no difference was noted between the values for the two groups: 90.47 % in Group 1 vs. 89.23 % in Group 2.

Apud Somfai T. et al. (2009) percentages obtained after thawing the straws in the two experimental groups differed. The percentage of oocytes recovered in the case of those vitrified in the 0 time germinal vesicle stage was 77.6%, while percentage of oocytes recovered in the case of vitrification post maturation was 93.8%.

Growing oocytes to maturation, and potential usage thereof for such assisted reproduction techniques, allowed development to stage metaphase II for 52.63% of oocytes in Group 1, and for 78.46% of oocytes in Group 2. Thus, vitrification of mature oocytes proves clearly advantageous, in terms of meiosis resumption, and as an expression of fertilization capacity.

Running research on oocytes collected from very young sows, i.e. before puberty, Diez et al. (2004) recorded values post cultivation, after thawing, such as 50.1% of oocytes reaching at metaphase II stage in Group 1 (oocytes vitrified immediately after collection, at germinal vesicle stage), and in the percentage of 77% oocytes that reached at metaphase II stage in Group 2 (i.e. oocytes vitrified post 48 hours maturation).

In above quoted study, cryoprotective substances used were ethylene glycol and dimethyl sulfoxide, as well as intracellular cryoproctectors, and sucrose as extracellular cryoprotectant.

As a processing technique, post straws thawing, followed by 24 hours maturation, the oocytes were denudated by vortexing for 25 seconds at minimum speed, while maintaining the two layers of cumulus cells around the oocyte. Diez et al. (2004) issue a number of statements as further detailed: cultivation of oocytes prior to vitrification is important because at such time oocytes have reached at cytoplasmic and nuclear maturation; during vitrification, the meiosis process is suspended; after warming, the meiosis activity is resumed; for the oocytes vitrificated in their early development stage -germinal vesicle-, the percentage of oocytes reaching the metphase II stage after warming is lower than in the case of oocytes vitrificated after maturation.

Table 1.

Oocytes characteristics at various steps

	Step	Number	Percentage
Group 1	Denudation and vitrification on day 0	42	100
	Thawing after 120 hours	38	90,47
	Germinal vesicle	39	90,47
	24 hours cultivation	38	90,47
	Metaphase II	20	52,63
Group 2	48 hours cultivation	65	100
	Metaphase II	65	100
	Denudation and vitrification	65	100
	Thawing after 120 hours	58	89,23
	24 hours cultivation	58	89,23
	Metaphase II	51	78,46

Table 2.

Germinal vesicle and metaphase II

	Number of oocytes	Germinal vesicle		Metaphase II	
		Nr.	%	Nr.	%
Group 1	42	38	90,47	20	52,63
Group 2	65	-	-	51	78,46

Table 2 is a clear representation of the fact that the protocol we used is suitable, because more than a half of the oocytes (52.63%) vitrified at the germinal vesicle stage have reached metaphase II. We can also see that the initial number of oocytes used in this study (42 vs. 56) had an impact on the percentage of oocytes that reached metaphase II stage (52.63% compared to 78.46%). In the process of vitrification, the most essential are the cryoprotective substances. In this study, by using ethylene glycol, in an adequate way, we avoided the toxic effects caused by overusing cryoprotective substances and also osmotic injury was prevented, due to the sucrose addition, which plays the role of an osmotic inhibitor. Thus, the mix of the two cryoprotective substances, ethylene glycol in a concentration of 45% and sucrose, is the most favorable in the process of oocyte vitrification, as they ensure the intact storage of oocytes and also their viability after warming.

Conclusions

Considering the sow oocytes with germinal vesicle, at thawing were not revealed differences among those freshly obtained or previously cultivated. Irrelevant to the vitrification moment, subsequently thawing all oocytes resumed the meiosis. Using both protocols generated a satisfying percentage (over 50%) of oocytes suitable for assisted reproduction techniques.

Aknowlegdements

The present research was carried in the Laboratory of Assisted Reproduction from the Horia Cernescu Research Unit established through POSCCE SMIS 2669 project.

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COMPARATIVE STUDY OF LAPAROSCOPIC SURGERY OF FEMALE GENITAL APPARATUS IN SOW AND BITCH

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Abstract

The advantages of using the laparoscopic surgry to the detriment of traditional surgery are reducing pain and post surgery complications and obtaining a better view of the area. The aim of this study is to evaluate the efficacy of using ultrasonic scalpel and bipolar scissors in laparocopic ovariectomy (OV) and ovariohysterectomy (OVH) in bitch and sow. Also, it has been tried to assess the degree of difficulty of the ovariectomy and the ovariohysterectomy, espectively in the two species. The study was conducted on 6 animals (two sows and four bitches). The introduction of the pneumoperitoneum induction was performed using the Veress needle. The surgeries were performed through the abdominal cavity using three trocars helping to introduce the laparoscope, an atraumatic forceps and the ultrasound scalpel or the bipolar scissors, following a previous skin incision. Using the ultrasonic scalpel successfully complements other instruments such as the traumatic forceps, scissors and haemostatic tool. Through its excellent haemostatic capacities there have been not registered significant blood loos as a consequence of the surgical removal of the ovaries or the uterus with the ovaries, when either the ultrasonic scalpel or bipolar scissors were used. Due to the difficulty of surgical laparoscopic removal of the sow genital apparatus, connected to the similarities in the the two species genital apparatus, it is reasonable to conclude that the surgery in sow is a model for the surgery in bitch.

Key words*: bitch, estrus control, laparoscopy, sows.*

Introduction

Even if the laparoscopic surgery dates back in 1985, yet very few veterinary doctors are familiar with the method. It is estimated that less than 1% of vets master this method in England (12). In our country this percent is considerably small. The reasons for this niche surgery not to be known are the high price of equipment and though the costs of surgery, longer surgeries, and the difficulty of reaching a corresponding ability to perform the surgery (he need of training on different types of simulators for a long period of time) (4).

The ovariectomy and the ovariohysterectomy are the most used methods for surgical control of dog population. Numerous scientific papers reveal the advantages of using OV detrimental to OVH (8, 14). Minimally invasive surgery, particularly laparoscopic ovariectomy, has many advantages over traditional open surgery laparoendoscopic using either the single - or 3 portal site laparoscopic approach; namely, less postoperative pain, low morbidity, smaller incisions, better viewing of the ovarian pedicle, less risk of complications associated with surgical manipulation of the abdominal viscera, and faster recovery to normal activity (2, 6). Based on a study by Devitt et al. (3) the laparoscopic sterilization was found to be 62% less painful than traditional surgery in dogs.

In surgery, two types of energy are used: electromagnetic energy used n monopolar (ME), bipolar (BE) and advanced bipolar and laser surgery and mechanical energy which includes manual and mechanical sutures and ultrasonic scalpel. The latter is a system capable of producing clinical effects of cavitations, proteic fusion, coagulation and cutting due to the ultrasound energy which amplified and released in the tissues. The ultrasonic scalpel is a cutting, haemostasis and dissection device operating at 55.5 kHz resonant frequency (1, 11).

Materials and methods

Initially, the training period was accomplished using a digital SIMBIONIX LAP Mentor simulator, equipment belonging to the Centre for Laparoscopic Surgery and Microsurgery "Pius Brânzeu" within the University of Medicine and Pharmacy "Victor Babeş" Timisoara and another simulator that was practiced on, namely 3-D MED, was the equippent of the Zooprophylactic Institute in Palermo.

The study was developed at the Experimental Zooprophylactic Institute "A. Mirror" in Sicily and in the CLC Horia Cernescu in Timişoara. 6 intact females were studied, two crossbreed of Large White with Landrace sows, weighing 35 kilograms and 4 crossbreed bitches, weighing between 16 and 27 kilograms. All females included in the study underwent complete physical examination and had no previous or current history of illness. The animals were divided into two groups G1 and G2. A sow (which underwent OV surgery) and two bitches (which underwent OV and OVH, respectively) took part in G1. In this group of animals the female genital tract excision was performed using ultrasonic scalpel (Ethicon Ultrasonic Device - HARMONIC ACE 7). G2 had the same display, a sow (OV surgery) and two bitches (OV and OVH surgery, respectively). In G2 the female genital removal was performed using bipolar scissors (STORZ).

Pre-anesthesia was performed using ZOLETIL 50 VIBRAC, a product containing a combination of an analgesic opioid (tiletamine) and a benzodiazepine anesthesic (Zolazepam) in a dose of 10 mg / kg body weight administered intramuscularly. The induction occurred after the administration of propofol (2 mg / kg body weight) (Rapinovet 10 mg / ml). Maintaining of the anesthesia in all subjects in both groups was achieved with isoflurane (ISOFLO) and 100% oxygen administered by inhalation.

Preparing animals for surgery consisted in a diet based on fluids 12 hours before surgery. After the preanesthesia, the animals were intubated using a laryngoscope. Size 7 endotracheal tube placement was made after a preliminary lifting the animals epiglottis using the laryngoscope. The animal fur was trimmed and the abdomen skin was disinfected with betadine. The animals were restrained on the surgery table in the supine position, slightly inclined (10 - 15^{o}), with the hindquarters located above (Trendelenburg position) and limbs in extension. The urinary bladder was emptied by catheterization. To be noted that in the porcine species the bladder catheterization is very difficult, which is why only one of the two females could be catheterized.

The auricular vein (in sows) and the cephalic vein (in the bitches) were catheterized to enable administration of the anesthetic agents and fluids during surgery. Each subject was administered therapy with liquid, Ringer's lactate at a rate of 10 ml / kg / h.

The anesthesia monitoring of the subjects was carried out by assessing the vital parameters such as oxygen saturation, pulse, heart rate.

There were used two video monitors, one fixed of laparoscopic surgery belonging to Storz and the other mounted on a wheel winch, the image sending via wireless technology, surgery table electrically operated drive, electro-surgical unit (electrocautery) with electricity monitoring system, laparoscopic equipment mounted on a wheel winch and composed of a light source, gas insufflators , camera recorder on hard drive.

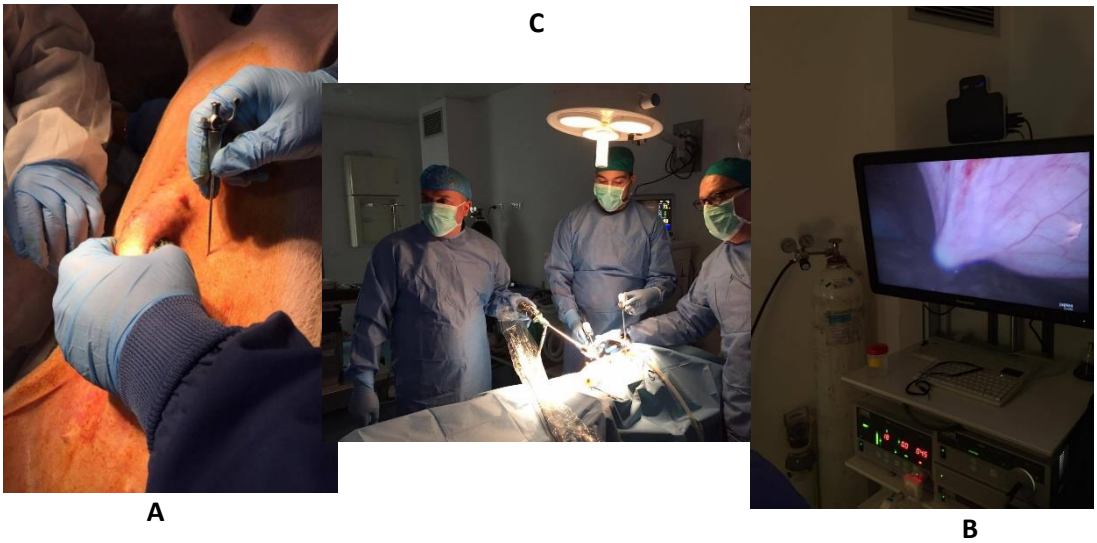


Fig. 1 **A** Introducing the pneuoperitoneum using a Veres needle, **B** Inserting the cannulas under endoscopic control, **C** Positionng the cannulas and the doctors during the surgery

The pneumoperitoneum induction was preceded by making an incision of about 3 mm at the umbilical scar of the bitch and easy aside the umbilical scar of sows (because of a recent surgery). The pneumoperitoneum induction was performed using the Veress needle (Fig 7) after a prior handhold of the abdominal wall and its pulling up. The pneumoperitoneum was established with an electronic insufflator to 10 -12 mmHg with a flow rate of 1 L / min Using CO2. After the pneumoperitoneum was induced, the Veress needle was withdrawn through the same hole and a 5 mm trocar was introduced. Throgh this trocar the laparoscope (STORZ, forward-oblique telescope Hopkins II 300, 5mm diameter) was inserted helping to visualize the abdominal cavity. The laparoscope used was one of 30 o and it is connected to a miniature camera and a cold light source, the camera control unit automatically taking parameters brightness and contrast settings. In the case of our surgeries, a laparoscope having a thickness of 5 mm was used.

The other two cannulas are introduced by visual contact, pressing with them on the abdominal wall and viewing the cone obtained by the pressure. (Fig. 1B). Provided that the endoscope is endowed with a light source, through transparency one can view any larger blood vessels in the abdominal wall. It is preferable that these vessels flagged by the light source to be avoided by the trocar.

After exploring visualy the abdominal cavity, the uterine horns were tried to be identified, this stage representing the initial one in the ovaries identification. After locating a uterine horn or uterine body it was proceeded to the genitalia visualising, using the a traumatic forceps, until the ovary is identified. Once the ovary is located, one of the atraumatic forceps is withdrawn and in its place the bipolar scissors or ultrasound scalpel were introduced in order to initiate the hemostasis. To initiate the electrical or ultrasonic coagulation the ovary is held with one atraumatic forceps and the suspensory ligament of the ovary is viewed upon, that being the place where the haemostasis begin.

In the OV method, the excision procedure by electrical coagulation started at the suspensory ligament of the ovary, continued in the mezovarium and ended at the own ovary ligament. In the OVH method, the excision was carried out starting the electro-coagulation to

the suspensory ligament of the ovary (Fig. 2 A) was continued at the mezovarium, mesometrium and the uterus was cut at the uterine body level. Due to the haemostasis performed by the electric scalpel it is no longer necessary to suture the uterine stump as it us in the classical surgery.

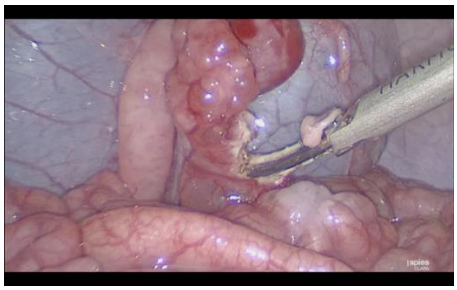
In females o which was carried out the OVH removal of the ovaries and uterus, or only ovaries in case of the females subject to OV method, any of these removals were performed after a prior expansion of the hole created by the introduction of the trocar. Widening the hole was done by using a scissors or a haemostatic forceps. After fixing one end of the uterine horn with a forceps, a portion of it is brought into the surgery spot. From now on, the genital is clutched to extract.

The abdominal incision was closed in two layers using a 3\0 USP braided absorbable material (3/0 Surucryl; SURU Int, India) and a simple interrupted suture pattern.

The aim of this study is to evaluate the effectiveness of using the ultrasound scalpel (Ethicon Ultrasonic Device - HARMONIC ACE +7) and the bipolar scissors (Storz) in OV and OVH in bitch and sow. Also, it has been tried to assess the degree of difficulty of an OV or OVH, respectively in the two species.

Results and discussions

For the both species studied, the ovary excision was made after a preliminary identification of the genital tract. In sows, the uterine horns are located with more difficulty, because the uterine horns show similarity with the small intestine.



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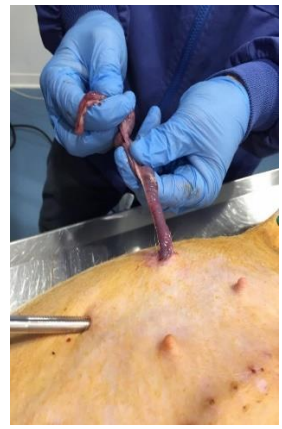

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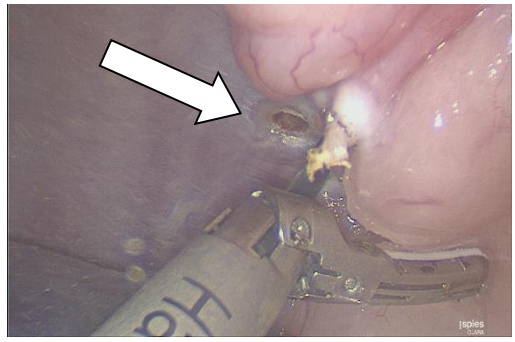

C

Fig. 2 **A**. Exposure and coagulation of the ovarian pedicle area accomplished by traction of the proper ovarian ligament. **B** Removal of the uterine horns and ovarys **C**. Peritoneus burning during surgical procedure.

Because of the anatomical features of the female genital of the sow, the bladder catheterization is more difficult compared to bitch. The bladder could not be emptied by catheterization before surgery to one of the studied sows, which is why we found a slight difficulty in manipulating genitalia. Because of this through the use of ultrasonic scalpel a burn has been created, at the peritoneal level, the burn having a diameter of about 2-3 mm (Fig. 2 A).

According to data in the studies, the use of coagulation instruments can accidentally create burns to the visceral peritoneum or adjacently. This accident is indicated in the studies as one of the most common complications of laparoscopic surgery. The most complications in the laparoscopic surgery are related to abdominal cavity access and pneumoperitoneum establishment, haemorrhage, perforation viscera and tissue damage due to energy application (7). We believe that this accident was due to faulty perception of the depth of the work area from the vet, a fact noticed by other clinicians in the studies (2, 4, 9, 11).

The burns at the peritoneal area can create more pain to the patient after the surgery (2, 5). Burning in the peritoneum is considered a less serious accident compared to the burn in viscera. For example, the burn of the small intestine can cause injuries that will later produce stenosis, with all the shortcomings that occur. Production of injuries in a tense bladder due to its content or the intestine injury could cause the cavity organ to be damaged with major repercussions on the health of the animal (5, 10).

Using the ultrasonic scalpel allowed the use of a single instrument with clamping, cutting and coagulation properties. Due to the capacity of this haemostatic forceps to achieve a good haemostasis, there was no significant blood loss. Haemostatic forceps, due to its ability to achieve good haemostasis were not registered significant loss of blood, thus the vascular ligatures proving unnecessary. Both bipolar vessel sealer and the ultrasonic scalpel, which facilitates sealing and dividing the ovarian pedicle, has been shown in both groups (G1 and G2) to be feasible, safe and reduce surgical times in both the OV and OVH approach.

Because the sow uterine horns are longer, the OVH technique was estimated to be more difficult than the technical OVH in bitch.

Any attempt to learn a certain laparoscopic surgical technique must be preceded by a "workout" of the surgeon on the simulator to strengthen coordination brain - hands, so necessary in this technique.

Laparoscopic surgery entails challenges relating to instrumentation and optics and surgeon undertaking laparoscopic are required to have specific hand-eye coordination skills. These skills, including altered depth perception and the operation of long instruments with a fulcrum effect are not learned by performing conventional open surgery (14).

Conclusions

1. Because the laparoscopic surgical technique of ablation of the female genital sow has a higher degree of difficulty, along with similarities between the reproductive apparatus anatomy of the two species, the sow can be a model for the surgery of the female genitalia of the bitch.
2. There were no significant differences regarding the use of bipolar scissors or scalpel ultrasound on the proteic coagulation.

Acknowledgments

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staff members of the *Institutului Zooprofilactic Experimental din Sicilia "A. Mirri"* particulary the Laparoscopic Unit.

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EVALUATION OF THERAPEUTIC EFFICIENCY OF ELECTROSTIMULATION IN DOGS USING ELECTROMIOGRAPHY

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Abstract
Neuromuscular electrostimulation uses a low electric current which allows recruitment and muscle contraction in patients who have lost the ability to voluntary muscle contraction as a result of a neurological disease (Dolhem, 2008). The aim of our study was to evaluate the effect of electrostimulation on atrophied and denervated muscles, using electromyography. The study was conducted on a group of five dogs which were presented with various neurological disorders in the Medical Clinic of the Faculty of Veterinary Medicine Iasi. Clinical and radiological examinations revealed inflammatory and degenerative processes and intervertebral compactions. A protocol of 30 physiotherapy sessions was initiated, each session using techniques like electrostimulation, passive/active exercise and therapeutic massage, and every 10 sessions, electromyography was used to assess the response to therapy. The results were the progressive increasing of muscle tone and clinical improvement.
***Key words**: electrostimulation, electromyography, dog, physiotherapy*

Introduction

Neuromuscular electric stimulation (NMES) use a low-level electrical current which, through alpha motor nerve stimulation, allows recruitment and muscle contraction in patients who have lost the ability to voluntarily contract muscles, as a result of neurological disorders manifested clinically by paresis or paralysis (Dolhem, 2008). Therefore, the primary therapeutic goal of NMES, regarding recovery, is to strengthen the muscle, followed by a wide range of other therapeutic benefits, such as: reduction of neurogenic muscle spasms, increased mobility of joints, blood flow stimulation, edema and pain sensitivity reduction, increased wound healing, sensory awareness improvement and function recovery (Carroll et al.., 1992; Pape et al., 1993; Taylor et al., 1993; Zupan et al., 1993).

In neurology, the use of NMES has two major objectives, namely to support muscle recovery and endurance from flaccid motor paralysis associated with peripheral motoneuron damage and to reduce muscle spasm in spastic paralysis associated with damage to the central motoneuron. (Baxterand McDonough, 2007). In order to achieve the desired result with electrostimulation, we concomitantly used in our study kinetotherapy techniques such as massage and exercises with specially designed physiotherapy balls and balance platforms.

Material and methods

Our study consisted of five cases, selected within the casuistry of the Medical Department within the Veterinary Medicine Faculty of Iasi, dogs aged between 2.5 years and 14 years, all showing various neurological disorders and muscular atrophy of the hind limbs, associated with difficulty to maintain weight on the hindquarters.

Clinical, radiological and hematological findings confirmed the presence of either aseptic inflammatory processes or demineralization and compaction of the vertebral spine.

During the neurological examination, it was observed that all of the patients included in the study presented walking incoordination of the hind limbs (dismetria - spinal ataxia). The patients could sustain their weight when all of the four legs were in a fixed position, but they were losing the ability to constantly sustain their weight on the hind legs during walking. In

standing position they tended to cross the hindlimbs. Reflectivity was normal on the hind legs, but the thigh muscles were visibly atrophied.

Regarding working materials, we used the Neuropack S machine, MEB 9400 K for electromyographical investigations and Intelect® Vet for electrical stimulation, special balls for physiotherapy and balance platforms.

Electromyography implies recording the electrical activity of the muscle, generated spontaneously or consecutive to muscular contraction.

Electric biopotentials were recorded using the Electromyography (EMG) program of a Neuropack S, MEB 9400 K (Nihon Kohden) electric diagnosis device. The examined areas were shaven, degreased using alcohol and Skine Pure (Nihon Kohden) abrasive paste, and the surface electrodes were applied using Elefix gel.

The electromyographical test was conducted on the following muscles: deltoid, triceps, extensor carpi radialis and brachial biceps in the anterior limb, and femoral biceps, quadriceps and cranial tibial in the posterior limb. The active electrode, i.e. the electrode that collects the bioelectrical data, consisting of a single-use bipolar concentric needle (50 x 0.45), was inserted through the skin in various areas and at different depths in the examined muscle, whereas the reference electrode was placed in the motor point of each muscle. In order to gather information as accurate as possible on the clinical status of each of the examined muscles, they were tested in 5-10 areas.

Interpretation of results is carried out after recording the electrical activity which is displayed on the oscilloscope. The presence, size and shape of the curved lines (action potential) provides information on the muscle's ability to respond to nervous stimuli. Every muscle contraction that produces a potential of action, and the muscle fiber size influences the rate (frequency of action potential formation) and the size (amplitude) of the action potential.

Intelect® Vet is a revolutionary product for veterinarians who provide physical therapy to animals. The device is equipped with two independent channels of electrotherapy and includes four types of currents - Interferential, Premodulated, Russian, High Volt - and an ultrasound probe with a frequency of 1 and 3 Mhz used in the rehabilitation of patients with orthopedic or neurological problems. Thus, the device offers four methods of therapy in a secure system, namely: electrical stimulation, ultrasound stimulation, STIM combination and ultrasound, and laser therapy.

With the purpose of rehabilitating the patients, 30 sessions of physiotherapy were performed using electrostimulation techniques, therapeutic massage, passive range of movement and active range of movement (physical exercises).

Regarding electrostimulation, a program to reduce muscle spasms and pain relief was used during the first 10 sessions, followed by 20 sessions of stretching for muscle toning. Stretching or controlled extension of the muscles and joints bring amazing benefits to any organism; it reduces muscle tension, improves blood and lymphatic circulation, as well as joint mobility. It also contributes to a better rest and relaxation. Massage is a technique of exercising pressure on the cutaneous, subcutaneous and muscle tissue. The pressure applied to the tissue has multiple effects on tissular movement and blood and lymph circulation. Thus, it carries many therapeutic effects regarding the muscle and connective tissue and painful sensitivity.

Neuromuscular electric stimulation was applied transcutaneous to the muscle motor points, after the area of interest was previously trimmed and disinfected and a small amount of special electrotherapy gel was applied to the adhesive electrodes to facilitate the transmission of electricity.

Results and discussions

The improvement was tested by performing an electromyographic evaluation every 10 sessions.

The electrophysiology supported the prognosis and guided the physiotherapy in spinal conditions, revealing abnormalities that objectify damage to the nerve's roots and/or peripheral nerves. Therefore, during electromyography, in all patients, paths of neurogenic type were registered, characterized by spontaneous activity ranging from moderate to severe in the examined appendiceal muscles, mainly consisting of giant potential (fig. 1) and fasciculation potential (fig. 2) (before physiotherapy).

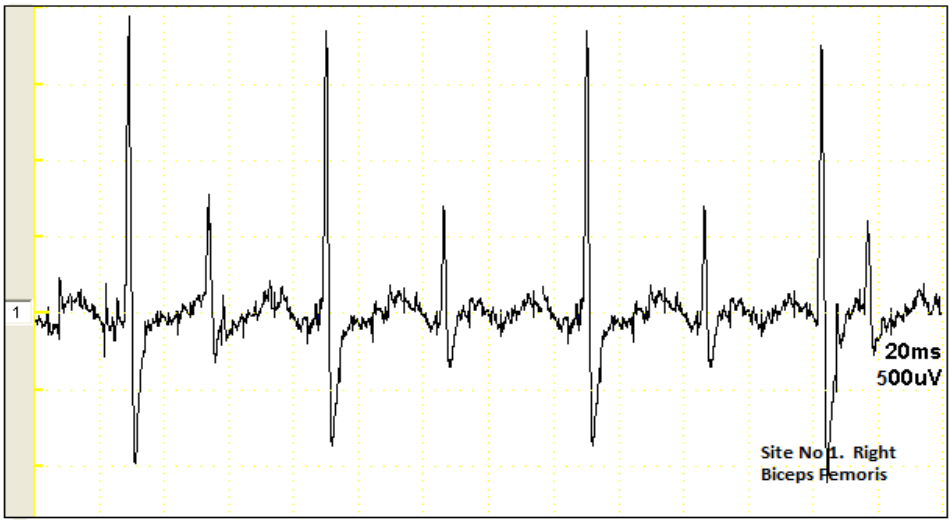

Fig. 1 Giant potentials. Dog, 7 years old

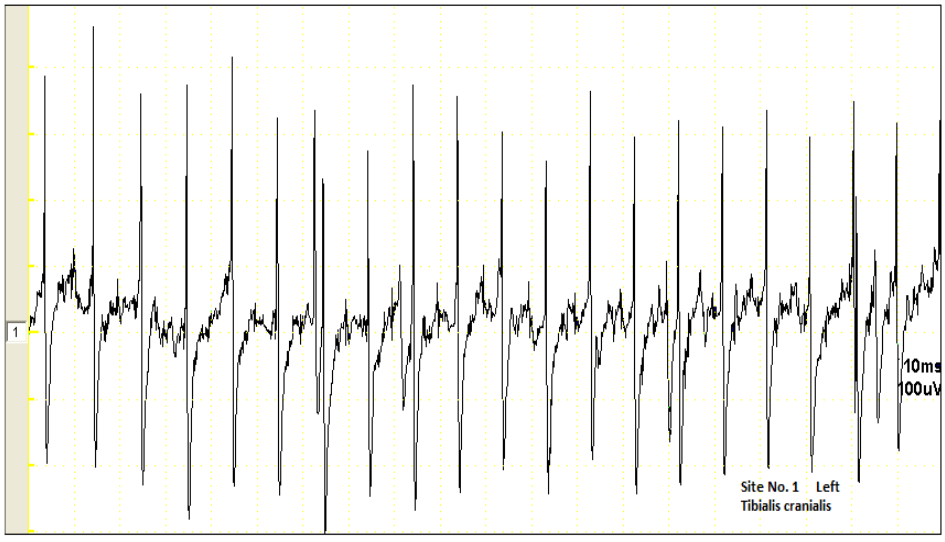

Fig. 2 Fasciculation potentials. Dog, crossbreed, 9 months old

The electromyograms have highlighted neurogenic routes consisting of slow sharp waves confounding with potential fibrillation (fig. 3), giant potential confounding slow positive waves (fig. 4) and complexes of repetitive discharges (fig. 5), in all dogs after 10 sessions of physiotherapy. After the 30thsession, in 4 out of 5 patients, electrical silentium was registered

(Fig. 6). The fibrillation potential and the positive sharp waves derived from the same pathological changes. Both arise from spontaneous combustion of individual muscle fibers as a result of the destabilization of their sarcolemma. They occur in denervation and usually have a bi- or triphasic form, its amplitude ranging from 100 to 300 μV, with a duration of 1-5 miliseconds and a regular rhythm of approximately 13 Hz. It is considered a muscular fiber potential which has lost its ability to accommodate and it is in a permanent depolarization-repolarization state. Its sound is similar to the noise of frying an egg or to the noise of raindrops falling on a tin roof. A significant reduction in fiber is an indicator of the successful commencement of the motor nerve reinnervation. (Aminoff, 1999; Cuddon, 2002).

Applying a passive range of motions, we stimulated and rebuilt the affected limb joint mobility. The exercise induced by active movements, such as using the balance platforms and therapeutic balls, helped patients to regain proprioception and balance. Therapeutic massage performed using anti-inflammatory gels obviously helped to stimulate blood circulation (Zbângu et al., 2014). In our study it was observed sequentially that, with each session of physiotherapy, there was a clear improvement of the general condition and, therefore, an increase of the muscle tone. Electrophysiological testing was used to observe the improvement of the nerve pathways of affected peripheral nerves.

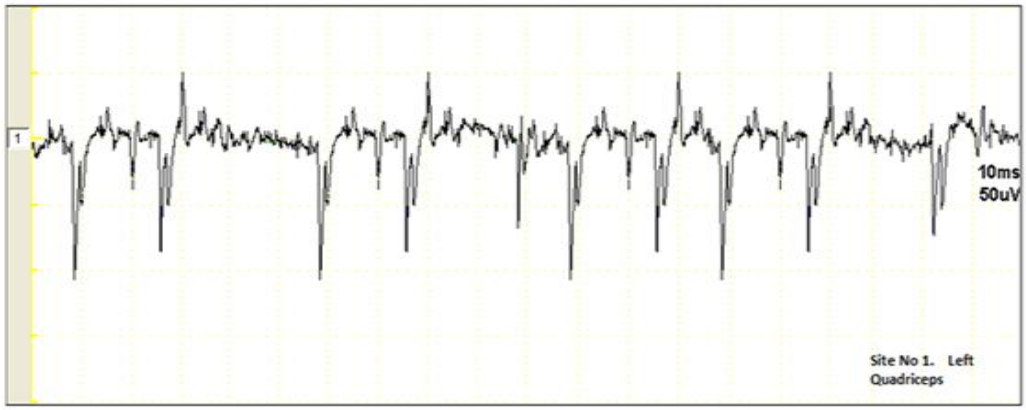

Fig. 3 Sharp slow waves intricate with fibrillation potentials. Female, 5 years old.

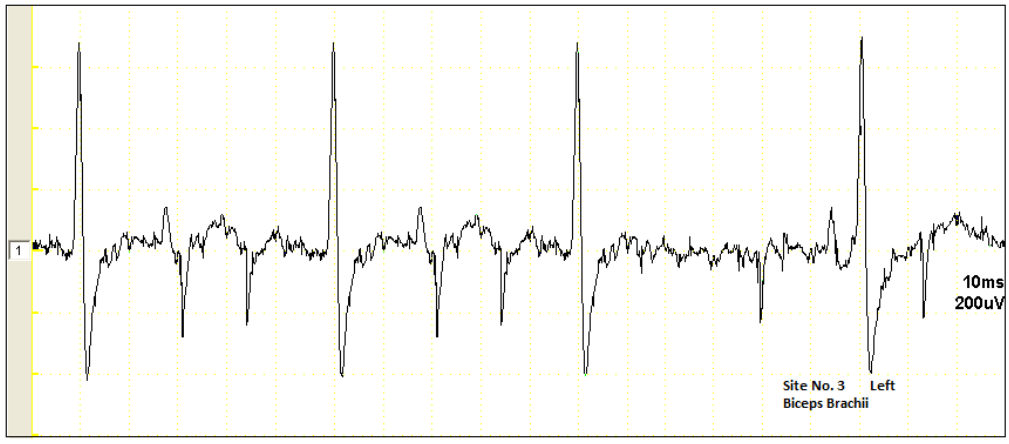

Fig. 4 Giant potentials intricate with positive slow waves in a Pekingese dog

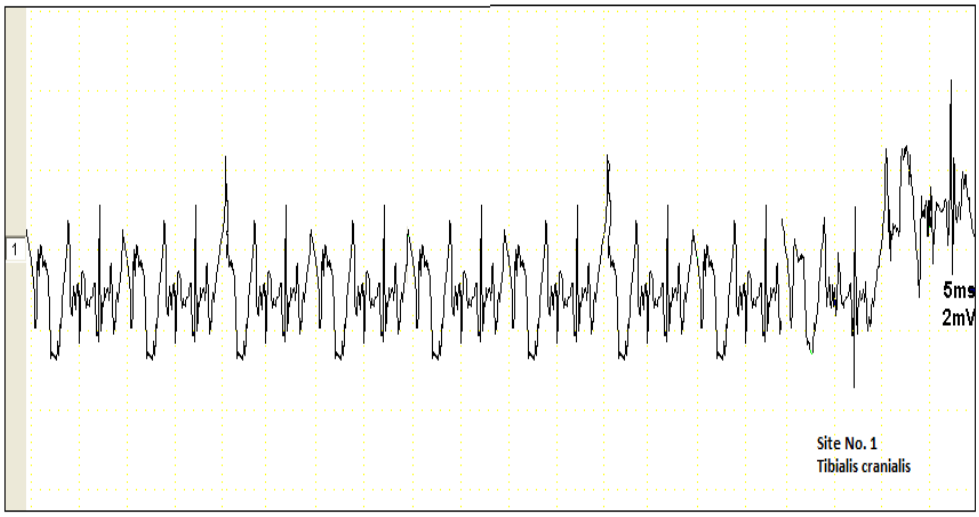

Fig. 5. Repetitive discharge complexes. Crossbreed dog, 2 years old. Vertebral fracture

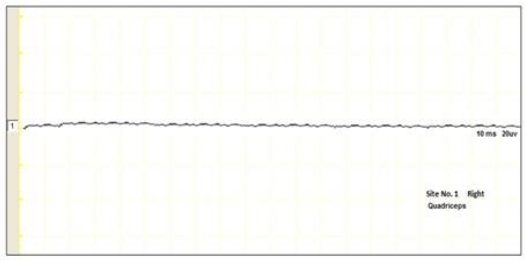

Fig. 6. Electrical silentium after the 30thsession of electrostimulation

Conclusions

Electrostimulation induced artificial muscle contraction, allowing to manage muscle spasm and increase muscle tone.

Electromyogram allowed to regularly evaluate the clinical status of these patients, guide and justify the treatment. Every 10 sessions a general improvement could be observed and, at the end of the 30 physiotherapy sessions, in 4 out of 5 patients, electrical silentium was registered, demonstrating the benefits of this type of therapy.

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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *DERMANYSSUS GALLINAE* INFESTING LAYING HENS IN ROMANIA: PRELIMINARY DATA

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Abstract

Dermanyssus gallinae (Mesostigmata: Dermanyssidae) is a blood-feeding ectoparasite causing important economic losses, with a high prevalence in poultry farms in Europe. The present study aimed to perform morphological and molecular characterization of D. gallinae infesting laying hens in farms in Southern Romania. The morphological identification of the mites was carried out by using specific keys. All mites morphologically identified as D. gallinae were subjected to molecular analysis by PCR, targeting the nucleotide sequence of ribosomal internal transcribed spacers (ITS) and the mitochondrial cytochrome oxidase subunit I (cox1). The morphological identification of the parasites reveled: the genitoventral shield of parasite posteriorly rounded with one pair of seta; the sternal shield wider than longer; the anal shield with three setae. The PCR reaction returned a single band for each sample of D. gallinae, whose length was 750 bp and 550 bp for of ITS-and cox1-targeting PCR, respectively. Altogether, the findings represent a basis for further studies for a better understanding of the epidemiology of red mites infestation in different types of housing systems used to raise laying hens in Romania.

Key words: *Dermanyssus gallinae,* morphological and molecular identification, cox I, ITS region, Romania.

Introduction

Dermanyssus gallinae is a hematophagous ectoparasite of poultry responsible for huge financial losses in different European countries. Prevalence of *D. gallinae* was reported in 80%-90% of poultry farms in Europe (such as in Italy, Serbia, The Netherlands, United Kingdom, Montenegro) and Japan (Sparagano et al., 2009).

The parasite leads to high economic losses in poultry farming with estimated annual costs of 130 million euros throughout the European Union alone (Van Emous, 2005).

In Romania, Magdaş et al. (2006) reported the prevalence of infestation with *D. gallinae* of around 90%, both in poultry farms and private households.

The mites are responsible for the lack of sleep, increased self-pecking in infested laying hens and severe infestations can lead to anemia or death (Kilpinen et al., 2005). Also, mite infested chickens show pruritus, ecchymosis; massive infestation (when the parasite may enter in the nasal cavities) led to nasopharyngitis (Mitrea, 2011).

The poultry red mite has different avian species hosts such as: domestic or wild doves, canaries or other birds that may also be found close to laying hens farms; however, the most commune host is the domestic hen (*Gallus gallus domesticus*) (Roy et al., 2009). *D. gallinae* may also attack non-avian hosts such as rodents, horses or humans causing dermatitis and pruritus (De Luna et al., 2008)

The parasite could act as reservoir hosts for pathogenic agents such as bacteria, *Escherichia coli, Erysipelothrix rhusiopathiae, Borrelia anserina, Listeria monocytogenes, Salmonella gallinarum, Coxiella burnetii,* and viruses - *Newcastle disease virus* (*NDV*) (Moro et al., 2009).

Other pathogens isolated from *D. gallinae* mites collected from private yards in Romania (Cluj county) included opportunistic bacteria such as *E. coli*, *Micrococcus* spp.,

Corynebacterium spp., *Staphylococcus* spp., *Streptococcus spp., Bacillus cereus* (Magdaș et al., 2006).

Morphological identification of *D. gallinae* can be performed using taxonomic keys (Moss`s keys), light microscopy and scanning electronic microscopy (LM and SEM) pictures (Di Palma et al., 2012).

Nowadays, different methods, especially molecular genetics techniques for identification of parasites, for detection of drug-resistant strains of parasites, or in control of infestations are used worldwide (Mitrea, 2002), having a substantial impact both in fundamental (systematics, epidemiology, ecology, and population genetics) and applied (diagnosis, and control) fields of veterinary parasitology. Therefore, molecular tools (eg. polymerase chain reaction) play an important role in epidemiology, ecology, population genetics (Ionita et al., 2008), and they are used in order to investigate host specificity, host range or infection routs (Roy et al.,2009; Øines and Brännström, 2011**)**.

Molecular characterization of *D. gallinae* is based on genes sequencing like mitochondrial cytochrome oxidase subunit I (COI) gene, 16S rRNA and nuclear internal transcribed spacers (ITS) region; it is also useful for revealing hybridization between different lineages (Roy et al., 2010). In Romania, such molecular studies have not yet been performed.

The present study aimed to perform a morphological and molecular characterization of *D. gallinae* infesting laying hens in farms, in Southern Romania.

Materials and methods

Collecting mites and morphological identification

Mites were collected from three poultry farms in Sothern Romania during the period November 2015 to April 2016. The method for collecting the parasites was performed using corrugated cardboard traps. All mites were preserved in 70% ethanol at room temperature.

The morphological identification of mites was performed on stereomicroscope using specific morphological keys (Di Palma et al., 2012).

Mites DNA Preparation

For mite DNA extraction, three pooled adult mite samples, identified as A, B, C, were used. Up to 20 individual mites from each pooled sample were used for DNA extraction. For this, the mites were prepared according to the manufacturer's instructions (PureLink® Genomic DNA Kits, Thermo Scientific, Milan, Italy). The purified genomic DNA was stored at 4°C until use.

Polymerase chain Reaction (PCR), purifying and sequencing

All mites morphologically identified as *D. gallinae* were subjected to molecular analysis by PCR, targeting the nucleotide sequence of ribosomal internal transcribed spacers (ITS) and the mitochondrial cytochrome oxidase subunit I (*cox1)*. The PCR for *coxI* gene and ITS region was conducted using thermal cycles previously determined according to the primers and the expected amplification product (Øines and Brännström, 2011).

The PCR products were visualized by ethidium bromide staining.

In order to obtain the DNA for sequencing, the PCR products were eluted from the agarose gel, ligated in a specific vector for cloning, and the recombinant plasmids were used to transform *Escherichia coli* Mach1 cells (Lodish et al., 2000). The recombinant plasmids were extracting by using PureLink™ Plasmid Miniprep Kit (Thermo Scientific, Milan, Italy).

Results and Discussions

Morphological study

In the present study, all mites collected from the three laying hens farms were morphologically identified as *D. gallinae* adults. The morphological examination of collected mites revealed: the presence of the styliform cheliceral articles (Figure 1); the genitoventral shield posteriorly rounded with one pair of seta (Figure 2), and the sternal shield wider than longer (Figure 3). Also, the anal shield has three setae (Figure 4).

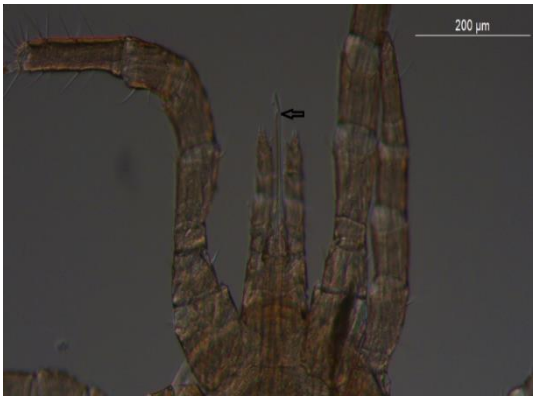

Fig. 1. The cheliceral article (on the arrow) and the palps of *D. gallinae*

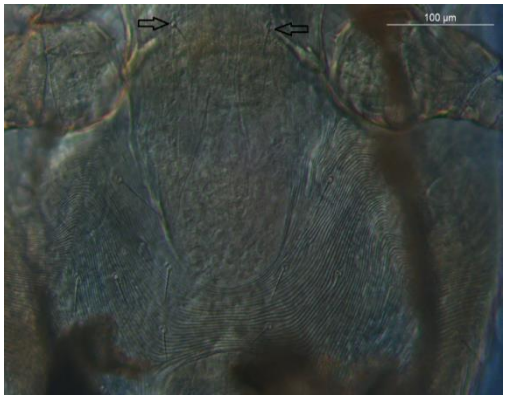

Fig. 2. The genitoventral shield of *D. gallinae* with one pair of setae

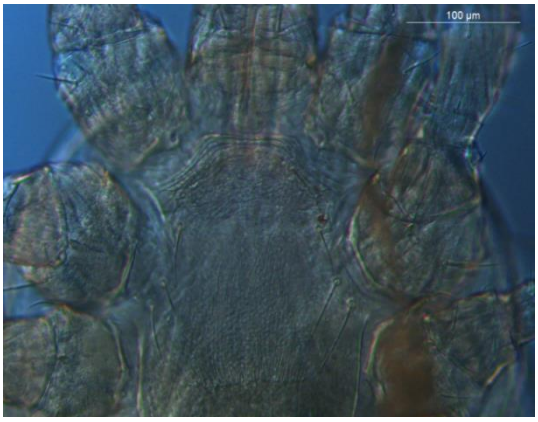

Fig. 3. Sternal shield (wider than longer) of *D. gallinae*

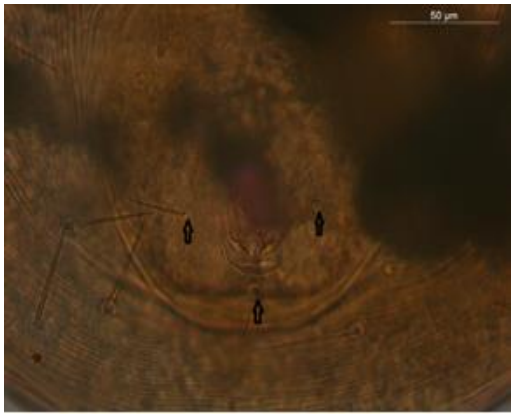

Fig. 4. Anal shield of *D. gallinae* with three setae

The PCR amplicons of *D. gallinae* were subjected for sequence analysis of cytochrome oxidase subunit I (*coxI*) and internal transcribed spacer (ITS).

The electrophoresis reveled a single band for each sample (A, B, C) and the length of the ITS band was 750 bp and 550bp for *coxI* gene (Figure 5).

The elution of DNA from agarose gel revealed a length of 500 bp of DNA samples (A, B, C) in order to add in ligation mix (Figure 6).

After transformation, the electrophoresis performed in order to verify the presence of recombinant plasmids, showed that the samples B and C were positive for recombinant plasmids. The A sample was negative, probably due to low concentration of PCR product (Figure 7).

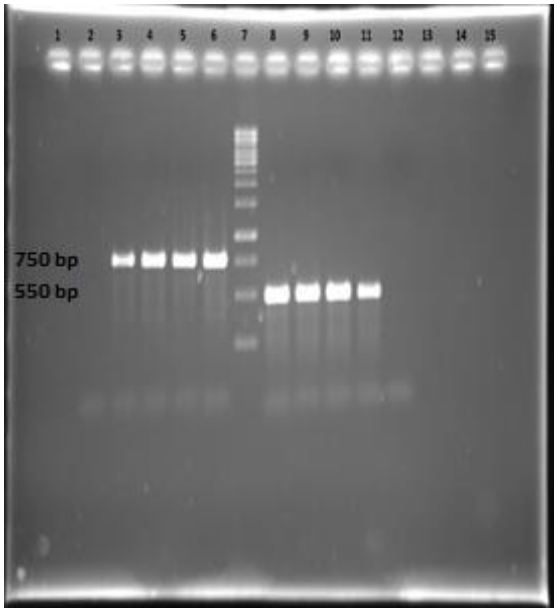

Fig. 5. Agarose gel electrophoresis of PCR; ITS (bands 4-A, 5-B, 6-C); *coxI* (8-A, 9-B, 10-C) from samples of *D. gallinae.* Bands 3, and 11: positive controls, 2 and 12: negative controls; band 7: DNA ladder

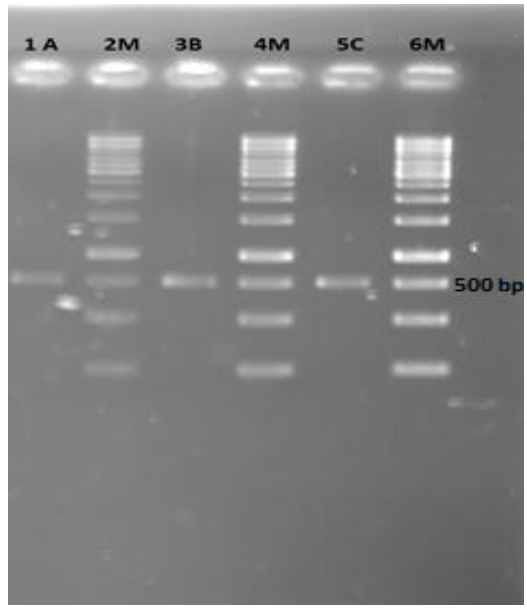

Fig. 6. Agarose gel with the bands for ligation (DNA samples - 1 A, 3 B, 5 C) Bands 2M, 4M and 6M represent the DNA ladder

Plasmids purification was performed for obtaining high purity plasmid DNA. The agarose gel stained with ethidium bromide showed that the length of the plasmids band (3B and 5C) for sequencing was 3.5 kb (Figure 8).

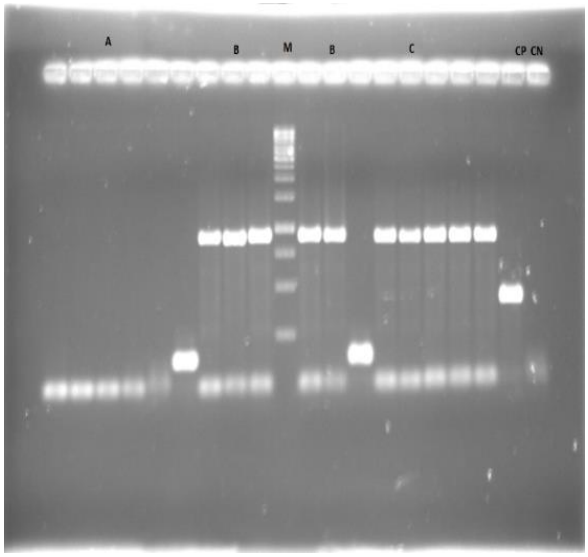

Fig. 7. Recombinant plasmids: sample A negative; sample B and C positive

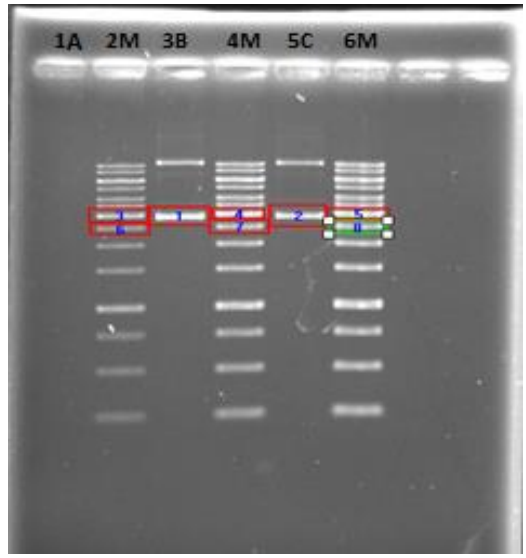

Fig. 8. Electrophoresis – the length of high purity plasmids for sequencing (samples: 1A, 3B, 5C); bands 2M, 4M, 6M: DNA ladder

The morphological identification of *D. galline* is necessary because this species may be confused with *Orinthonyssus sylviarum* (northern fowl mite) which has the same host and environment (Di Palma et al., 2012). In the present study, following Moss` keys, all mites were identified as *D. gallinae*.

Also, a correct identification of *D. gallinae* is needed in terms of applying an appropriate treatment in infestations with this parasite (Di Palma et al., 2012).

Of the 25 species included in the genus *Dermanyssus* (Duges, 1834), 14 species shows similarities in morphology to those of *D. gallinae*, this sometimes leading to erroneous identification of the poultry red mite. Therefore, molecular tools are used for phylogenetic analysis of *D. gallinae* (Roy et al., 2009).

The molecular characterization of *D. gallinae* based on nucleotide sequences has been performed in Europe, the United States, Brazil, Australia and Japan (Chu et al., 2015).

Molecular epidemiological studies revealed intra- and international migrations of the mite in different countries (Sweden, Norway, Italy, France) (Marangi et al., 2014; Øines and Brännström, 2011; Roy et al., 2009; Roy et al., 2011).

The phylogenetic study of *the cox I* sequences of *D. gallinae,* performed in different regions in Italy, revealed the presence of the two major haplogroups A and B (Marangi et al., 2014). In Norway and Sweden, the phylogenetic analysis of *D. gallinae* revealed 32 haplotypes encountered in two major haplogrups (A, B). Both countries have haplotypes from both haplogrups, this thing suggesting that *D. gallinae* from this two countries have a commune origin (Øines and Brännström, 2011). The study conducted by Marangi et al. (2009) showed that all UK samples of *D. gallinae* had a similar phylogeny and a distant relationship with the samples from Italy and France.

In Japan, Chu et al. (2015) in their study regarding the molecular characterization of *D. gallinae,* revealed that the haplotypes from A and B haplogroups, in both *coxI* and 16S rRNA genes were closely related to those found in Europe.

Regarding the exchange of mites between wild and domestic birds, data from Norway and Sweden, suggest that wild birds are not a reservoir for infestation with *D. gallinae* (Øines and Brännström, 2011).

Conclusion

This study represents the first step towards molecular characterization of *D. gallinae* in Romania. All mites morphologically identified as *D. gallinae* were characterized by sequence analysis of the cytochrome oxidase I and ribosomal internal transcribed spacer regions. A phylogenetic analysis will be carried out and a comparison between the mites found in Romania and the ones from other European countries will be performed. Altogether, the findings represent a basis for further studies for a better understanding of the epidemiology of red mites infestation in different types of housing systems used to raise laying hens in Romania.

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STUDY ON ENDOPARASITES OF CATTLE AND SHEEP FROM A SILVOSTEPPE AREA IN SOUTHERN ROMANIA: PRELIMINARY DATA

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Abstract

Endoparasite infection in ruminants has major economic impacts worldwide due to the losses caused by reduce the rate growth and productivity such as reduced milk and meat productions, and reproductive potential of animals. Geographical location, climatic conditions, age and anthelmintic therapy status of animals represent some of the risk factors for parasite infections. The aim of this study was to investigate the endoparasite fauna in cattle and sheep grazed in a silvosteppe area from Southern Romania. The study was carried out during April 2015 - May 2016. A total number of 51 dairy cattle (aged between 4 months and 10 years), and 63 sheep (of different breeds, aged between 6 months and 3 years) were included in the study. Animals displayed no clinical signs. For the both cattle and sheep, deworming was not carried out 2-3 months prior of the study. Fecal samples were collected and examined, first grossly, then using a sodium chloride flotation and sedimentation techniques followed by microscopy for identification of parasitic elements (helminth eggs, protozoa oocysts), Baermann method for detection of lungworm nematodes. The most prevalent parasite infections in cattle and sheep, respectively, were with strongyles (76.50% and 92.06%), including Nematodirus spp., followed by Fasciola hepatica (25.50% and 34.92%), Eimeria spp. (19.60% and 23.81%), Dicrocoelium lanceatum (5.90% and 42.85%.), and Moniezia spp. (3.90% and 19.04%). Additionally, sheep were positive also for lungworm larvae (36.50%). These findings indicate that internal parasites control should be implemented in the study area in order to avoid the productivity losses and to improve the animal welfare.

Key words*: cattle, sheep, endoparasites, Romania.*

Introduction

Cattle and sheep have an important economic role worldwide. Gastrointestinal parasites in livestock, such as protozoan, nematodes, cestodes and trematodes play an important role for animal health status and productivity (Bussieras and Chermette, 1994; Choubisa and Jaroli, 2013; Ananta et al., 2014). All grazing animals can be infected with different species of parasites although their patogenicity varies with the present number of species and susceptibility of the host (Taylor, 2010). Studies have shown that gastrointestinal parasites are the most serious problems of economic losses both in ruminants farms and household (Ng'ang'a et al., 2004; Odoi, 2007).

In cattle, gastrointestinal infection can cause weight loss in young growing calves, reduced milk and meat production, reduced work capacity, reduction in food intake, villous atrophy, anemia. Similarly, in sheep, gastrointestinal helminth infection can cause poor wool or meat quality, anemia, weight losses, mortality (Mitrea, 2011; Choubisa and Jaroli, 2013).

Geographical location, climatic conditions, age and anthelmintic therapy status of animals represent some of the risk factors for parasite infections and the prevalence of this varies from region to region (Tramboo et al., 2015).

The aim of the present study was to investigate the endoparasite fauna in cattle and sheep originated and grazed in a silvosteppe area from Southern Romania.

Materials and methods

A total number of 51 dairy cattle (aged between 4 months and 10 years) from households and 63 sheep (of different breeds, aged between 6 months and 3 years), selected from 5 farms (between 12-15 animals per farm), were included in the study. Animals displayed

no clinical signs. All animals originated from a silvosteppe area in Southern Romania; they were grazed on natural pastures starting from April until October, and housed during the winter. For the both cattle and sheep, deworming was not carried out 2-3 months prior of the study.

Fecal samples were collected and examined, first grossly, then using a sodium chloride flotation and sedimentation techniques followed by microscopy for identification of parasitic elements (helminth eggs, protozoa oocysts), Baermann technique for detection of lungworm nematodes (Ionita and Mitrea, 2013).

Results and discussion

The copro-parasitological examination revealed for the both species investigated, cattle and sheep, a diverse endoparasitic fauna, comprising protozoan and helminths (trematodes, cestodes, and nematodes), located, according to the parasite species, in the gastro-intestinal tract or respiratory apparatus. The main parasitic stages observed at microscopical examination included: eggs of digestive strongyles (fig. 1), including *Nematodirus* spp. (fig. 2), larvae of pulmonary strongyles, ooccysts of *Eimeria* spp. (fig. 3), eggs of *Moniezia* spp. (fig. 4), *Fasciola hepatica* (fig. 5) and *Dicrocoelium lanceatum* (fig. 6).

The most prevalent parasite infections in cattle and sheep, respectively, were with strongyles (76.50% and 92.06%), including *Nematodirus* spp., followed by *F. hepatica* (25.50% and 34.92%), *Eimeria* spp. (19.60% and 23.81%), *D. lanceatum* (5.90% and 42.85%.), and *Moniezia* spp. (3.90% and 19.04%) (Table 1). Additionally, sheep were positive also for lungworm larvae (36.50%). *Eimeria* infection was prevalent in young animals, while *D. lanceatum* was dominant in adults.

In cattle, gastrointestinal infections with a single species of parasites were found in 56.90% (29/51), while multiple gastrointestinal infections (two or three species of parasites) were identified in 29.40% (15/51) of the examined samples. The associations between *Eimeria* spp. + strongyles; *F. hepatica* + strongyles; *Moniezia* spp. + strongyles; *F. hepatica* + *D. lanceatum; F. hepatica* + strongyles + *D. lanceatum* were registered in cattle.

In the present study, infection with one species of parasite in sheep was 49.20% (31/63) and multiple endoparasite infections were found in 50.80% (32/63). In sheep, the multiple gastrointestinal infections were as follows: *Eimeria* spp. + strongyles; *F. hepatica* + *D. lanceatum; Eimeria* spp. + lungworm larvae; *F. hepatica* + *D. lanceatum* + strongyles*; Eimeria* spp. + strongyles + *Moniezia* spp.; *Eimeria* spp. + strongyles + lungworm larvae.

Analyzing the findings of the present study, it is emphasized that strongyle infections are highly prevalent (over 70%) in grazing cattle and sheep in Southern Romania. These data are in line with previous studies conducted in other area of Southern Romania (Valcea county) that have revealed the highest prevalence of gastrointestinal nematodes in sheep and cattle (ranging from 71.5% to 92.8%) (Mitrea et al., 2008a,b). Similarly, study from Dobrogea area (Ardeleanu et al., 2002), reported a prevalence of gastrointestinal strongylidosis in sheep between 70% - 100%. In contrast, Gherman et al. (2004), in a study conducted in Bistrita Nasaud County, registered a smaller prevalence of strongylidosis (30% - 35%).

The results of the study conducted by Gorski et al. (2004) showed that *Eimeria* was found in 34% of sheep in Poland, while in our study coccidia were found in a slightly higher prevalence (up to 50%). However, it was mentioned that in our study eimeriosis was prevalent in young animals. This fact has been documented also by Cozma and Titilincu (2007), reporting as highly prevalent eimeriosis in lambs, with an extensivity of 95%; in their study, 26% of the infected lambs presented diarrheic syndrom and a mortality of 16% was registered.

Table 1.

Prevalence (number positive; %) of endoparasites fauna in cattle and sheep, originated from a silvosteppe area in Southern Romania

Host species	**Parasite species**					
	Eimeria spp.	*Fasciola hepatica*	*Dicrocoelium lanceatum*	*Moniezia* spp.	Digestive strongyles	Lungworm nematodes
Cattle n=51	10 (19.60%)	13 (25.50%)	3 (5.90%	2 (3.90%)	39 (76.50%)	0
Sheep n=63	15 (23.81%)	22 (34.92%)	27 (42.85%)	12 (19.04%)	58 (92.06%)	23 (36.50%)

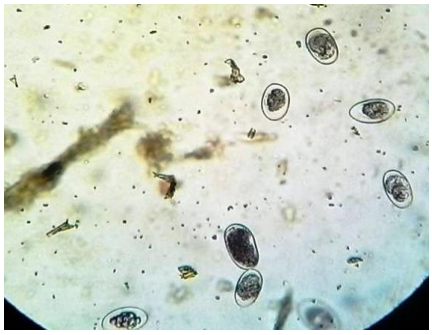

Fig. 1. Strongyle eggs identified using flotation technique in sheep (x10)

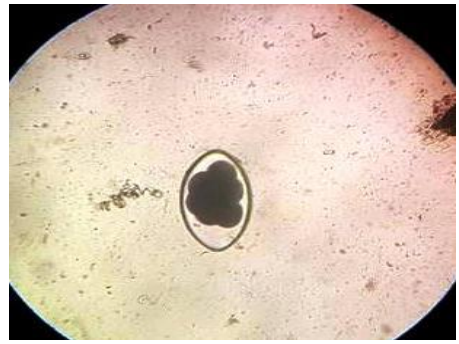

Fig. 2. *Nematodirus* spp. egg identified using flotation technique in cattle (x10)

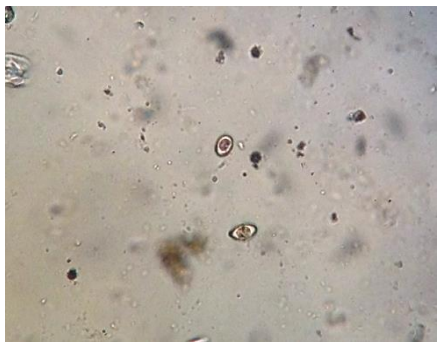

Fig. 3. Oocyst of *Eimeria* spp. identified using flotation technique in sheep (x10)

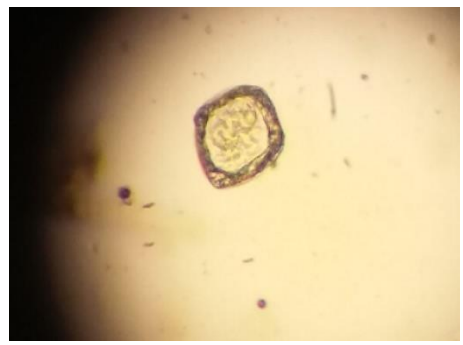

Fig. 4. *Moniezia benedeni* egg identified using flotation technique in sheep (x20)

Fig. 5. *Fasciola* spp. egg identified using sedimentation method in cattle (x10)

Fig. 6. *Dicrocoelium* spp. egg identified using sedimentation method in cattle (x20)

There is well documented that strongyle infections are very common in ruminants, in which the rates of infection varies from 40 to 95 % (Bussieras and Chermette, 1994; Koinari et al., 2012; Squire et al., 2013). The high prevalence of strongyle infections may be due to the pour growing and hygienic conditions, feeding, watering systems and lake of anthelmintic treatment (Chihai et al., 2011).

A morphological study conducted by Indre et al. (2011) showed that the highest prevalence of gastrointestinal nematodes in sheep in the Timis County was represented by *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (100%), and the smaller prevalence was registered for *Nematodirus spathiger* (6.66%). In another studies, Iacob (2007), reported that the gastrointestinal species identified in sheep belonged to genera: *Trochostrongylus, Nematodirus, Ostertagia, Haemonchus, Strongyloides, Moniezia* and *Eimeria*.

Fluke infections are also very common in grazing ruminants, as previous have been reported in different studies. Therefore, the prevalence of *F. hepatica* and *D. lanceatum* in cattle and sheep varied between 42.8% - 66.6% and 16.6% - 21.4%, and between 42.8% - 57.1% and 28.5% - 71.4%, respectively (Mitrea et al., 2008a,b).

In a similar study conducted in cattle grazed on steppe and meadow ecosystems in Moldova, similar prevalence values for *F. hepatica* (46.0%) and *D. lanceatum* (63.0%) were found, showing increased infection levels with the age of animals (Chihai et al., 2011).

With regard to the tapeworm infections, this was more frequent found in sheep in this study, with the prevalence value registered of 19.04%. These findings are in line with results of other studies, where infestations with *Moniezia* spp., in sheep from three counties in Southern and North-Eastern Romania, had a prevalence varying from 14.2% to 21.4% (Mitrea et al., 2008a).

Based on all the study findings it can be assumed that the high level of endoparasite infections in ruminants grazed in the investigated area could be explained by intense rates and permanent contamination of pastures with parasitic stages, associated with climatic factors that are favorable for their maintaining and developing. Moreover, the specific conditions of different ecosystems are important factors not only for geohelminthoses but also for biohelminthoses, as they can influence the presence and distribution of the intermediate and complementary hosts. Besides these, other factors such us management factors, parasitological control programs could maintain a high pressure for parasite infections in grazed animals (Mitrea, 2002).

Conclusions

The results of this survey indicate that the environmental conditions of studied areas are favorable for acquiring different parasite infections of grazing cattle and sheep. Therefore, these findings indicate that internal parasites control should be implemented in the study area in order to avoid the productivity losses and to improve the animal welfare.

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THE ANALYSIS OF SEVERAL FACTORS THAT INFLUENCE THE BREASTFEEDING CAPACITY AT THE PIC SOWS

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Abstract

Because of the technological, nutritional and sanitary-veterinary conditions which are provided nowadays, the number of piglets has increased significantly at parturition and to a lesser extent at the weaning. In the last three decades, it has been achieved an increase of only 0,5 piglets weaned/sow/reproductive cycle, and the main losses were recorded due to the insufficient consumption of milk. Our study is based on the context of the data described above and it was conducted on a batch of lactating sows with suckling piglets in a PIC multiplication farm. It was used an experimental protocol based on the logistics and the recording systems that were already existing in that unit farm, a component of the EUROHYB intensive system. The essence of the study consisted of the evaluation of the sows breastfeeding capacity, on the basis of a score which was given at the weaning and at the end of the previous lactation and it was finally analysed in correlation with fecundity and fertility. The researches were organised under the form of regular investigations, focused on monitoring the "nests" of the lactating sows with suckling piglets, the statistical analysis of the recorded data and the evaluation of the influence of the genotypic, phenotypic and environmental factors over the sows breastfeeding capacity and the growth and health indices at suckling piglets. The statistical analysis of the influence of genetic, phenotypic and environmental factors revealed significant correlation between the weight of the batch at parturition and at 21 days (rfxy=0,494), respectively between the number of living piglets and the size of the batch at weaning (rfxy=0,487), respectively between the number of weaned piglets and the weight of the batch at weaning (rfxy=0,593). We have ascertained that there is a strong phenotypic correlation (rfxy=0,767) between the total number of functional nipples and the number of weaned piglets. We have also observed that the frequency of the requested pairs of nipples has decreased in the order 1 to 8, indicating a positive correlation between the weight of the piglets batch and the quantity of the milk consumed (rxy=0,686). Therefore, according to the regression bxy=5,055, to a functional additional nipple corresponds an extra piglet/batch and an increase of the weight of the batch with 5 kilograms at weaning. The optimization of the morpho-productive features, of the genetic and health indices at PIC sows determined the increase of the milk production level and implicitly, the increase of the breastfeeding capacity, which had led to the reduction of the losses caused by milk subnutrition at suckling piglets.

Keywords*: lactation, PIC sows, suckling piglets*

According to the current estimates, the performances of reproductive sows can reach up to 2,5 parturitions / year, with 41 weaned piglets (Ognean L. *et al*, 2015). It is noticed that the achievement of high productive and reproductive perfomances of the sows are due to both high level of feeding and maintaining standards, which are applied, and the implementation of several standards with great relevance, regarding the management of gestation, breastfeeding and weaning stages (Vlasiu A. *et al*., 2012). All these things lead finally, to the increase of the milk production and implicitly, to the increase of the sows breastfeeding capacity, respectively the increase of the number and weight of weaned piglets (Mabry J. W. *et al*., 1996). Regarding the lactating sows, the establishment of the energy and nutritive requirements is mainly based on the milk production level, because of the enhancement of the metabolism, with the predominance of the catabolic processes (Olmos-Hernandez A. *et al.*, 2010).

Therefore, the lactating sows suffer important weight losses that commonly reach 13-15% during an 8 week lactation. The poor feeding conditions cause much more significant losses, that can reach up to 40%, with devastating consequences on the productive

performances of the sows (Sarandan H. *et al*., 2009). As it is well known, highly productive sows can produce 350-400 L milk in an 8 week lactation, requiring both adequate feeding and watering (Ognean L. *et al.*, 2015). The water consumption of the sows during the lactation period is much higher than during other physiological states, reaching up to 25-30 L/day (Sarandan H. *et al*., 2009). From the latest studies, focused on the increase of the milk production at sows , a major impact had those which were based on using pocine somatotropin or on stimulating the secretion of prolactine hormone at sows (Olmos-Hernandez A. *et al.*, 2010).

The present research subscribes the theoretical framework described above and it underlines the correlative analysis of the breastfeeding capacity and of the main productive and reproductive indices, in a batch of breeding sows from o PIC multiplication farm.

Materials and Methods

The study was focused on investigating a batch of lactating breeding sows (n=205) from a PIC multiplication farm and the suckling piglets (n=3955), which had resulted from the total annual parturitions (n=430). The conducted investigations and researches were focused on monitoring the evolution of the genotypic, phenotypic and environmental factors with major impact over the reproduction and lactation at PIC sows, respectively over the growth and health of the suckling piglets. In addition, we have also evaluated the influence of several risk factors over the productive performances of the sows and the piglets.

The research was conducted through several investigations, which were made in the nests of lactating sows and suckling piglets and it was focused on the evaluation of the growth index at suckling piglets (by measuring their weight), the health surveillance and on monitoring the risk factors that may occur during the stages of the maternity: parturition, breastfeeding and weaning. In order to realise this survey, we have implemented a protocol based on the logistics, the materials and monitoring systems which were already existing in the investigated farm, a component of the EUROHYB intensive system.

The main goal of our research was to implement a program used in PIC farms for the evaluation of the sows breastfeeding capacity, quantified through the correlation between weight losses (established by means of a maintenance score, which was given at weaning and at the end of the previous lactation) and milk production (estimated by converting the suckling piglets rate into the consumed milk). At the same time, a correlative analysis was made between the evolution of the prolificacy and productive longevity indices of the sows from the breeding bath. Finally, all data resulted from the conducted investigations and the BLUP index of the sows were the basis of the statistical analysis of the influence of several phenotypic, genotypic and environmental factors over the main couple of characters that influence the breastfeeding capacity, including some indices regarding the growth and the health of the suckling piglets.

The use of this genetic study program allowed us to realise a relevant statistical analysis concerning the correlative action of the genetic, phenotypic and environmental factors, based on the evaluation of the milk production and quantifying into the capacity of breastfeeding.

Results and Discussions

The analysis of the influence of the genetic, phenotypic and environmental factors over the sows breastfeeding capacity and the main biometric and health indices in the lots of suckling piglets offered the required data, in order to evaluate the risk factors that may affect these parameters. The correlative action of the several genetic, phenotypic and environmental factors was evaluated by means of programs used in the genetic study, regarding the sows milk production and it revealed the existence of various factors, which influence directly the

breastfeeding capacity. In this regard, we correlated the breastfeeding capacity with several genetic, phenotypic and environmental factors due to the monitoring activity, which was performed over a long period of time.

Therefore, as it is presented in Table 1, the statistical analysis revealed strong correlations between the weight of the piglets batch at parturition and its weight at 21 days (rfxy = 0,494), between the number of living farrowed piglets and the size of the batch at 21 days (rfxy = 0,487) and also, between the number of piglets from the batch at 21 days and the weight of the batch at 21 days (rfxy = 0,593). A great impact over the breastfeeding capacity had the couple of characters, represented by the total number of functional nipples and the number of piglets from the batch at 21 days and moreover, between the last two, it was confirmed a significant phenotypic correlation (rfxy = 0,767). Thus, according to the regression (bxy = 5,055), an additional functional nipple materializes into an extra piglet/batch and a heavier weight of the batch at 21 days with more than 5 kg. We have also noticed that an important correlation was made between the total number of piglets at parturition and the number of piglets at weaning due to the corroboration of the collected data from the investigated farm with the data collected from 16 other populations (rfxy = 0,660).

Table 1.

The correlations of several phenotypic, genotypic and environmental factors with the main couples of characters which influence the breastfeeding capacity at PIC sows

Couple of characters	Correlation			Regression
	Phenotypic	Genotypic	The environmental	
Total no. of nipples / **No. of functional nipples**	0,463	0,459	0,469	0,696
No. of functional nipples / **No. of piglets/batch at 21 days**	0,767	0,169	0,826	1,046
No. of functional nipples / **Weight / batch at 21 days**	0,451	0,360	0,443	5,055
Weight / batch at calving / **Weight / batch at 21 days**	0,494	0,551	0,529	3,158
No. of living piglets at calving / **No. of piglets/batch at 21 days**	0,487	0,764	0,526	0,889
No. of piglets/batch at 21 days / **Individual weight at 21 days**	-0,336	-0,247	-0,317	-0,613
Daily consumption of food / **Weight / batch at 21 days**	0,630	0,439	0,321	1,412

The prolificacy is a productive index, extremely useful for the evaluation of the reproductive sows and according to that, the number of breastfeeding piglets can influence the milk production even more than the quantity of the food. This correlation is reflected by the results of our study and in accordance with it, the sows with 12-15 suckling piglets can produce with 25-35% more milk than those with 8-10 piglets. The data also show that in the first 3-4 days of life, the small lots of piglets can not consume the entire amount of milk, which is produced by the sow, and as a consequence, the milk retention is the main factor that decreases the stimulation of alveolar tissue. Taking into consideration this principle, in the investigated farm, the new-born piglets are re-located in order to standardize and enlarge the batches.

The productive longevity indicates the limit age up to which the reproduction of sows is profitable and evolves in linked correlation with the body structure of the animal and

considerably influences the milk production. Regarding our investigated farm, it is required that the majority of sows to be reformed after the fourth lactation (over the fourth rank of parturition), as it is shown in the genetic criteria established by the BLUP index. We can also notice that in our investigated farm, this index is updating after each parturition in order to improve the genetic heritage by using substituted gilts and to highlight the genetic progress in the farm. Therefore, several females (after the sixth rank of parturition) were maintained in the batch and in the previous parturitions, they have achieved performances situated at the level or above the average population. The increase of breastfeeding capacity has been highlighted by the evolution of the lactation curve, which consists of an upward phase (7-10 days), a plateau phase, of about 10 days and a downward phase, with a sudden decrease around the weaning.

Summarizing all the results obtained from the investigated farm, we can confirm the existence of a high level of milk production, positively correlated with the prolificacy and the breastfeeding capacity of the PIC sows. The analysis of productive and reproductive indices has emphasized an increase of the parturition rate (85%; 2,2 births/year), of the number of births of piglets /sow/year (27,1) and the achievement of the average weight of piglets at birth (1,7 kg) and at weaning (7,5 kg). The overall analysis correlated with achieving first fertile mating at the optimum age (233-238 days), respectively, at the 348-353 days, at first parturition have contributed in ensuring a high level of milk production (7,9 L/sow/day).

In this PIC farm, the batch consisted of 2463 swine and the annual evolution of mortalities underlined 360 cases of deaths; if we reported the recorded mortalities to the total number of born piglets, it would represent 10,2 %. The losses had the following percentage distribution on different categories, such as: suckling piglets (45%), growing youth (40%), youth in testing (7%) and commercial pigs (8%). After analysing the causes of mortality regarding the suckling piglets we were able to determine the following hierarchy, consisting of the major technological and pathological factors involved in producing those deaths: piglets crushed by the mother sows (55%), diarrhea syndromes (35 %), starvation (7%) and other causes(3%).

The investigations concerning the breastfeeding behavior of the new-born piglets (the first 72 hours) from 20 nests revealed that those piglets, with the body weight below the average of the lot have achieved smaller gains than the average bodyweight of the lot. This thing indicates that they had the competition for the most milk productive nipples and thereby, the consumption of both colostrum and milk has decreased. Regarding the usage of the nipples, depending on their anatomical position, we have found out that there is a directly proportional correlation and the nipples with a lower milk production are less preferred by the piglets. Actually, the usage of the nipples has decreases proportionally with the decline of milk production from the first toracal pair to the inguinal one. Therefore, the underweight piglets manage to feed themselves only from the nipples with a lower production of milk, and so, a relevant correlation (rxy = 0,686) was observed between the body weight of the piglets and the quantity of the consumed milk. Another studies from the same field showed that the most vigorous piglets took hold of the toracal nipples, with the highest milk production (Rada *et al*., 2010).

The MMA syndrome (mastitis–metritis–agalactiae) is incriminated as a pathological factor that influences significantly and directly the breastfeeding capacity; regarding our farm situation, only three cases of MMA syndrome were diagnosed from the entire batch of lactating sows. Some researches have ascertained that this syndrome can lead to a decrease of milk production up to 40% at the sows in the first three days of lactation and it can also double the percent of mortalities and produce important decreases of the daily average rate (Rada *et al*.,

2010). One of the most severe effects of this syndrome is the production of lipopolysaccharides and their release from the fetal circulation into the colostrum (Klaver J. *et al.,* 1981).

The losses regarding the young growing piglets appeared after their weaning (usually after 6-10 days), mainly because of the mistakes which had been done in the weaning procedure and the technological measures that had been implemented since the first week of life. The individual sheets of animals had a major influence on ensuring the efficacy standards in this farm, that are used for monitoring the morpho-productive and reproductive indices at the sows from the breeding batch and the substituted gilts.

According to the consulted references, an essential index, used for monitoring the risks that can affect the lactation of the sows is the lactation curve with its particularities. The major risks occur more often during the upward phase and the plateau phase, even though the milk production increases all along these phases and it decreases around weaning. Another noteworthy researches have underlined, just like we did, that the small lots of piglets did not consume the entire amount of milk produced in the first 3-4 days and therefore, it will be produced an adversely impact on the galactopoiesis at sows (King Rh., 2000).

The correlation between the genetic breed characteristics and the maintenance and feeding standards provided on the farm was the basis of the significant achieved performances at gilts from the line L03, that are also known for the superior level breastfeeding capacity.

Some researches show that the cumulative action of several risk factors badly influence the milk consumption during the first days of life, leading to the decrease of the energetic and plastic intake, with morphological and functional changes which affect the integrity of the mucosa of the small intestine, responsible for producing the diarrhea syndromes (King Rh., 2000). Quite often, the new-born piglets may be affected by the hypoxic syndrome, that is characterised: by the decrease of vitality, because of the subnutrition determined by disrupting the mother-piglet relationship; the energetic and plastic intake, the capacity of feeding and morpho-functional integrity of the digestive tube and annex glands are also affected (Vlasiu A. *et al.,* 2012).

Conclusions

1. The implementation of several reducing measures of the action of the risk factors has determined considerable increases of the milk production, prolificacy and breastfeeding capacity in PIC sows;
2. Providing the optimal zoo-hygienic and the feeding conditions led to increased parturitions (85%), prolificacy (12,7 %), number of births/year (2,11%), number of piglets/year/sow (27%);
3. The statistical analysis of the action of the genetic , phenotypic and environmental factors revealed significant correlations between the weight of the lot at parturition and the weight of the lot at 21 days (rfxy=0,494), respectively between the number of living piglets and the size of the lot at 21 days (rfxy= 0,487);
4. The mortalities prevailed in suckling piglets (45%), followed by growing youth (40%), youth in testing (7%) and commercial pigs (8%);
5. The frequency of the usage of the nipples pairs decreased in the order 1-8, resulting a positive correlation between the body weight of the piglets and the amount of consumed milk (rxy= 0,686);
6. The decrease of milk production in " over 6 parturition rank" confirmed the fact that the advanced age is one of the risks factors, justifying the efficacy of the sows capitalization during the fifth lactation;

7. It was proved that the increase of the piglets number in the batch has stimulated the galactopoiesis and the sows with 12-15 piglets produce with about 25% more milk than those with 8-10 piglets;
8. The productive longevity in PIC sows was assessed at four lactations, and after that, most of the reforms occurred due to the genetic , productive and health criteria;
9. The mortalities of suckling piglets occurred mainly because of crushing the piglets by the mother sows (55%), diarrhea syndromes (33%) and they were less caused by starvation (7%);
10. The smaller gains made by new-born piglets with the weight below the lot indicated that they had lost the competition for the high milk productive nipples and implicitly, the decrease of the milk consumption.

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CLINICAL REMISSION OF TYPE II DIABETES AT A FEMALE DOG AFTER SURGICAL STERILIZATION

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Abstract

In dogs and cats develops more frequently the type II diabetes, non-insulin-dependent, generated by peripheral resistance to insulin and often associated with hypercholesterolemia and obesity. In this study we analyzed the beneficial influence of sterilization on the evolution of canine diabetes. The studied case was a overweight female dog (German Sheperd, 9 years old, 33 Kg), that has presented for 8 months diabetic symptoms, dominated by hyperglycemia (160-186 mg/dL) inconsistently associated with glucosuria and constantly with polydipsia and polyphagia. In time these events have worsened despite the implementation of a rigorous program for the control of diabetes, based on appropriate measures of diet, exercise and socializing. Moreover, the emergence of severe genital complications (pyometra/polycystic ovaries) resulted in a emergency extirpation of the whole genital apparatus. During the post-operative examinations, within 3 weeks a significant amendment of diabetic symptomatology was noticed, following that, within two months the blood glucose decreased to 110 mg/dL and the main blood-biochemical parameters returned to physiological limits. In another few weeks the blood sugar level stabilized around 75 mg/dL and diabetic manifestations resolved completely. In conclusion we believe that the results of this case study are a relevant information for clinicians regarding the use of which require sterilization, or other pathologies related to therapy in clinical remission to normalize glycemia and some forms of canine diabetes.

Keywords*: control, diabetes, female dog, sterilisation*

Modernization and evolution of human caused important changes to the way of life and to the activity of people and also to their cohabitation relationships with their pets. Thus, in recent decades it has greatly increased interest in animal welfare and the general in pets welfare in particular (Dombay E., 2011). These developments led to changes in common diseases in pets, because if in the past were infectious nature diseases, now are likely metabolic and neoplastic ones, having similar forms to those found in humans. In the front stands of dismetabolics is diabetes, which is the most common disease found in humans and with a rising incidence in carnivores and other animals. This metabolic disease has a pale clinical course, especially in the early stages, which makes diagnosis and confirmation of it to raise issues. Regardind the study on diabetes in different animal species, research has primarily interested in dog, cat, pig, sheep, goat and monkey and quite sporadic in fox, dolphin, hippo or elephant (Roep B.O. and Atkinson M., 2004). The research of diabetes in dogs and cats have a major concern for humans and is focused on addressing the complexity of the factors and ethiopathogenetic's mechanisms related to the lifestyle and the diet, general health and the physiological conditions of risk, such as pregnancy and reproductive function in general (Fleeman M. and Rand J.S., 2001). All these converge towards developing a prophylactic-therapeutic conduct adopted to the predominate risk factors, reaching a real development of protocols for monitoring, supervision and treatment of the animals predisposed or affected by various forms of diabetes (Fleeman M. and Rand J.S., 2001). In the context of what was presented, it is enroll the motivation of this case study, whose purpose is focused on informing clinicians about the complexity and diversity of palliative therapeutic formulas that can lead to remission of clinical forms of canine diabetes (DZC).

Material and method

The case taken in study was a bitch (Cora) German Shepherd breed, 9 years old and weighing 34 Kg. The animal received a special life regime in terms of movement and socialization being maintained in the yard with plenty of space and free movement regime, supplemented with regular walks. The feeding included constantly two portions per day and relied on pelleted feed, with average protein completed with one appreciable proportion of the cooked food from owners' cuisine.

From the development of the health of this female it is to note the presence of a generalized dental aplasia and the early hormonal infertility, which started after her only gestation at 11 months old, with only 2 puppies, feeding and foaled natural and normal. Also remember that the sexual activity of this female who in the meantime was diagnosed with polycystic ovaries, and it was characterized by prolonged estrous cycles (18-22 days) accompanied by heavy hemoragic and with increased frequency leakage (sometimes repeating at every 4 months).

Noteworthy is also the development of two tumoral formations of relatively stable nature: one, richer vascularized at the level of the glandular parenchyma of the first right abdominal mamelon and another, devoid of vascularization and benign, near the left mamelon groin. The patient also presents locomotor disorders manifested by difficult raising and walking, with periodic aggravation, due to spondylosis, clearly diagnosed during radiological examination. The patient was taken in study based on the results recorded during the questionnaire we implemented in the clinic for Canine Diabetes (DZC) screening, which revealed that during the last month it showed diabetic symptoms, dominated by hyperglycemia (160-186 mg/dL) associated with inconstant glucosuria and constantly polydipsia and polyphagia. In time, these events have worsened despite the implementation of a rigorous program of diabetes control, based on appropriate measures of diet, exercise and socialization, completed by administration of the product Diavit for 3 months. This is an herbal supplement (Morar R. and Dana Liana Pusta, 2004). Moreover, the emergence of severe genital complications (pyometra/polycystic ovaries) resulted in the emergent extirpation of the whole genital apparatus. 6 months before and after surgery weekly clinical evaluations were performed, completed by rapid testing of glycemia (with equipment Accu-Chek Active) and every 2 months biochemical testing (the analyzer Abaxis Vet Scan), hematologic (with analyzer Abacus Junior Vet), cardiac (ECG) and ultrasound complex. The genital tract harvested after surgical removal has undergone a thorough morphophatogic examination, also including histopathological investigations, focused on highlighting possible tumor formations.

Data from evaluations and tests were analyzed statistically for this purpose using the usual tests of biostatistics.

Results and discussions

Following the first evaluation we found that the patient has received a very good regime of movement, being permanently free in the yard and walked out occasionally. Therefore we excluded the involvement of a sedentary lifestyle and stress factors generated by this in the onset of diabetes for this patient. We also note that we have not found an account of the development of diabetes in the patient's family because during our inquiries no case of confirmed disease or clinical symptoms was identified. In contrast, we consider that the use of dried fodder and, especially, remnants of food in the kitchen in the patient's alimentation had a major impact in the onset of the disease along with hormonal disorders, infertility and disruption of consecutive estrous cycles.

Regarding the onset and development of diabetes in this female German Shepherd of

9 years a few aspects are noteworthy. At the onset of symptoms that led to suspecting the disease (polyphagia and especially polydipsia), it was recorded a glucose level of 163 mg/dL. At the subsequent application of the questionnaire for Canine Diabetes screening (DZC), it was confirmed the information already submitted. In addition to that, it was found out that the female passed through a single gestation/lactation at 11 months. It was also confirmed the daily regime of free movement in the yard and socializing with another pet (a cat) and the clinical history previously mentioned. During the blood and biochemical complex tests conducted during the following days we noticed only a granulocytic neutrophils (75.8%) and glycemia level of 185 mg/dL (Tab. 1 and 2). Under these circumstances was established the treatment with Diavit (3 + 3 tablets) and hipoglucidic diet with pelleted feed for Type II Canine Diabetes (DZC), excluding totally the feeding with kitchen scraps. We managed to stabilize the glycemia around 185 mg/dL. Meanwhile, during the ultrasound it was diagnosed a significant polycystic ovary, the start of pyometra, the 2 mammary tumoral formations and during the radiological investigations the progression of thoraco-lumbar spondylosis with "parrot beaks". In less than a month after these investigations, the patient made a sudden superacute crisis spurt, which began by vomiting and was manifested then by immobility, deep deviation, anorexia and low grade fever. In the next 2 days was established a symptomatic treatment, using nutrient infusion, rehydration and cardio-circulatory support, filled in with antibiotic therapy. Through this therapeutic protocol we have not managed to stabilize the patient and not to risk a more severe deterioration in health status it was proceeded to urgently total sterilization operation (ovariohysterectomy). Laparotomy surgery showed the onset of sero-fibrinous peritonitis, which explained the severity of the crisis triggered previously. The patient has undergone surgery and postoperative treatment well, following that within a week to restore fully to overall condition the temperature, the appetite and the vital functions. Assessments made at one month postoperatively revealed its framing in physiological limits for blood counts, for blood chemistry and cardio-vascular (Tab. 1 and 2; Fig. 1), except for total platelets which decreased and resolved in the following two months. Surprisingly, at the same time it was normalized also the glycemia levels which dropped to 74 mg/dL and diabetic symptoms (polydipsia and polyphagia) faded. Histopathological examination of the excised genital tract outlined a lesional array of bilateral polycystic ovaries and pyometra in early stage, with no evidence of tumoral formation. At the next 3 months monitoring the health of the patient was maintained at stabilized level and everything is back to normal, except for the locomotor symptoms given by spondylosis. We also note that among the therapeutic measures that were implemented was maintained the hypoglucidic diet, being recommended the a regular monitoring of health status with frequent assessment of glycemia.

Table 1.

Dynamics of haematological parameters during patient monitoring time period

Parameters	**Recorded values**			**Measurement units**	**References**
	Initial	***Preoperative***	***Final***		
WBC	10.23	13.70	9.39	x 10^9/L	6.00-17.00
LYM	2.02	1.10	5.52	x 10^9/L	1.00-4.80
MID	0.45	1.14	0.30	x 10^9/L	0.20-1.40
GRA	7.76	11.46	3.57	x 10^9/L	3.00-11.40
LY %	19.7	8.0	58.8	%	12.0-30.0
MI %	4.4	8.3	3.2	%	2.0-10.0
GR %	75.8	83.7	38.0	%	60.0-70.0
RBC	19,8	8.56	6.93	x 10^{12}/L	5.50-8.50
HGB	9.2	14.8	15.7	g/dl	12.0-18.0

HCT	31.17	44.30	44.23	%	37.0-55.0
MCV	66	67	64	fl	60-77
MCH	19.5	22.5	22.6	pg	19.5-24.5
MCHC	29.5	33.4	35.4	g/dl	32.0-36.0
RDWc	14.4	14.9	15.5	%	
PLT	784	472	97	x 10^9/L	200-900

Initial - 10.12.2015; *Preoperative* - 19.02.2016; Final - 29.06.2019

Table 2.

The dynamics of blood biochemical indices during the patient monitoring time period

Parameters	Recorded values			Measurement units	References
	Initial	***Preoperative***	***Final***		
GLU	185	111	74	mg/dL	60-110
BUN	6	7	17	mg/dL	7-25
TBIL	0.3	0.4	0.3	mg/dL	0.1-0.6
CA	<4.0	10.3	<0.4	mg/dL	8.6-11.8
TP	6.4	6.1	5.7	mMol/L	5.4-8.2
ALB	3.7	3.4	3.1	g/dL	2.5-4.4
ALT	71	80	86	U/L	10-118
CRE	0.7	0.8	0.9	mg/dL	0.3-1.4

Initial - 10.12.2015; *Preoperative*- 19.02.2016; Final - 29.06.2019

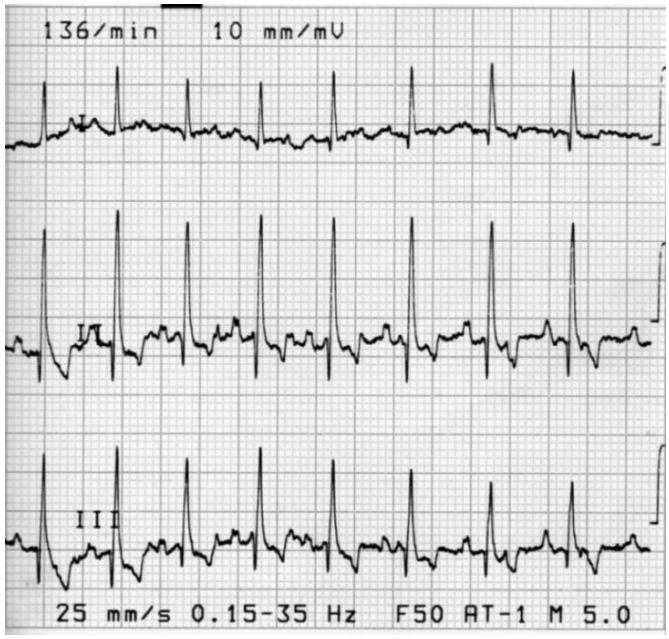

Figure 1. Preoperative appeareace of ECG, showing normal morphology of each of wave ant the lack of cardiac dysfuctions

According to bibliographical data, the incidence of diabetes in pets is growing, and it is appreciated between 0.2% and 1% at dog (Hess RSET al., 2000), which is a relevant argument to convince vets clinicians to accept that the evolution of this disease is a real problem for dogs and cats. Such a conception is essential for shaping as early diagnosis and for appropriately and effectively treatment. This is essential to ensure animal welfare and owners' comfort.

In terms of age as contributing factor in canine diabetes (DZC), the data indicate long intervals between 2.5 and 12 years, with a peak of susceptibility between 6-9 years (Hess R.S.et al., 2000). Most of the research in the field have investigated the impact of overweight and obesity major, known as factor in onset of diabetes in humans, and including canine diabetes (DZC). Thus, some recent studies show that over 50% of dogs and cats between the ages of 5-10 years are obese, their life span is 15% lower than those of normal weight (Scherk Margie, 2012). So overweight or obese cats are 2 to 4 times more likely to develop diabetes than those with normal weight (Rand J.S. et al., 2004). We must remember that there are studies according to which dogs have the ability to offset the excess fat tissue by increasing insulin production (Rand J. S. et al., 2004).

Interesting data also provided a study focused on assessing overweight as a contributing factor in diabetes taking up the correlation owner-dog, according to which 44% of dogs susceptible to diabetes belong to overweight or obese owners, 25% are diabetic with normal weight and belong to owners of normal weight and 31% of diabetics are owned by healthy propretors with normal weight (BO Roep and M. Atkinson, 2004).

Research on the influence of pregnancy and sterilization reveals that females with blood glucose levels above the normal rule passed one or two gestations before being sterilized (S.D. Greco, 2001). Also relevant is the data which shows the favouring influence of pyometra and ovarian cysts in the onset of diabetes, associated with other pathologies such as acute renal failure or heart failure (B.T. Larson et al., 2003).

Among the commonly used therapeutic formulas in prevention and control of Canine Diabetes (DZC), the one based on Diavit is already well known, also being an alternative to synthetic antidiabetic products implemented in pets' medicine. (Lantus commercial products or Caninsulin).

Finally we believe it could be relevant to canine diabetes (DZC) detection criteria ADA (American Diabetes Association) indicated in the diagnosis of diabetes in humans. They are based on develop symptoms (polyuria, polydipsia, weight loss unexplained), accompanied by glucose values "random" at or above 200 mg/dL, basic glucose at or above 126 mg/dL, and the tolerance test glucose (75 g of glucose, 2 h) equal to or more than 200 mg/dL.

Conclusions

1. According to the recorded data of the investigated patient the evolved pathologies involved in triggering the canine diabetes (DZC).
2. Female sterilization produces significant changes in the hormonal profile, which can cause significant fluctuations of glycemia, with hyperglycemia frequently.
3. The history of feeding the patient with predominantly dry pelleted feed and leftover food from the owner's kitchen has greatly increased susceptibility to diabetes.
4. For this diabetic patient the postoperative feeding has proved very beneficial and comfortable a hypoglucidic diet with a rich containing in proteins.
5. Post-sterilization remission of the diabetic symptoms for this patient reveals that is essential to treat the related various diseases to normalize blood glucose level.
6. An efficient management in controlling of this diabetes disease it is attribute hormonal

and emotionally balance and physical exercise provided, along with proper diet based on fiber with a hypoglycemic effect.

7. bună eficienţă în controlul acestui caz de boală diabetică atribuim echilibrului hormonal, emoţional şi fizic asigurat, alături de regimul alimentar adecvat bazat pe fibre cu efect hipoglicemiant.
8. We believe that this case study is a relevant information for clinicians regarding the use of sterilization which require therapy or other pathologies related to normalize glycemia and clinical remission of some forms of canine diabetes (DZC).

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STUDY ON ANTHELMINTIC EFFICACY OF PRODUCTS BASED ON IVERMECTIN, FENBENDAZOLE AND PYRANTEL IN CONTROLLING DIGESTIVE PARASITOSIS IN EQUINES FROM N-E AREA OF ROMÂNIA, USING FECRT TEST

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Abstract

Digestive helmints represent an important cause of the equine pathology, among these ones the most representative are the nematodes, from the Strongylidae family, Parascaris equorum and the cestodes such as Anoplocephala perfoliata. The measures to control these parasitosis are mainly based on regular deworming programs using different antiparasitic compounds. There are numerous studies, both nationally and internationally certifying a wider or narrower spread of resistance to some of the substances used for this purpose, especially in some species strongyls and other digestive helminths. The efficacy of treatment with products used in this study was tested on a herd of 56 horses copies of the territory Iași, Suceava, Bacău and Neamț, and the test used was the FECRT. The results showed a high efficiency of all three antiparasitic substances. In the case of ivermectin, FECRT average values were between 98.33% and 100%, for fenbendazole average was 97.11% and 98.21% for pyrantel. Thus, it found a high degree of safety regarding installation chemoresistant, at least for a certain period.

Key words: *horses, digestive parasitosis, treatment efficacy, chemoresistance*

Introduction

Horses can be infested with over 150 species of intern parasites (Güiris *et al*., 2010). Intestinal helmints represent an important cause of the equine pathology, among these ones the most representative are the nematodes, from the *Strongylidae* family, and the cestodes such as *Anoplocephala perfoliata* (Osterman Lind *et al*., 2007; Bonneau *et al*., 2009). The digestive pests can alter behavior, fertility, physical condition, foals' growth, immunity deficiency to other pathogen organisms or performance aptitudes (Cernea, 2008).

The large strongyles and the cyathostomins are among the world's most significant parasites that infect horses (Riccardo *et al*., 2010). Among these, recently, the greatest attention has been given to cyathostomins due to the fact that they are considered the main intestinal parasites of horses, with a colossal spread (Morariu *et al*., 2007; McWilliam *et al*., 2010). However, strongyls carried out on hosts a variety of pathogenic actions, especially given to large number of parasite species and to many stages of development taking place in the same host in different body tissues and organs. Sometimes there is a high percentage of morbidity and even mortality, especially among youth treated, thus causing multiple damages of medical, economic and animal welfare concerns (Covașă, 2011).

Internationally, the study of equine strongylidosis is a topical issue, particularly regarding the implementation of effective measures to control these morbid entities. Antihelminthic treatments are the basis for these purpose, so that it requires ongoing assesment of results abtained following administration of antihelminthic substances (Covașă *et al.*, 2012).

There are numerous studies, both domestically and internationally, proving a wider or narrower spread of resistance by the parasite involved. Therefore, in this study we aimed to evaluate the effectiveness of commonly used anthelmintic substances in combating digestive parasitosis of horses from N-E region of România, namely ivermectin, fenbendazole and pyrantel, to detect the phenomenon of chemoresistence. For this, we resorted to using qualitative and quantitative coproparasitological techniques, respectively FECRT test (*Faecal egg count reduction test*).

Material and methods

Observations on the effectiveness of treatment with antiparasitic substances were conducted on a herd of 56 horses copies of the territory Iaşi, Suceava , Bacău and Neamț. Three groups are represented by the following horse farms: livestock farms Rediu, Iaşi - 6 animals , Rădăuți Stud - 30 animals and Sports Club Blăgeşti, Bacau - 7 animals. Animals in the last batch, the number 13, came from extensive system of Iaşi county, the villages Popeşti and Sineşti.

Treatment effectiveness was verified in a comparative way for the following antiparasitic substances: ivermectin, fenbendazole and pyrantel (as pamoate) in combating *Strongylidae* infestations. For this purpose, the test used was FECRT (*Faecal egg count reduction test*) after treatment.

Efficacy of ivermectin was checked in horses from two lots: Stud farm livestock Rădăuți and Rediu. In the first group, antiparasitic product was used as an oral paste, *Noromectin* 1.87 % at a dose of 200 mcg/kg. Rădăuți stud horses were dewormed with *Ecvirom-I* product in the form of oral suspension containing ivermectin 0.2 g/100 ml (1 g/50 ml syringe), and praziquantel 2.5 g/100 ml (1.25 g/50 ml syringe), the ivermectin dose administrated being the same, respectively 0.2 mg/kg .

The fenbendazole effectiveness has been verified in horses from the extensive system in Iași county, following administration of the product *Panacur* oral paste (18.75 %) at a dose of 7.5 mg/kg .

In the lot consists from horses of Sports Club Blăgeşti, Jud. Bacău, it was tested the effectiveness of pyrantel pamoate, using the product *Pyratel EQ* oral paste containing 132 mg/mL of pyrantel pamoate, the dose administrated being 6.6 mg/kg.

The treatments were performed in the morning and from each animal were harvested samples (approximately 150 g each) from the feces freshly removed the day before administration of the substance antiparasitic (day 0), and in the near future, namely 24 hours, then 3, 5, 7 and 14 days.

To identify the *Strongylidae* parasitic elements, we used the Willis method, as a ovoscopic method, and Baermann, as a larvoscopic method. As a quantitative method, MacMaster method was used to determine the parasitic load for each exemplary, pre- and post-treatment, respectively O.P.G. (eggs/gram of feces).

Subsequently, based on O.P.G. values, it was done the *faecal egg count reduction test*-FECRT. Anthelmintic efficacy of the product was calculated using the formula:

E % = (OPG before treatment (day 0) – OPG on day 14/OPG on day 0) x100

Results and discussions

Based on coproparasitological analysis, we identified *Strongylidae* parasitic elements, namely eggs in various evolution stages and different sizes and L3 infestant larvae.

In horses from the groups treated with ivermectin, the mean values of the parasitic load degree of pre-treatment day (day 0) were between 50 and 800 O.P.G. Approximately 24 hours after antiparasitic drug administration were obtained higher values, between 150 and 1200, thereafter decreasing gradually, all the values are negative on day 7.

Following administration of ivermectin is observed intensification of eggs removal immediately after treatment, as an effect of antiparasitic substance, followed by progressive decrease in the number of eggs in feces. Negative values of O.P.G. occurred since the 5th day post-treatment, on day 14 the eggs recurrence was observed in the faeces.

The FECRT results after treatment with ivermectin are shown in Table 1 and Table 2.

Table 1.

FECRT values obtained after treatment with ivermectin in horses from Rădăuți Stud

Crt. no.	Mean OPG day 0	Mean OPG day 7	FECRT %	Mean OPG day 14	FECRT %
1.	150	0	100	0	100
2.	200	0	100	0	100
3.	200	0	100	0	100
4.	350	0	100	0	100
5.	250	0	100	0	100
6.	300	0	100	50	83.33
7.	200	0	100	0	100
8.	200	0	100	0	100
9.	50	0	100	0	100
10.	650	0	100	0	100
11.	150	0	100	0	100
12.	200	0	100	0	100
13.	200	0	100	0	100
14.	350	0	100	50	83.33
15.	250	0	100	0	100
16.	100	0	100	0	100
17.	550	0	100	0	100
18.	300	0	100	0	100
19.	150	0	100	0	100
20.	200	0	100	0	100
21.	300	0	100	0	100
22.	400	0	100	50	83.33
23.	450	0	100	0	100
24.	350	0	100	0	100
25.	100	0	100	0	100
26.	150	0	100	0	100
27.	200	0	100	0	100
28.	150	0	100	0	100
29.	200	0	100	0	100
30.	250	0	100	0	100
Average	251.66	0	100	5	98.33

The obtained results demonstrate a good activity of ivermectin in the treatment of strongylidosis, in the first group FECRT average values showing a 100% efficiency (Covașă *et all*., 2012), and in the second, a 98.33% efficiency.

Recorded data show a low probability of resistance installation, FECRT values being much higher than limit of 85% which could lead to suspicion emerging phenomenon of resistance.

Table 2.

FECRT values obtained after treatment with ivermectin in horses Livestock farms Rediu

Crt. no.	Mean OPG day 0	Mean OPG day 7	FECRT %	Mean OPG Ziua 14	FECRT %
1.	800	0	100	0	100
2.	500	0	100	0	100
3.	450	0	100	0	100
4.	350	0	100	0	100
5.	350	0	100	0	100
6.	100	0	100	0	100
Average	425	0	100	0	100

The FECRT results for fenbendazole treatment are shown in table 3.

Table. 3.

FECRT values obtained after treatment with fenbendazole

Crt. no.	Mean OPG day 0	Mean OPG day 7	FECRT %	Mean OPG Day 14	FECRT %
1.	350	0	100	0	100
2.	400	0	100	50	87.5
3.	700	0	100	0	100
4.	400	0	100	0	100
5.	450	0	100	0	100
6.	550	0	100	0	100
7.	500	0	100	0	100
8.	650	0	100	50	87.5
9.	400	0	100	0	100
10.	750	0	100	50	87.5
11.	550	0	100	0	100
12.	400	0	100	0	100
13.	400	0	100	0	100
Average	500	0	100	11.53	97.11

It is noted the same trend of O.P.G., negative values recorded from the 5th day after the treatment, on day 7 all the samples being negative. The obtained result of FECRT, 97.11 %, show also for fenbendzole a good efficiency without manifestations of the phenomenon of resistance.

Table 4 shows the dynamics of *Strongylidae* egg coproeliminations in horses treated with pyrantel and FECRT values obtained from them.

Also in the case of pyrantel, the results obtained from treated horses show good efficacy, the mean value of FECRT touching 98.21 % of percentage.

It thus appears that the three antiparasitic substances used to control the horses strongylidosis of these lots shows a high degree of safety regarding resistance installation, their rational use in the future ensuring the opportunity of effective control of these parasitosis.

Table 4.

FECRT values obtained after treatment with pyrantel

Crt. no.	Mean OPG day 0	Mean OPG day 7	FECRT %	Mean OPG day 14	FECRT %
1.	350	0	100	0	100
2.	400	0	100	0	100
3.	700	0	100	0	100
4.	400	0	100	0	100
5.	450	0	100	0	100
6.	550	0	100	50	87.5
7.	500	0	100	0	100
Average	185.71	0	100	7.14	98.21

Worldwide, anthelmintic therapy, especially the strongylidosis, faced with the increasing emergence of chemoresistant populations to probenzimidazole and benzimidazole derivatives, tetrahydropyrimidines even to macrocyclic lactones (Moore, 2000). Resistance to benzimidazole has been reported in North America, South America, South Africa, Australia, New Zealand, Turkey and several European countries (Kaplan, 2002; Çirak, 2004). If we analyze the spreading situation of anthelmintic substances resistance in Europe, we can notice a slight extension of the phenomenon from west to east, affecting countries with tradition in raising horses, like England, Holland and Germany (Morariu, 2007). Thus, in some of these countries benzimidazoles resistance of cyathostomes reaches 70% or more in horse farms (Kuzmina *et* Kharchenko, 2008). Recent studies have shown the installation of benzimidazole resistance in Greece, Ukraina and Turkey (Papadoupoulos *et al.*, 2000; Çirak *et al.*, 2004; Kuzmina *et* Kharchenko, 2008).

In our country, Cernea *et al.* (2004), noted this phenomenon to horses from Bistrița Năsăud lands, where was reported cyathostomes resistance to albendazol treatment.

Due to high effectiveness and broad spectrum of action, ivermectins tend to become the most widely used class of antiparasitic substances. In Europe, as in our country, there does not exist dates showing the emergence of strongyls resistance to treatment with ivermectin (Covașă *et al.*, 2012). However, there are recent studies which showed only reduced efficacy of these compounds by reducing the period of eggs recurrence in faeces after treatment (Von Samson-Himmelstjerna *et al.*, 2007; Lyons *et al.*, 2008; Edward *et* Hoffmann, 2008; Molento *et al*., 2008; Riccardo *et al.*, 2010; Lyons *et al.*, 2010). This was particularly highlighted for ivermectin, maintaining good efficacy for moxidectin (Lyons, 2010). It is considered that the O.P.G. from feces must be negative or very low for 8 weeks after treatment with ivermectin and 10 weeks or more in the case of moxidectin (Lyons *et al.*, 2010).

In exchange was reported in several studies, ivermectin resistance of *P.equorum*, both both in America and in Europe and Asia (Boersema *et al*., 2002; Hearn *et* Peregrine, 2003; Kaplan *et al*., 2006; Stoneham *et* Coles, 2006; Schougaard *et* Nielsen, 2007; Slocombe *et al*., 2007; Von Samson-Himmelstjerna *et al*., 2007; Lindgren *et al*., 2008; Molento *et al*., 2008; Sakhaee *et al*., 2011; Laugier *et al*., 2012).

In terms of *Strogylidae* tetrahydropyrimidines resistance, it has been described in countries such as Norway, Denmark and the US (Kaplan, 2002; Kaplan *et al*., 2004; Von Samson-Himmelstjerna *et al*., 2007), especially those in the cyathostomins group.

In most regions of the globe it has been reported a predominance of a small group of species (10-12) in the *Strongylidae* population of domestic horses subjected to anthelmintic treatments (Collobert-Laugier *et al*., 2002; Osterman-Lind *et al*., 2003; Kuzmina *et al*., 2005;

Kuzmina *et* Kharchenko, 2008). Ten cyathostome dominant species (*C. nassatus*, *C. catinatum*, *C. calicatus*, *C. ashworti*, *C. longibursatum*, *C. goldi*, *C. pateratum*, *C. minutus*, *C. coronatus*, *C. leptostomum*) were identified as resistant to benzimidazole in various countries (Tolliver *et al*., 1993; Lyons *et al*., 1996; Kaplan, 2002). Beside them, the species *C. labiatus* was found resistant to benzimidazole in Olanda and Sweden (Eysker *et al*., 1988; Osterman Lind, 2007).

Therapy failure caused use of drug combinations which originally had a high efficiency, then in most cases ascertaining the emergence of the phenomenon of multiple resistance. In this situation finding new molecules against which resistant strongyls manifest sensitivity, is an ongoing concern and urgent topical. Currently trying to find new ways to fight this phenomenon, one of the possible alternatives being phytotherapy (Cernea *et al*., 2009).

Conclusions

1. A special emphasis in combating of resistance phenomenon, falls on screening tests of resistance, screening which must precede performing of anthelmintic treatment.
2. Benzimidazoles represent the most widely used group of antiparasitic substances until recently to combat digestive parasitosis and serous of horses, with a relatively broad spectrum. However, numerous studies have revealed a massive increase in resistance to many compounds of this class, particularly in the *Strongylidae* case.
3. If internationally numerous cases of chemoresistance have been reported, in our country this is still a weak widespread phenomenon, but ongoing.
4. We consider that low frequency of *Strongylidae* or other helmints resistance installation phenomenon to macrocyclic lactones and other antiparasitic substances has the right cause the recent use of these substances in antiparasitic treatment unlike most Western countries where they were put on the market longer.
5. The efficacy study of antiparasitic substances used in our country against strongylidosis and not only, reveals a suitable anthelmintic activity, without manifestations of resistance in the studied areas, even 100 % given as the FECRT test values.
6. We believe that the absence of resistance to ivermectin in this case are due to regular annual deworming and alternately plan in semi-intensive system, and in extensive system due to sporadic carrying out of these treatments.
7. As demonstrates in many previous studies, ivermectin remain, especially in our country, a higher class of antiparasitic compounds, both in terms of spectrum and treatment effectiveness.
8. In addition to chemical treatment of digestive parasites in horses, research in this field has expanded to other alternative means, one of which being phytotherapy , sometimes with promising results.
9. Investigations into the effectiveness of parasitosis treatments and other control measures, is a main direction in the future in equine parasitology and beyond.

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SEROLOGICAL SURVEY ON EXPOSURE TO *BARTONELLA HENSELAE* OF DOMESTIC CATS IN BUCHAREST AREA: PRELIMINARY DATA

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Abstract

Bartonella henselae, the causative agent of cat scratch disease (CSD) in humans, is a Gram-negative facultative intracellular bacterium. Domestic cats are the main reservoir for B. henselae; infected cats are usually asymptomatic, but sometimes they can present non-specific symptoms like: lethargy, fever or anorexia. They may have recurrent periods of bacteraemia, which may last from several months to years. For detection Bartonella spp. infection, direct methods (bacterial isolation, molecular biological methods, Polymerase Chain Reaction) and indirect methods (serological tests: IFA, ELISA and Western immunoblot) are available. In Romania, data about B. henselae infection in cats are scarce. Therefore, this study aimed to investigate the exposure to infection with B. henselae of domestic cats in the urban area of Bucharest (South-eastern Romania). For this, a total number of 50 cats, including stray and owned cats, of common breed, and different age (from 7 months to 15 years), were included in the survey. Cat blood samples were collected and serological tested for the presence of B. henselae IgG antibodies using an indirect IFA test. Plasma samples were initially tested at 1:50 dilution and then the positive samples were tested at 1:100 dilution. The slides were read using a fluorescence microscope at 400x magnification. For the cats examined in the present study, the seroprevalence of B. henselae was 56% (28/50). Of the seropositive cats, 21 were owned cats. There were no statistically significant differences between young cats (with age under 1 year) and older cats (p=0.118). Further investigations are planned on the both IFA positive and negative samples, using blood cultures and molecular methods, for confirmation and identification of B. henselae. In conclusion, the results of the present study show a high exposure to B. henselae infection of cats on Bucharest area. Also, the findings indicate that pet cats pose great risk for B. henselae infection this being important in the transmission of the infection in humans (zoonotic risk).

***Key words**: Bartonella henselae, IFA, domestic cats, Bucharest.*

Introduction

All species of the *Bartonella* genus are part of the small Gram-negative facultative intracellular bacterium (Sander et al., 1998); they are immobile, with lengths of 1-2 μm, are oxidase, catalase, urease and indol negative (Engelkirk and Duben-Engelkirk, 2008). This bacterium infect and persist in erythrocytes and endothelial cells in domestic and wild mammals worldwide (Mietze at al., 2011).

Bartonella henselae is the causative agent of cat scratch disease (CSD) in humans. Regnery et al. (1992) have identified *B. henselae* antigens seroactivity in 88% of 41 human patients with suspected CSD. Typical of the CSD, it denotes a "self-limiting" disease, characterized by fever and lymphadenopathy associated with cat scratch or bite (Breitschwerdt, 2014). Atypical manifestations of CSD included tonsillitis, encephalitis, cerebral artery, transverse myelitis, hepatitis and/or splenic granulomatous, osteolysis, pneumonia, pleurisy, and idiopathic thrombocytopenic purpura (Zangwill, 2013).

Domestic cats are the main reservoir for *B. henselae* (Staggemeier et al., 2010). Cats contamination is realized through the bite of hematophagous arthropods (e.g. fleas, ticks). Of these, *Ctenocephalides felis* has an important role in transmission of infection with *B. henselase* in cats (Schouls et al., 1999).

Natural infected cats are usually asymptomatic, but sometimes they can present non-specific symptoms like: lethargy, fever or anorexia; lymphadenopathy, stomatitis, neurological and renal disease are sometimes observed (Ebani et al., 2012; Guptill, 2010). They may have

recurrent periods of bacteremia, which may last from several months to years (Ebani et al., 2012).

For detection of *Bartonella* spp. infection in cats, direct methods (bacterial isolation, molecular biological methods, Polymerase Chain Reaction) and indirect methods (serological tests: IFA, ELISA and Western immunoblot) are available (reviewed in Nasoiu et al., 2015). For serological testing of animals, in order to detect *Bartonella* spp. antibodies, blood samples have been used, while for the identification of *Bartonella* species through molecular methods, the samples collected for analysis are: blood, lymph node aspirate, tissue aspirate, saliva, articular liquid, ocular exudate, or biopsy samples (Hardy, 2007).

In Romania, data about *B. henselae* infection in cats are scarce. A study on fleas conducted in the Czech Republic and Romania by Lawrence et al. (2015), using a diagnostic real-time PCR assay, proved that there were no positive fleas collected in Romania for *Bartonella* spp.

Cotar et al. (2011) in their study on patients with Q fever endocarditis from Romania reported, that some serum samples exhibited antiphase I *C. burnetii* IgG antibody titers >800, while none of samples has IgG for *B. henselae* or *B. quintana* (blood samples were tested using IFA test).

The aim of this study was to investigate the exposure to infection with *B. henselae* of domestic cats in the urban area of Bucharest (South-eastern Romania).

Materials and methods

A total number of 50 cats (33 females; 17 males), including stray (n=10) and owned cats (n=40), of common breed and different age (from 7 months to 15 years), were included in the survey. Data from gender, age and animal habitats (indoor, around the house or stray cats) were recorded.

Cat blood samples were collected and serological tested for the presence of *B. henselae* IgG antibodies using an indirect immunofluorescence antibody test (IFAT) according to the manufacturer instructions (MegaFLUO®Bartonellahenselae, DiagnostikMegacor, Hörbranz, Austria). IFA test seems to have a good specificity and is used widely as method of diagnosis (Lamas et al., 2008).

The samples were obtained by cephalic or saphenous venepuncture, collected in tubes with EDTA, centrifuged and kept at -20°C until they were used. Plasma samples were initially tested at 1:50 and then the positive samples were tested at a 1:100 dilution.

The examination of samples was performed using a fluorescence microscope (filter system for FITC) with 400 x magnification.

Interpretation of results was subjectively evaluated, using a scale from 1 to 4 depending on the specific fluorescence intensity. Samples with a ≥ 2 score were declared positive for infection with *B. henselae* (score 1: sample negative; ≥ 2 score: sample positive, depending on the intensity of specific fluorescence, score 2 meaning low intensity and score 4 meaning high intensity) (Ebani et al., 2012) (**Figure 1**).

Results and discussion

To detect *Bartonella* infection in cats we used an indirect immunofluorescence test for detection of specific IgG antibodies against *B. henselae* in plasma or serum of the cat. The fluorochrome reaction was observed by use of the fluorescence microscope.

The results were subjected for interpretation as follows:

- *Positive reaction: B. henselae* bacteria in the cytoplasm of infected cells show a yellow-green fluorescence, glowing intensely; IgG titers of 1:50 and higher, indicate the contact with the pathogen.
- *Negative reaction:* was considered when there were no clearly visible fluorescing bacteria in the cell cytoplasm.

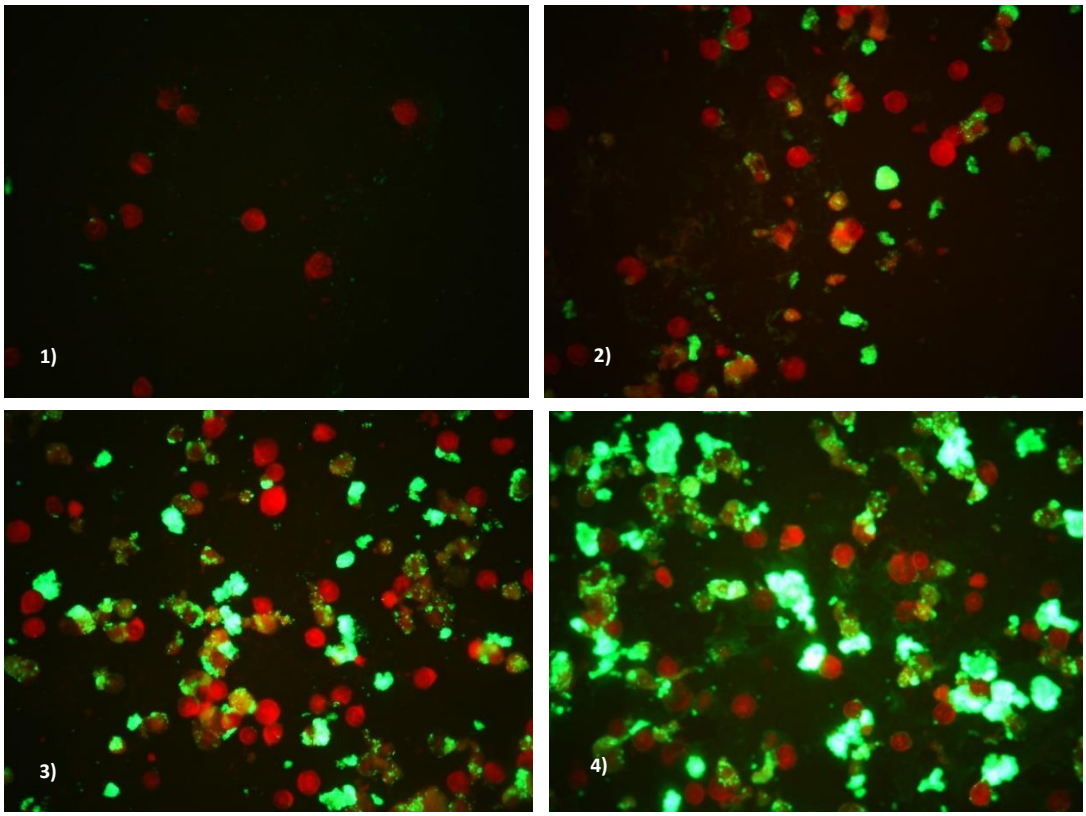

Figure 1. Interpretation of results depending on the specific fluorescence intensity:
1) score 1 – weak fluorescence (+/-); 2) score 2 – clearly visible fluorescence (++);
3) score 3 – bright fluorescence (+++); 4) score 4 – brilliant fluorescence (++++)

For the cats examined in the present study, the seroprevalence of *B. henselae* was 56.00% (28/50).

Depending on the origin and habitat of tested cats, the data showed no statistically significant differences between owned and stray cats ($P = 0.480$) (Table 1).

Also, there were no statistically significant differences between young cats (with age under 1 year) and older cats ($P = 0.118$) (Table 1).

B. henselae infestation rate was higher in females than in males, but no statistically significant differences between genders was registered ($P = 0.386$) (Table 1).

Table 1.

Seroprevalence for *B. henselae* infection in cats from Bucharest area, by IFA test

Animals category	Serologic testing			
Habitat	No. of cats tested	No. of positive cats	Prevalence (%)	*P*
Owned cats	40	21	52.50	0.480
Stray cats	10	7	70.00	
Age				
≤ 1 year	17	10	58.80	0.118
>1 year	33	18	54.70	
Gender				
Female	33	20	60.60	0.386
Males	17	8	47.10	

Cats included in this study did not show any clinical signs of infection with *Bartonella*. This fact supports the hypothesis that even cats are asymptomatic, they often represent a reservoir for *Bartonella* infection in humans (Ebani et al., 2012).

Exposure assessment to *B. henselae* infection of cats from different habitats is important in understanding the epidemiology of this bacterium. The results of this study show that *B. henselae* infection is widespread in both stray and owned cats, suggesting that these cats could represent a potential risk for human health in this area.

The seroprevalence obtained in the present study for *B. henselae* infection in cats in Bucharest (56.00%) indicate a high value compared to other European countries, such as Norway (0%) (Bergh et al., 2002), Switzerland (8.3%) (Glauss et al., 1999), Northern Italy (23.1%) (Brunetti et al., 2013) and similar with those in Denmark (45.6%) (Chomel et al., 2002).

Guptill et al. (2004) have performed studies to determine the prevalence of *B. henselae* bacteria in four areas in the United States (USA), where the prevalence was 51% of seropositive cats (138/271); the highest seropositivity was reported in Florida (67%) and California (62%) and the lowest seropositivity was found in Washington, D.C. (28%) and Chicago (12%) areas.

Al-Majali (2004) investigated the seroprevalence of *B. henselae* in domestic cats in Jordan, and an overall prevalence of 32.00% was found. He compared the positivity bacteria in three regions of the country, finding a higher percentage in the north (38%) than in the center (18%) and South (12%). This difference was due to more favorable conditions for the development and propagation of fleas in northern Jordan since the average rainfall and the temperature were higher than in other regions.

Switzer et al. (2013) highlight that cats who spend their whole lives outside and are exposed to massive flea infections and such habitats can be considered as predisposing factors for *B. henselae* infection (Switzer et al., 2013).

Regarding seropositivity between genders, in this study a slight superiority was evident in females. This statement coincides with the findings of Zaror et al. (2002) where the cats studied in Valdivia, Chile, 73.3% of females were positive. However, in another study conducted by Switzer et al., (2013) in Iraq, a higher prevalence was registered in males (63.3%), however it was not established statistically significant difference (Al-Majali, 2004).

The results from the present study showed no statistically significant association between age and *B. henselae* seroprevalence. Chomel et al. (1995) describe the fact that

seroprevalence increases in general with cats' age, while bacteraemia is most common in young cats (<1 year). Similar observations came from Argentina (Cicuttin et al., 2014), Denmark (Chomel et al., 2002), Phillippines, California (Chomel et al., 1999) and Pisa, Italy (Ebani et al., 2012).

Conclusions

The high seroprevalence of *B. henselae* in asymptomatic cats included in the present study indicates potential high risk for the transmission of infection in humans (zoonotic risk). Further investigations are planned on the both IFA positive and negative samples, using blood cultures and molecular methods, for confirmation and identification of *B. henselae*.

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COMPARATIVE RESEARCH REGARDING TWO METHODES FOR ESTRUS SYNCHRONIZATION IN POSTPARTUM DAIRY COWS BASED ON PROGESTERON (PRID) AND PROSTAGLANDIN F2α AND THE RELATIONSHIP WITH THE METABOLIC STATUS

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Abstract

The study was conducted between October 2015 and June 2016 in the zootechnical biobase of Research and Development Station Breeding Cattle, Dancu, Iasi on Romanian Black Spotted breed dairy cows, in free system of modernized shelter, with a capacity of 200 heads/shelter. The study aimed at establishing the effectiveness of two methods for the induction and synchronization of estrus in dairy cows, based on gestagens and prostaglandin F2α respectively, in relation to their metabolic status. Depending on the applied treatments, two experimental groups were established: E1 group, composed of 13 cows, of which 7 cows with corpus luteum and 6 cows without corpus luteum), which were treated with progesterone administrate through a intravaginal device (PRID) for 7 days and prostaglandin (PG)F2α 24 hours before the removal of device . E2 group, composed of 29 cows with a corpus luteum were treated with single dose of prostaglandin (PG)F2α.. The average interval from the calving to treatment was 89.60+6.607 days (E1 group), respectively 113.40 ± 5.851 days (E2 group). Blood samples for biochemical analysis (total seric protein, serum albumin, total cholesterol, GGT, ALT, AST, serum calcium and phosphorus) were collected from E2 group of cows. The measurements were made using automatic biochemical analyzer Cormay. The results were statistically analyzed and significant differences were determined by Student's test. Analysis of the results revealed the following: the expression of oestrus between 69.23% (E1 group) and 89.65% (E2 group); average intervals from treatment to first artificial insemination (1 IA) was between 5.53 ± 0.99 days (E2 group) and 8.66 ± 3.07 days, (E1 group); average intervals from treatment to conception was between 33.83 ± 12.05 days (E1 group) and 34.45 ± 10.22 days (E2 group); conception rate was between 46.15% (group E2) and 66.6% (E1 group). After estrus synchronization treatments with prostaglandin (PG)F2α the cows of group E2, with normal values of biochemical parameters had a higher conception rate, compared to cows with abnormal biochemical parameters (72, 72% vs 26.66%, P <0.01).

Key words: *dairy, estrus synchronization, prostaglandin, PRID intravaginal device, fertility*

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Introduction

Reproductive performance of highly productive dairy cows with high genetic value decreased in many farms, the main causes being the improper environmental and defective management (1,3,13,14,15). Detecting oestrus and performing insemination at the optimum time, is essential in the management plan of any dairy farm and failure in achieving this goal negatively influences both the conception rate and the reproductive performance of animals. In these circumstances, the oestrus induction and synchronization programs became an useful tool in large farms with dairy cows, increasingly being used due to their benefits for reproductive management (1,2,15).

By implementing these programs of oestrus synchronize, every animal from the farm may receive treatment, followed by a short insemination period (AI) after the detection of oestrus or the insemination may be performed at a predetermined time, without detecting the oestrus (programmed AI), thus the number and frequency of manipulation operations, as well as the presence of persons for oestrus detection will be reduced.

In countries with advanced zootechnical sector, oestrus synchronization is a biotechnology currently used for improving the reproductive management in cows. The benefits of this biotechnology reside in the reduction of time interval from calving to the first insemination (AI) and from calving to conception, with major impact on successive calvings interval. Additionally, the detection of oestrus may increase the number of births/year/reproductive life (1,7,8,9,10).

The traditional protocols are designed to control the corpus luteum on the ovary, while the newer protocols are meant to control the ovulation and/or follicular waves that occur on the ovary during the 21 days of estrous cycle. The basic principles of this biotechnology are to synchronize the oestrus in cows estrus, either by temporarily blocking the sexual cycle, either by stimulating the functional involution of the corpus luteum. For achieve these goals, the following hormonal products are recommended: steroids with gestagen effect, prostaglandins and hormonal associations between prostaglandin and gonadorelin, or between gestagens, prostaglandin and gonadorelin (1,2,5,8,9,10,14). The most effective biotechnical methods used to control the sexual cycle of cows are those that do not require more products administration and manipulations of females and their effect results in an effective synchronization of oestrus and higher fertility in cows.

The synchronization of cows oestrus with gestagens consist in the use of steroids with gestagenic action (progestin), which by blocking the preovulatory secretion of LH from pituitary, will inhibit the oestrus and ovulation. The most recommended methods of administration are vaginal spirals, ear and skin implants, or feed (1,2,8,10,13,16). The gestagens recommended for use in animal feed, particularly for induction and synchronization of oestrus in heifers are synthetic steroids (Chlormadinoacetat CAP, MAP-Medroxyprogesteroneacetate, Norgestomet), (7, 11,16). The synchronization of oestrus with prostaglandin consist in the use of prostaglandin F2α (Proliz, Prosolvin, Oestrophan, Estrumate, Dinolytic) in single or double doses with functional luteolytic effect of the corpus luteum (1,2,5,11,13,14).

The aim of this study was to determine the effectiveness of using the two methods described previously for the induction and synchronization of oestrus in dairy cows with gestagens and prostaglandin (PG) F2α in single dose and the furthermore to observed the possible correlations with the metabolic status of the cows treated with PG(F2α).

Material and method

The studies were conducted between October 2015- June 2016 in the zootechnical biobase of the Research and Development Station for Cattle Breeding, Dancu, Iasi, on dairy cows Romanian Black Spotted breed, which are serviced in free system of modernized shelter, with a capacity of 200 heads /shelter.

The treatments for inducing and synchronizing of oestrus to dairy cows were applied in October 2015- June 2016 period on two experimental groups: E1 group, composed of 13 cows, of which 7 cows diagnosed with a corpus luteum (E1a group, PRID+CL) and si 6 cows without corpus luteum (E1b group, PRID+no CL), which were treated with progesterone using an intravaginal device (PRID) and E2 group composed of 29 cows diagnosed with a corpus luteum, which were treated with analog prostaglandin (PG)F2α (Estrumate from Intervet International BV, Netherlands).

The intravaginal device was maintained for 7 days and 24 hours before the removal of the device, an intramuscular dose of prostaglandin (PG) F2α was injected. The average interval from calving to treatment was between 89.60 + 6.607 days (E1 group), respectively 113.40 ± 5.851 days, (E2 group).

From cows in E2 group, blood samples were collected for biochemical analysis, for the following parameters: total serum proteins, serum albumins, total cholesterol, liver transaminases (GGT, ALT, AST), calcium and serum phosphorus. The measurements were made using automatic biochemical analyzer Cormay. The results were statistically analyzed and significant differences were determined by Student's t test.

The effectiveness of the treatments applied in this study was established by determining the rates of expression of estrus after treatments, average intervals from treatment to first artificial insemination (AI) and from treatment to conception, conception rates on total IA and in different intervals of treatments. Additionally, the relationship between conception rates in cows of E2 group, which were treated with PGF2α, normal (E2a group) and abnormal biochemical parameters values (E2b group) was investigated.

Results and discussions

The analysis of these results showed a manifestation of oestrus in cows after treatments, which varied between 69.23% (9 of 13 cows), (E1 group, cows treated with progesterone, PRID) and 89.65% (26 of 29 cows), (E2 group, cows treated with prostaglandine (PG) F2α). We found the lower values of averages intervals from treatment to first IA in E2 group (PGF2α), compared to E1 group (PRID), (5.53 ± 0.99 days vs. 8.66 ± 3.07 days) and values close between the two groups of average intervals from treatment to conception (33.83 ± 12.05 days, vs. 34.45 ± 10.22 days), the differences being statistically insignificant.

Cows diagnosed with corpus luteum from E1a group,(PRID+CL) had a better response after treatments than cows without corpus luteum from E1b group (PRID+ no corpus luteum), which resulted in higher values of oestrus manifestation rate (100% vs. 33.33%, P <0.001), smaller values of average interval from treatment to first AI (5.57 ± 2.76 days vs. 19.50 ± 5.50 days) and lower values of average interval from treatment to conception (26.80 ± 11.98 days vs. 69 days), (Table 1).

Table 1

Procentage of cows detected in estrus and insemineted after treatments with progesterone (PRID) and prostaglandine (PG) F2α to estrus synchronize

Lots	Treatments	No. cows/lot	No. cows in estrus	% cows in estrus	Interval treatment to estrus + insemination (days)		Interval treatment to conception (days)
					average	variation limits	average variation limits
Lot E1 a 2-57	PRID +CL	7	7	100.00	5.57± 2.76	2-22	26.80 ± 11.98
Lot E 1 b 69	PRID + no CL	6	2	33.33	19.50 ± 5.50	14-25	69.00 ± 00.00
Lot E1 2-69 a+b	Total PRID	13	9	69.23	8.66 ± 3.07	2- 25	33.83 ± 12.05
Lot E2 2-107	PGF 2α	29	26	89.65	5.53 ± 0.99	2-19	34.45 ± 10.22

Conception rate of dairy cows after oestrus synchronization treatments had higher values with 20.51% in E1 group (PRID), as compared to E2 group (PGF2α), (66.66% vs. 46.15%, P <0.05). Cows diagnosed with a corpus luteum (PRID+CL) from E1a group, had higher values of conception rate with 54.76% as compared to cows without corpus luteum from

E1b group (PRID+ no corpus luteum), (71.42% vs. 16.66%, P<0.001), the differences being very significant.

Taking into account the different intervals from treatments to conception, the conception rate was higher with 16.66% in E2 group, varying between 0-30 days interval from treatment, compared to E1 group (66.66% versus 50.0%, (P<0.05) and higher values with 7.67% in E1 group (PRID) to 0-60 days interval (83.33% vs. 75.66%), compared with E2 group (PG F2α), with an average pregnancy index between 2 (E 2 group) and 2.3 (E1 group) (table 2).

Table 2

Pregnancy rates after treatments with progesterone (PRID) and prostaglandine (PG) F2 α to estrus synchronize

Lots	Treatments [a]	No. cows/lot	No. cows pregnant	% cows pregnant total,	% cows pregnant in different intervals from treatment			average pregnancy index
					0-30	31-60	over 60 days	
Lot E1a	PRID +CL	7	5	71.42***	50.00	33.33	0.00	1.6
Lot E1 b	PRID + no CL	6	1	16.66	0.00	0.00	16.60	3.0
Lot E 2.3 a+b	Total PRID	13	6	66.66 *	50.00	33.33	16.60	
Lot E2	PGF 2α	29	12	46.15	66.66	9.00	27.30	2.0

*P<0,05 diferente semnificative, ***P<0,001 very significant differences

This study aimed at establishing the effectiveness of oestrus synchronization treatment with prostaglandin F 2α in relation to animal's health. For this purpose we analyzed some biochemical parameters in cows in the experimental group and depending on the values registered, we divided the animals into 2 groups: cows with values within normal limits and cows with abnormal, according to data from the literature (6). Furthermore, we investigated the correlation between the percentage of cows in oestrus and the rate of conception after treatment in relation to their health, defined by the biochemical parameters.

Analysis of biochemical parameters determined from blood samples from cows in group E2 (PG F2α) revealed

that 44.83% (13 of 29 cows) had normal values of biochemical parameters and 55.17% (16 of 29 cows) presented abnormal values. The main biochemical parameters with significant differences as compared to the standard values were the following: total serum proteins (8.056 ± 0.249 g/ dl, versus 6.676 ± 0.136 g / dl, P <0.05), serum albumin (3.735 ± 0.158 g / dl, versus 3.023 ± 0.053 g / dl, P <0.05), Total Seric Cholesterol (197.118 ± 8.911 versus 203.86 ± 1.50, P<0,01), GGT (23.75 ± 1.117 UI, versus 18.69 ± 0.45 UI ,P <0.001), ALT (47.912 ± 4.254 UI, versus 25.61± 0.92 UI ,P<0.001), AST (86.575 ± 9.688 UI, versus 22.79 ± 0.74 UI, P <0.001), seric calcium (11.55 ± 0.384 mg / dl, versus 10.20 ± 0.29 mg/dl, P <0.05).

Analyzing the effectiveness of treatments with PGF2α to synchronization of oestrus in cows, according to the values of the metabolic status, a higher conception rate with 35.07% (P <0.01), compared to cows that had abnormal biochemical parameters (72.72% vs. 26.66%) was observed (table3).

Table 3

Normal (E2a) and anormal biochemical parameters values, (E2b) the percentages of cows with estrus and conception rate

Specification	UM	Statistical estimates of biochemical parameters								Differences E 2a – E2b	Statistical significance Student test	
		Cows with normal values, E2a				Cows with anormal values, E2 b						
		average ±	V %	minimum	maximum	Average ±	V %	minimum	maximum			
Samples	n	13				16					t	P≤
Total serum proteins	g/dl	6.676 ± 0.136	7.39	6.0	7,4	8.056 ± 0.249	12.387	6,4	9.3	- 1,38	3.108	P<0,05
Total serum albumin	g/dl	3.023 ± 0.053	6.35	2,7	3,5	3.735 ± 0.158	17.00	2,8	4.9	- 0,707	2.209	P<0,05
Total serum cholesterol	mg/dl	203.86 ± 1.50	2.65	199.50	216,60	197.118 ± 8.911	18.08	143.4	270.0	+ 7,76	3.515	P<0,01
GGT	UI/l	18.69 ± 0.45	8.84	15	21	23.75 ± 1.117	28.925	14.9	38.0	- 5,06	5.000	P<0,001
ALT	UI/l	25.61 ± 0.92	13.00	21	31	47.912 ± 4.254	35.515	34.3	90.1	- 22,30	14.50	P< 0,001
AST	UI/l	22.79 ± 0.74	11.82	20	26,80	86.575 ± 9.688	44.764	19.8	164.18	- 63,78	31.89	P< 0,001
Serum calcium, Ca	mg/dl	10.20 ± 0.29	10.30	8.60	12,90	11.55 ± 0.384	13.320	9.4	14.9	- 1,35	2.280	P>0,05
Serum phosphorus, P	mg/dl	5.30 ± 0.12	8.26	4.50	6,00	5.868 ± 0.218	14.876	4.0	7.9	- 0,59	1.329	P>0,05
Ca/P	mg/dl	1.916 ± 0.04	9.34	1.69	2,33	1.99 ± 0.075	15.16	1.58	2.5	-0,074	0.290	P>0,05
Detection of estrus and conception rate after treatment to estrus synchronization in cows												
cow with estrus and insemination after treatment	n	11				15						
	%	84,61				93,75						
Average interval treatment - first insemination	n	5.727±1.183	68.55	2	14	5.4±1.523	109.2	2	19	+0,32	0,26	P>0,05,
Average interval treatment-conception	n	38.5±12.877	94.60	3	107	21.25±12.22	115.1	2	53	+17,25	3,75	P<0,01
Pregnancy index	n	2±0.411	51.776	1	4	1.5±0.288	38.49	1	2	+0,75	0,773	P>0,05
Total pregnant	n	8				4						
	%	72.72				26,66				+46,06 P<0,01		

after treatments to synchronization of estrus with prostaglandin (PG)

Regarding the relationship between metabolic status and fertility of dairy cows, the literature indicates strong correlation. Animals with various metabolic disorders have reduced fertility. Thus, they have determined correlations between negative energy balance, which is installed in highly productive cows frequently and reduced fertility. Studies have shown that the negative energy balance, revealed by reduced serum levels of glucose, insulin, insulin-like growth factor-I (IGF-I) are responsible for delaying the first ovulations by inhibiting pulsatile secretion of LH, reducing the production of estrogen, reducing the concentration of serum progesterone, subsequently leading to reduce fertility. The protein base dietary stimulates high levels of milk production, but reduces reproductive performance in cows. High levels of protein in the diet of cows can increase the plasma urea, which negatively affects the uterine environment and fertility. During the first months of lactation, 5 to 10% of milk cows suffer from high yields of severe hepatic lipidosis and 30 to 40% from mild hepatic lipidosis. This means that almost 50% of these cows are at risk of developing metabolic disorders. When fat infiltrates the liver, liver damage occurs in tissues, and enzyme levels indicating liver injury (AST, GGT and GLDH) are generally high (4).

Comparing the results obtained in this study with those of other authors, we may affirm that the effectiveness of oestrus synchronization in cows with a corpus luteum treated with progesterone using an intravaginal device and prostaglandin F2α determined a conception rate higher with 10- 20% as compared with treatment with prostaglandin F2α only (8, 17).

These finding indicate that, in the absence of a corpus luteum, after progesterone therapy the fertility is reduced due to lower progesterone concentration and increased estrogen concentration. After the insemination of cows with a corpus luteum, conception rate is significant higher ($P < 0.01$), as compared to the cow without the corpus luteum (12).

Some data in the literature show that the effectiveness of treatments with prostaglandin F2α for the synchronization of oestrus in cows depends on the hormonal products which is used. Thus, analyzing the comparative efficiency of using the two prostaglandin cloprostenol and dinoprost, it observed that Cloprostenol has a longer half-life compared to dinoprost because it is more resistant to endogenous metabolism. In this idea, cloprostenol would reduce the time to complete luteolysis and increases conception rate compared to Dinoprost. Serum concentrations of progesterone at the time of PGF2α administration was positively correlated with luteolysis and pregnancy prediction.

In summary, cloprostenol induced a greater decrease in progesterone, P4 for the first 12 houres following treatment and subsequently a greater increase in estrogenes, E2 compared to Dinoprost, although there were no differences in these two products in conception rates % in cows with complete luteolysis, and pregnancy loss (5,11,14).

When the prostaglandin F 2α is used in the last stages of the luteal phase in cows (day 11 and 15 of the estrous cycle) signs of estrus are stronger and fertility rate is higher as compared to the administration of the prostaglandin F2α in the first part (days 6-9) of the phase luteal (13). Other authors also have showed that the effectiveness of treatments with prostaglandin F2α depends on the time of application of treatment in relation to the phases of oestrus, in the early stage and middle stage, corresponding to the luteal phase shorter, the rate of conception is lower compared to the late phase of oestrus (11).

In most cases, cattle fertility after oestrus synchronization treatments using different protocols, depends, beside the correct administration of the treatment, on other factor such as environmental conditions, nutrition and metabolic health of the animals.

Conclusions

1. The manifestation of oestrus in cows using two therapeutic protocols gave better results in cows from E2 group, treated with PGF2α compared to cows of E1group, treated with progesterone, PRID (89.65% versus 69.23%), with lower values of averages interval from treatment to the first insemination (5.53 ± 0.99 days versus 8.66 ± 3.07days) and close average interval from treatment to conception (33.83 ± 12.05 days in E1 group and 34.45 ± 10.22 days in E2 group), the differences being statistically insignificant.
2. Cows diagnosed with corpus luteum from the group E1a, (PRID+CL) have a better response after treatment compared with of cows without corpus luteum in group E1b (PRID+no CL), which resulted in a higher rate of manifestation of estrus ($P < 0.001$), the lower average interval from treatment to conception and higher conception rate ($P<0.001$).
3. Conception rates after treatments to synchronization of estrus in cows registered higher values in E1 group (PRID) compared to E2 group (PG F2α), ($P < 0.05$).
4. Cows of E2 group, treated with PGF2α, with normal values of biochemical parameters have higher conception rate ($P < 0.01$) compared to cows with anormal values.
5. Cows fertility after treatments for oestrus synchronization depends on the methods used, the moment of application of treatments in relation to the phases of the estrous cycle, cyclicity activity, nutritional management and metabolic animal health.
6. Programs for the induction and synchronization of oestrus in large farms for dairy cows has great practical importance, contributing to the detection oestrus of cows, shortening postpartum insemination interval, reducing the interval between calving and first insemination and the interval between calving and conception and increasing the rate of conception, with major impact on reproductive performance of animals.

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EFFECTS OF GRANULOCYTE-COLONY STIMULATING FACTOR ON BONE MARROW MORPHOLOGY FOLLOWING CYCLOPHOSPHAMIDE INDUCED NEUTROPENIA IN RATS

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Abstract

Granulocyte-colony stimulating factor is a glycoprotein that stimulates synthesis of granulocytes, especially of neutrophiles. It can be used to correct myelosupression associated with long-term chemotherapy or in the treatment of neutropenia. The aim of our study was to assess the effects of G-CSF on bone marrow after cyclophosphamide induced neutropenia in rats. The study was conducted on 24 female Wistar rats divided in 3 experimental groups; the control group, group of cyclophoshamide treated animals and the group of animals that were treated with Granulocyte-colony stimulating factor after neutropenia induction with cyclophosphamide. Cytological exam of bone marrow aspirates and histological exam from sternal bone marrow were realized using routine techniques. Examination of the aspirates taken from the femoral bone marrow and of the histological sections taken from the sternum showed a dramatic reduction in the number of myeloid precursors in individuals of group 2 which have been subjected to cyclophosphamide-induced myelosuppression, while the administration of G-CSF to the individuals of group 3 induced marked proliferation of the myeloid precursor cells, correcting the myelosuppressive effect of the cyclophosphamide In conclusion, G-CSF can be used for the stimulation and mobilization of myeloid progenitor cells from the bone marrow.

Keywords*: myelosuppression, neutrophil, growth factor*

Granulocyte-colony stimulating factor (G-CSF) is aglycoprotein that belongs to the family of colony-stimulating factors, along with macrophage colony stimulating factor and granulocyte macrophage colony stimulating factor (Cetean *et al.*, 2015). G-CSF is secreted by fibroblasts and endothelial cells from the bone marrow, but also by cells of the immune system like monocytes.

As a major role, granulocyte-colony stimulating factor regulates the differentiation of hematopoietic cells in bone marrow. It has been also shown that G-CSF accelerates leukocyte and neutrophil recovery after high-dose chemotherapy. In the present time, high-dose chemotherapy is still associated with prolonged periods of myelosuppression and absolute leukopenia that can last for 8 to 10 days (Peters, 1993). G-CSF has also a great role in the dendritic cell activation, cells with a great importance in the immune response initiation (Cetean *et al.*, 2015).

The production of this growth factor is stimulated mainly by bacterial lipopolysaccharides, so it is secreted mainly in bacteria induced inflammation, but also in other conditions like sterile inflammation (Saba *et al.*, 2002, Peters *et al.*, 1993).

G-CSF acts by binding to his specific receptor on responsive cells which are represented by variety of myeloid progenitor cells.

The most important role of the G-CSFs is the synthesis of granulocytes, which includes neutrophils, eosinophils, and basophils. These cells are essential in the immune response. The most sensible granulocytes to the action of G-CSF are the neutrophils, the growth factor

stimulates the production, mobilization and survival of these cells (Cetean, *et al.*, 2015).

G-CSF is already used in human medicine to minimize chemotherapy-induced myelosuppression and it's also used in veterinary medicine in the treatment of clinical neutropenia (Fernandez *et al.*, 2007).

Cyclophosphamide is an alkylating agent of the family of oxazaphosphorines, which works by adding an alkyl group to the guanine from the structure of DNA and blocks DNA replication by interchain and intrachain cross-linking. It is a synthetic antineoplastic drug with various life-threatening side effects (Murali and Kuttan, 2015).

Cyclophosphamide is used for the treatment of various neoplastic processes (eg, lymphoma, leukaemia, Langerhans cell histiocytosis, intracranial tumors like: astrocytoma, glioblastoma, meningioma) and in various autoimmune diseases due to the strong immunosuppressant action (*eg*. lupus erythematosus, rheumatoid arthritis) (Murali and Kuttan, 2015).

One of the most important side effect of cyclophosphamide administration is neutropenia, which may predispose patients to infections with opportunistic bacterial and fungal agents (Martin *et al.*, 1997; Hellmich *et al.*, 1999).

The aim of our study was to assess the effects of G-CSF on bone marrow after cyclophosphamide induced neutropenia in rats.

Materials and methods

The study was conducted on 24 female Wistar rats (5 months old), with an average weight of 250–300 grams, that were divided in 3 experimental groups. The animals were purchased from the Laboratory animal facility of the "Iuliu Hatieganu" University of Medicine and Pharmacy of Cluj-Napoca, Romania and the *in vivo* study was realized in the same place.

During the study, all the animals were kept in special cages, in an artificially illuminated room (12 h dark/12 h light cycle), at a temperature of 22–23 °C and at 50%–60% humidity. Standard pelleted diet and water *ad libitum* were administered. All the experiments were approved by the Ethical Committee on Animal Welfare of "Iuliu Hatieganu" University and complying with Guidelines in the Use of Animals in Toxicology.

Experimental design: the animals were randomly divided in 3 experimental groups (n=8), the first one was represented by the control group (group1); the second one by the cyclophoshamide treated group (induced neutropenia) (group 2) and the third one by the cyclophoshamide and G-CSF treated group (group 3).

Animals from the control group did not receive any treatment.

To induce neutropenia (group 2 and 3), the cyclophoshamide was administered in a unique dose of 50 mg/kg intraperitoneally.

Animals from group three received 30 μg/kg G-CSF (Filgastrim) administered subcutaneously starting from 48 hours after cyclophosphamide administration, 11 days once a day.

At the end of the experimental period (14 days after initiation), the animals were killed by cervical dislocation and immediately necropsied.

For the histological study, the sternum and the femur of the animals were fixed in 10% buffered neutral formalin, decalcified in a mix of 8% formic and 8% clorhidric acid for 24 hours and embedded in paraffin.

Sections were made at 4 micrometers and the slides were stained by Haematoxiline–Eosine (HE) method.

The slides were examined under a BX51 Olympus microscope and images taken with an Olympus UC 30 digital camera.

Sections were examined by an independent observer blinded to the experimental

protocol.

Cytological exam was also performed; aspirates were realized from the femoral bone marrow and stained by Wright-Giemsa method.

The sections and aspirates were assessed for their cellularity on a 6-point scale (0–5), with 0 indicating no particles present, 1 hypocellular, 2 low but normal, 3 normal, 4 high but normal and 5 hypercellular (Teg *et a.l*, 1999) and the data were analyzed statistically using ANOVA two way test. Standard deviation was also calculated. The myeloid/erythroid ratio was also assessed.

Results and discussion

All animals survived the experiment. No relevant gross lesions were observed during necropsy.

Microscopically we evaluated bone marrow sections by assessing cellularity, adipose tissue distribution, the number of megakaryocytes and the erythroid/myeloid ratio.

There were significant differences between individuals of the three experimental groups regarding the evaluated parameters.

Animals from the control group presented normal histological and cytological features regarding cellularity, the number and distribution of megakaryocytes. Also the erythroid/ myeloid ratio was normal, showing a mild myeloid predominance.

On sections from the cyclophosphamide treated group (group 2) there is a dramatic reduction in the number of myeloid precursors and the number of megakaryocytes and a proportional increase in the number of erythroid precursors, the myeloid/erythroid ratio showing marked erythroid predominance. The amount of adipose tissue was also reduced.

Animals of group three presented a marked increase in cellularity and in the number of myeloid precursors, with evident myeloid predominance.

Table 1

Cellularity and myeloid/erythroid ratio assessment (mean ±SD)

Group	Group 1	Group 2	Group 3
Cellularity	5,14 ± 0,69	4,28 ± 0,75	5,71 ± 0,48
Myeloid/erythroid ratio	Mild myeloid predominance	Marked erythroid predominance	Marked myeloid predominance

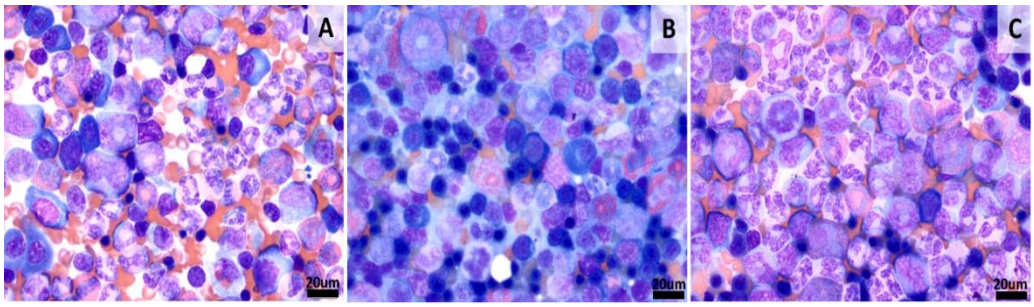

Fig.1. Cytological exam of bone marrow aspirate from different experimental groups. (A) Control group, normal cellularity and myeloid/erythroid ratio; (B) Group 2, high cellularity, marked erythroid predimonance; (C) Group 3, high cellularity, marked myeloid predominance; Wright-Giemsa x1000, Scale bar=20 µm.

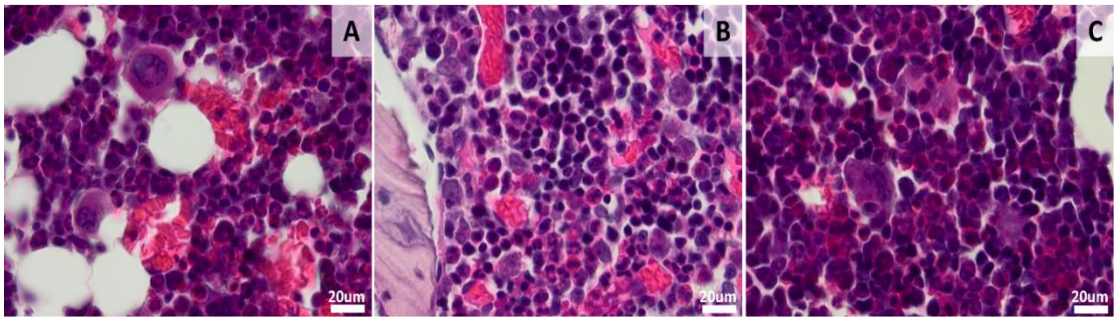

Fig. 2.Histology of sternal bone marrow sections from different experimental groups.(A) Control group, normal myeloid/erythroid ratio, adipose tissue distribution and megakaryocyte number and morphology; (B) Group 2, decreased cellularity and adipose tissue content, marked erythroid predimonance; (C) Group 3, increased cellularity, decreased adipose tissue content, marked myeloid predominance; HE x1000, Scale bar=20 µm

The main side effect of the majority of anticancer drugs is myelosuppression, which limit their use in the usual therapeutic dose and can reduce the frequency of administration. Death during an episode of severe myelosuppression is usually due to either bleeding or due to septic processes. The proliferation of hematopoietic progenitor cells is controlled by different growth factors, the production of neutrophilic granulocytes being stimulated by G-CSF (Lemoli and D'Addio , 2008).

Examination of the aspirates taken from the femoral bone marrow and of the histological sections taken from the sternum showed a dramatic reduction in the number of myeloid precursors in individuals of group 2 which have been subjected to cyclophosphamide-induced myelosuppression, while the administration of Filgastrim to the individuals of group 3 induced marked proliferation of the myeloid precursor cells, correcting the myelosuppressive effect of the cyclophosphamide.

Previous studies showed that G-CSF administration can correct cyclophosphamide induced neutropenia in animal models or in human patients (Teg *et al.*, 1999; Murali and Kuttan, 2015). In human medicine, this drug is used in a series of diseases like non neutropenic patients infections (pneumonia); infertility; several neurological disturbances; therapy of acute myocardial infarction; regenerative medicine (skeletal muscle) (Cetean *et al.*, 2015).

Although the assessment of the effects of granulocyte colony stimulating factor on animals that suffered treatment with cyclophosphamide was semi quantitative, we were able to highlight the main changes and benefits induced by this growth factor.

Conclusions

Filgastrim has remarkable effects in the treatment of cyclophosphamide induced myelosuppression.

G-CSF can be used for the stimulation and mobilization of hematopoetic stem cells from the bone marrow.

The use of G-CSF in veterinary medicine can be a very important therapeutical method in the recovery of bone marrow after chemotherapy, to reduce the duration of neutropenia, and in other conditions like bacterial infections, neurological diseases or muscle regeneration.

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IMAGING, NECROPSYC AND HISTOPATHOLOGICAL FINDINGS IN AGGRESSIVE MAMMARY TUMOR IN ONE CAT

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Abstract

The rate of cancer diagnosis in cats is increasing, and one of the most common type is of mammary gland tumors. Mammary neoplasis are clinically diagnosed, radiology and ultrasound revealed, and confirmed by necropsy examination and through histopathology. One intact female cat, 9 years old, with 10 cm ulcerated mammary tumor and with severe clinical condition, was brought to the Radiology service for imaging examination metastasis check up. Considering the critical condition, the radiological findings, and the clinical tumoral staging, with the consent of the owner, the cat was directed to the anatomopathology department for euthanasia, and for necropsy procedures. Pathological findings that were discovered on survey radiographs included: increased radiopacity in the pulmonary projection area, mild pleural effusion, radiopaque structure defined in the ventral abdominal wall, with decrease radiodensity middle area, and radiopaque kidney projection area. During necropsy, macroscopically was identified: aggressive mammary tumor invasions, pulmonary metastasis, kidney changes and ovarian cysts. Histophatological changes consists of: diagnosed mammary adenocarcinoma consisted of polymorphic cells with vacuolated cytoplasm and hyperchromatic nuclei, with frequent mitosis and with necrosis areas. Also, subpleural pulmonary metastasis with compact tumoral cells areas, hepatic congestion injury and fibrous nephritis were encountered. Mixed mammary adenocarcinoma exhibit a complex histological pattern, with an aggressive clinical behaviour, associated with a reserved or bad prognostic. Mammary tumours are one of the most frequent neoplasia in female cats; therefore, these tumours represent a serious problem in veterinary medicine.

Keywords: *adenocarcinoma, cat, mammary, tumor, radiography*

Introduction

To understand cancer, we must realize that it is not a simple or a freestanding disease, rather the term "cancer" is an umbrella term that describes a large number of pathological processes whose only common feature is uncontrolled cell growth and proliferation. The prevalence of cancer in pets is growing steadily, for a variety of reasons, some still being studied, and it is one of the major morbidity and mortality causes in cats and dogs.

The rate of cancer diagnosis in cats is somehow high, and one of the most common type is of mammary gland tumors. Mammary neoplasis are clinically diagnosed, radiology and ultrasound revealed, and confirmed by necropsy examination and through histopathology.

Materials and Methods

One intact cross bread female cat, 9 years old, with 10 cm ulcerated mammary tumor and with severe clinical condition, was brought to the Radiology service for imaging examination metastasis check up.

The superinfected mammary mass corresponded to the inguinal pair of mammary glands, causing pain when palpated, presented increased consistency and lacked mobility. The patient exhibited severe dyspnea, being only half conscious and found in a state of advanced weight loss.

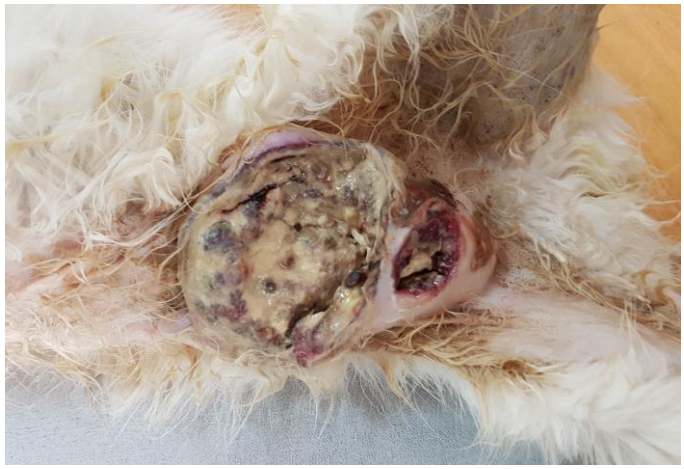

Fig.1. Clinical image. Mammary tumor, ulcerated, superinfected, inguinal location. FMV Iași.

Following clinical examination and medical history assessment, radiological examination was employed, using two lateral positions, thoracic and abdominal, operations carried out with maximum care in order to avoid deterioration of the vital signs of the animal.

Considering the critical condition, the radiological findings, and the clinical tumoral staging (IV), with the consent of the owner, the cat was directed to the anatomopathology department for euthanasia, and for necropsy procedures.

Necropsy was performed by opening main anatomical cavities (thoracic and abdominal) and by examination of internal organs, following pathological modifications, as well as accumulation of pathological materials and fluids.

In order to establish a certain diagnosis, morphological samples were taken, followed by their preparation and special histopathological examination.

Results

Pathological findings that were discovered on survey radiographs included: increased radiopacity in the pulmonary projection area, mild pleural effusion, radiopaque structure defined in the ventral abdominal wall, with decrease radiodensity middle area, and radiopaque kidney projection area.

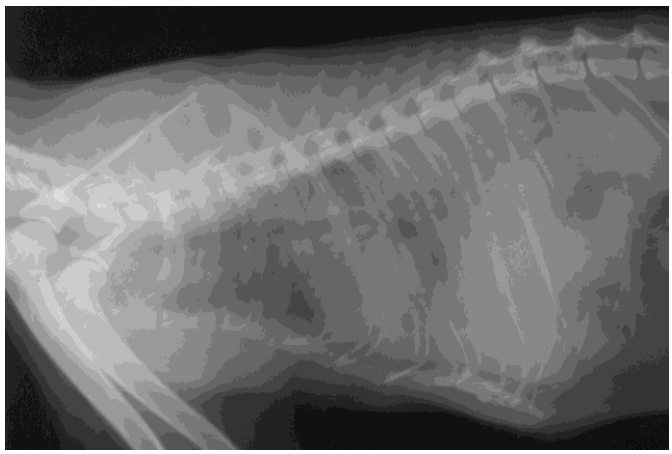

Fig.2. Radiological image. Thorax – lateral radiograph. Massive pulmonary infiltration in diaphragmatic lobes, pleural effusion, reduced respiratory space. FMV Iași.

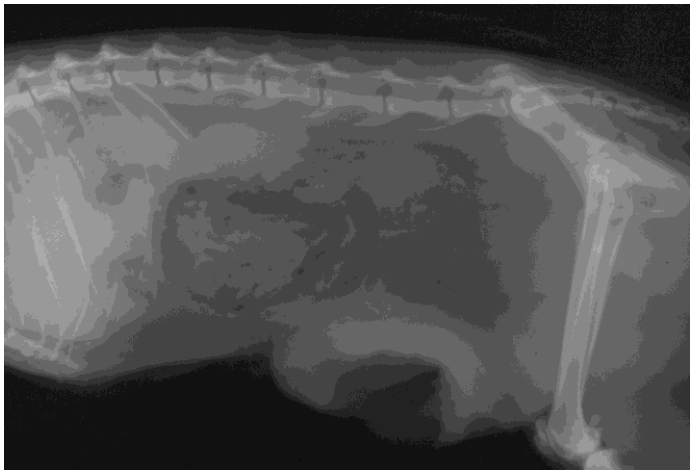

Fig.3. Radiological image. Abdomen – lateral radiograph. Increased radioopacity over the renal projection area. Ventrally situated radioopaque mass, well differentiated, presenting a central radiotransparent area, corresponding to the tumoral mammary mass with central area of necrosis. FMV Iași.

During necropsy, macroscopically was identified: aggressive mammary tumor invasions, pulmonary metastasis, kidney changes and ovarian cysts.

Fig.4. Necropsy image. Thorax. Nodular lung metastases. FMV Iași.

Histophatological changes:

- diagnosed mammary adenocarcinoma consisted of polymorphic cells with vacuolated cytoplasm and hyperchromatic nuclei with star or spindle appearance, with frequent mitosis and with necrosis areas. Capillary neoformation, intratumoral hemorrhages and neutrophil infiltrations were found.

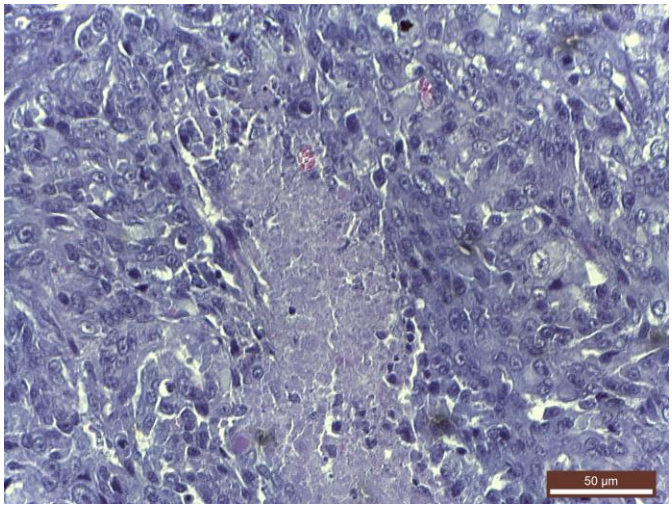

Fig.5. Histopathological image. 50 µm, HE stain. Mammary adenocarcinoma consisting of polymorphous cells, anisokaryosis and necrosis areas. FMV Iaşi.

- pulmonary metastasis consisting of compact areas of tumoral cells, extended areas of necrosis, as well as tumoral cells of acinar structure, with areas of pulmonary edema and atelectasis due to compression of pulmonary alveoli located at the edge of the metastasis.

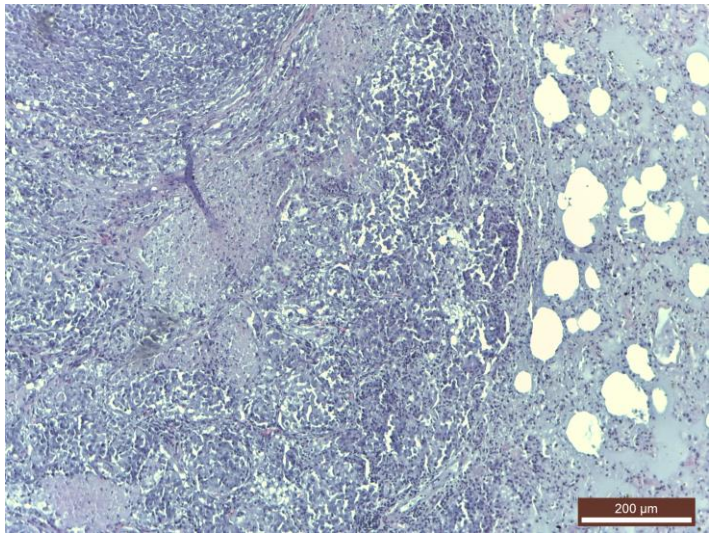

Fig.6. Histopathological image. 200 µm, HE stain. Compact lung metastasis, alveolar atelectasis and pulmonary edema. FMV Iaşi.

Also, hepatic congestion injury and fibrous nephritis were encountered.

Conclusion

Mixed mammary adenocarcinoma exhibit a complex histological pattern, with an aggressive clinical behaviour, associated with a reserved or bad prognostic. Mammary tumours are one of the most frequent neoplasia in female cats; therefore, these tumours represent a serious problem in veterinary medicine.

In any type of mammary mass, regardless of its dimensions, early examination using clinical and imaging methods is recommended, but also by cytological and histopathological investigations, in order to establish a diagnosis and subsequently an adequate therapeutic conduit.

Avoiding administration of hormonal products and consulting the specialist veterinarian upon noticing any modification in mammary glands are recommended, thus helping avoid tumoral advancement towards stages III and IV.

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