UNIVERSITATEA DE ŞTIINŢE AGRICOLE ŞI MEDICINĂ VETERINARĂ "ION IONESCU DE LA BRAD"
IAŞI – ROMÂNIA

LUCRĂRI ŞTIINŢIFICE

SERIA MEDICINĂ VETERINARĂ
VOL. 56 (1 – 2)

ISSN 1454-7406
Editura „ION IONESCU DE LA BRAD" Iaşi
2013

Responsabilitatea privind conţinutul articolelor, inclusiv traducerea acestora în limba engleză, revine exclusiv autorilor.

The entire responsibility for the content of papers, including the English translation, belongs to the authors.

CUPRINS

BIOCHEMICAL EFFECT OF TAURINE ON EXPERIMENTALLY HYPERGALACTOSEMIC RATS

Omayma. A.R. Abou Zaid[1], Faten Zahran [2] and Heba, A.M.Awad [3]
[1, 3]Biochemistery Department, Faculty of Vet. Med. Moshtohor, Benha University, Egypt;[2]Biochemistery Department, Faculty of science, Zagazig University, Egypt;omayma.ragababozaid55@gmail.com

Abstract

In recent years, there has been a great demand for natural products that have possible preventive action against diabetes and its secondary complications. Current diabetes research makes use of hypergalactosemic rat as experimental models of type I diabetes mellitus.Keeping this in mind, our study was undertaken to investigate the influence of taurine on galactose and glucose levels, oxidative stress markers and lipid profile in galactose-induced hypergalactosemic rats. 70 weaning albino male rats divided into four groups were used in this study. Rats treated daily with 30% galactose (6 g/kg) orally for 80 days (group C; n=25) to induce hypergalactosaemia. Control rats received normal chow diet (group A; negative control group, n=10). Rats treated with 4% taurine (0.8 g for each rat daily) (group B; positive control group, n=10). The fourth set of rats received 30% galactose and 4% taurine (group D; n=25). Taurine administration started after 80 days from the beginning of the experiment and continue for 21 days. Galactose and glucose levels as well as body weights were estimated weekly to monitor hypergalactosaemia and hyperglycaemia induced by galactose feeding. Serum were collected at the end of the experiment to determine insulin, lipid profile, total protein content, antioxidant enzymes (superoxide dismutase (SOD), catalase(CAT) and glutathione peroxidase (GSH-Px) activities), and reduced glutathione (GSH) levels, while glycogen estimated in liver tissues of rats. In addition HbA1c was determined in whole blood of rats. The results of this study imply that treatment of rats by taurine can control, to some extent, galactose-induced hypergalactosaemia, hyperglycaemia and its attendant complications.

Keywords*: galactose; experimental hypergalactosaemia; taurine; diabetes mellitus; antioxidants.*

Introduction

Galactosemia considered as one of the most mysterious of the heavily-researched metabolic diseases. It is among the most common carbohydrate metabolism disorders and can be a life-threatening illness during the newborn period (Fridovich-Keil J. Walter., 2008). It is a group of diseases marked by high levels of blood galactose resulting from the inability to use galactose to produce energy (Berry *et al.*, 2006). This inability of the body to metabolize galactose, causes the accumulation of galactose-1-phosphate in the body and this causes damage to the liver (cirrhosis), kidneys (fanconi syndrome), central nervous system (mental retardation), eyes (development of cataract), ovaries (primary or secondary amenorrhea) and other body systems (Garden *et al.*, 2000). It has been estimated that hereditary intolerance to galactose occurs in approximately one in 18,000 infants. It occurs in approximately 1 out of every 60,000 births among Caucasians, while the rate is different for other groups.

Galactosemia is inherited in an autosomal recessive manner. It affects both boys and girls equally. Infants with galactosemia appear normal at birth, however symptoms usually appear a few days to two weeks after initiating milk feedings. The early clinical features of severe galactosemia include liver dysfunction, manifested by jaundice and hypoglycemia; neurological findings of irritability and seizures; and gastrointestinal findings of poor feeding, vomiting and diarrhea (Segal S. 1995). Galactosemia can kill quickly; it should be considered in any infant with nonglucose reducing substances in the urine. It is important to emphasize that testing of urine with glucose oxidase (Clinistix, Tes-tape) will not detect galactose. This is a strong argument for continued use of the older methods for the screening of urine for reducing substance (Benedict or Fehling test, Clinitest) (Gubbels *et al.*, 2008). There are 3

forms of the disease: galactose-1 phosphate uridyltransferase deficiency (classic galactosemia, the most common and most severe form), deficiency of galactose kinase, and deficiency of galactose-6-phosphate epimerase) (Jacobson *et al.*, 2011).

High blood sugar is toxic to many parts of the body, and the eyes are no exception. Indeed, people with diabetes and galactosemia are 25 times more likely to go blind than those who do not have the disease. Elevated plasma glucose or galactose in the blood results in accumulation of sorbitol or galactitol in the lens. They are difficult to diffuse out of lens, causing increased osmotic pressure. High glucose also causes nonenzymaticglycation of lens proteins. All these cause an opaque cloudiness of the lens known as cataract. Galactitol is not further metabolized and diffuses out of the lens very slowly. Thus, hypergalactosemia is even more likely to cause cataract than hyperglycemia.

However, galactose, has been used to study the development of hyperglycemia & its complications due to its ability to increase glycation (Ramana *et al.*, 2008). The abnormalities detected in experimental galactosemic animals including retinopathy, cataracts, neuropathy, nephropathy and generalized basement membrane thickening are more compatible with findings in experimental diabetes mellitus than in human galactosemia. Because patients with galactokinase deficiency fail to manifest the central nervous system (CNS) and ovarian complications which characterize classic galactosemia. So, Current diabetes research makes use of hypergalactosemic rat models to reproduce pathologic seen in type I diabetes mellitus (Mary Otsyula *et al.*, 2003).

Taurine(2-aminoethane sulphonic acid) is the most abundant free amino- acid present in animal tissues and is involved in the development and function of many organs such as brain, , liver, kidney, eye and heart (Huxtable RJ. 1992). Taurine is found to stimulate glycolysis and glucogenesis & also reported to have insulin-like action (Henry *et al.*, 1974). So many beneficial effects of taurine supplementation in DM have been recently suggested as a result of its hypoglycaemic (Taurine decrease the concentration of glucose &fructosamine and increase the contents of insulin & glycogen in the liver and has a positive effect on β-cell function), antioxidative&nephroprotective effects (Katarzyna Winiarska *et al.*, 2009).

Materials and Methods

***Animals*:** 70 male albino rats weighing 175-200 g were used in this study obtained from Laboratory Animals Research Center, Faculty of Veterinary Medicine, Benha University, Egypt. Rats were housed in separated metal cages 10-25 per cage and kept under the same constant environmental and nutritional condition through out the period of investigation; water was supplied *ad Libitum*, in the special laboratory animal room at Faculty of Vet. Med. Moshtohor, Benha University.

***Chemicals*:** Galactose and taurine were purchased from El-Goumhouria Co. for trading chemicals, medicines and medical appliances, Egypt. Other chemical and kits purchased from Segma (USA).

Nutraceuticals preparation:

Preparation of 4% Taurine: 0.8 gram of Taurine was dissolved in 3 ml distilled water and given as oral daily dose for each rat in (B & D groups) for 21 days.

Preparation of 30% Galactose: 6 g/kg of galactose was dissolved in 3 ml distilled water and given as oral daily dose for each rat in (C & D groups) for 80 days according to [8] & [13].

Experimental design:

Seventy male rats were divided into 4 groups placed in individual cages and classified as follows:

Group (A): served as negative control (n=10), left untreated & fed on chow diet and saline.

Group (B):considered as positive control group (n=10), rats fed on chow diet & 4% taurine added to the drinking water.

Group (C): untreated hypergalactosemic group (n=25), rats fed on chow diet and 30% galactose for two months & receiving no therapy.

Group (D):hypergalactosemic rats treated with taurine (n=25), rats fed on chow diet and 30% galactose& 4% taurine added to the drinking water.

The administration of taurine started after 80 days from the beginning of the experiment. Control animals received physiological saline alone (Thirunavukkarasu *et al*., 2004).

Blood sampling:

blood samples were collected after overnight fasting by ocular vein puncture from all rats (control and experimental group) weekly along the duration of experiment, at 2,4,6,8,10 and 12 weeks from the beginning of the experiment. Directly, after rats were anaesthised using diethyl ether, blood samples were collected from all rats and divided into two parts for determination of the following *Biochemical parameters:*

- **Serum samples:** to determine SOD, GPx, catalase activities and GSH level, galactose, glucose, total cholesterol, HDL, LDL, TG, total protein and insulin.
- **Edta samples:** to determine HbA1c.

Tissue sampling:

The second part was used for preparation of tissue (liver) homogenate with 0.9% saline using electrical homogenizer, centrifuged at 3000 r.p.m for 15 minutes, the resulting supernatant were collected and used for estimation of glycogen concentration. Livers from rats were preserved at -20ºC until performing the investigations.

Statistical analysis:

Statistical analysis was done using SPSS software version 15. The inter-group variation was measured by one way analysis of variance (ANOVA) followed by Post Hoc LSD test. Results were expressed as mean ± SEM. The mean difference is significant at the 0.05 level (Snedecor and Cochran (1989)).

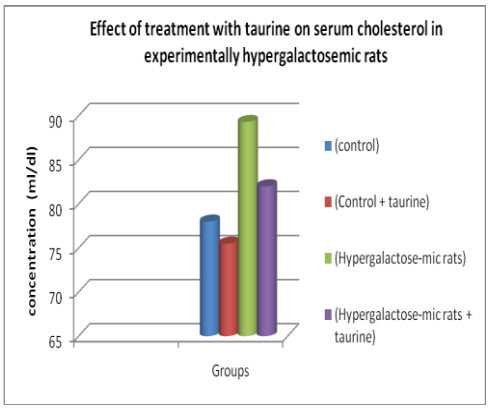
Results

Effect of taurine on changes of antioxidants, lipids profiles, galactose, glucose, glycogen and insulin in normal, hypergalactosemic and taurine-treated hypergalactosemic albino rats.

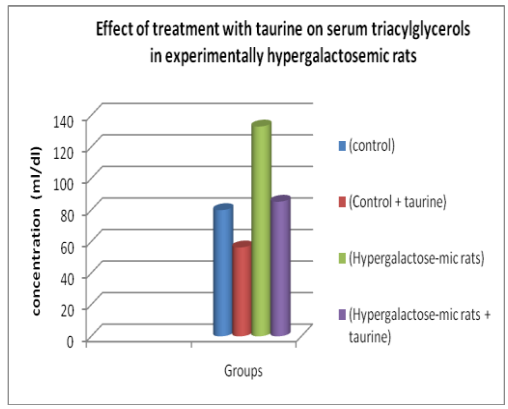
Taurine treatment induced alteration in plasma levels of lipid parameters. As seen in Fig.1.a-d, hypergalactosemic rats showed non-significant increase in cholesterol, TG, LDL and decrease inHDL compared to normal non hypergalactosemic rats. taurine treatment normalized the changes induced in hypergalactosemic rats.

Moreover, taurine decreased the significant increase in galactose and glucose concentration inhypergalactosemic rats (Fig.1.e-f). glycogen and insulin levels was decrease in hypergalactosemic rats and return to normal values by treatment with taurine(figure 1.g-h).

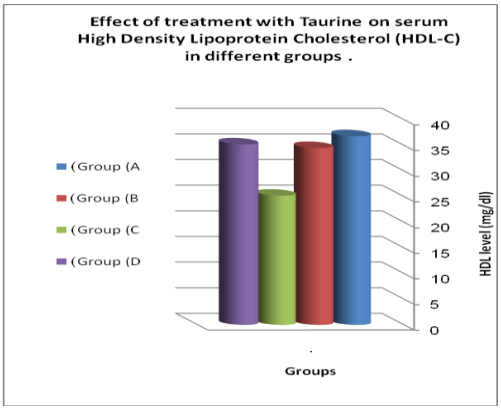
Also, taurine treatment induced changes in antioxidants parameters. As seen in (Fig.1.i-l), hypergalactosemicrats showed significant decrease in catalase, SOD, GSH-px activities and GSH level compared to normal non hypergalactosemic rats.



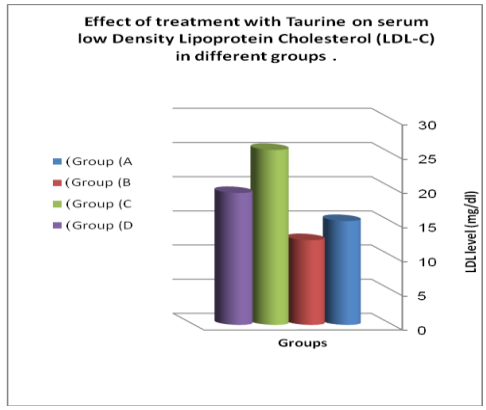
A



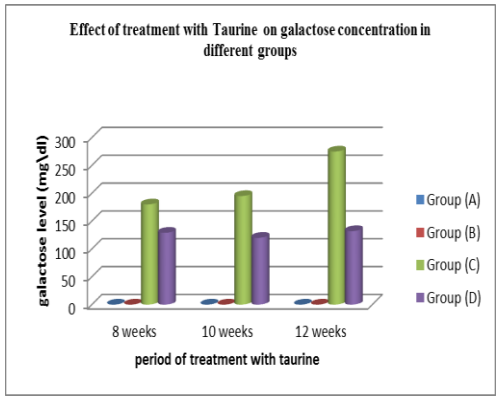
B



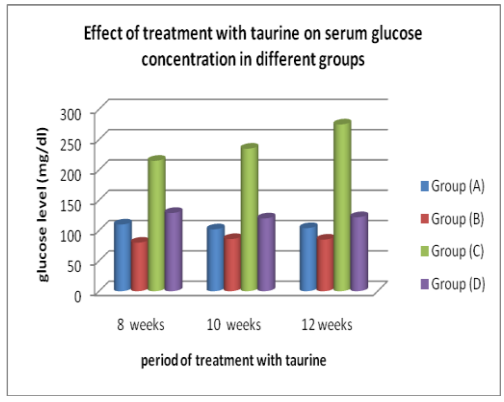
C



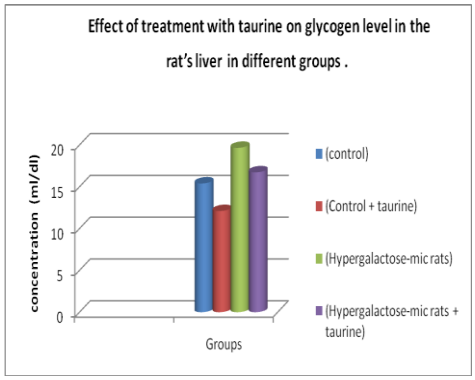
D



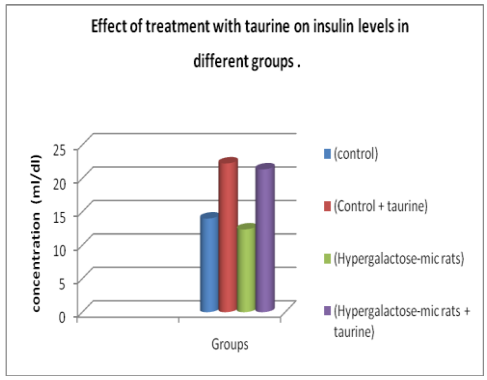
E



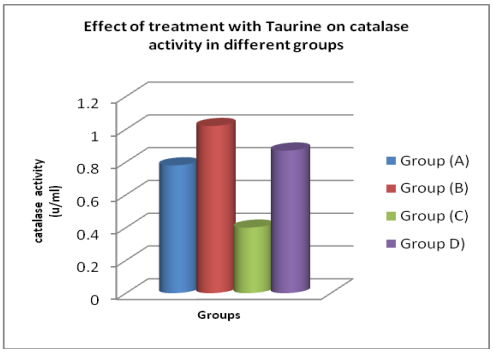
F



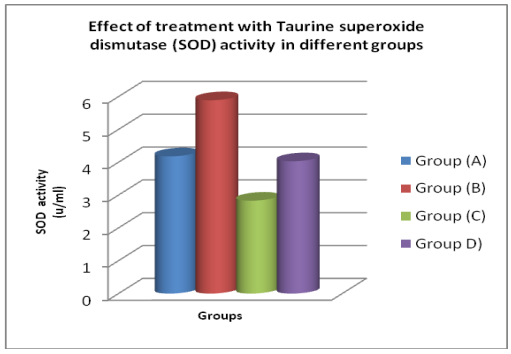
G



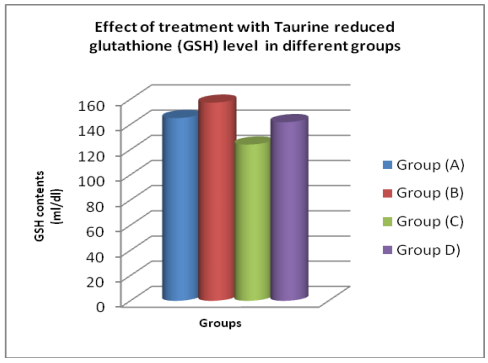
H



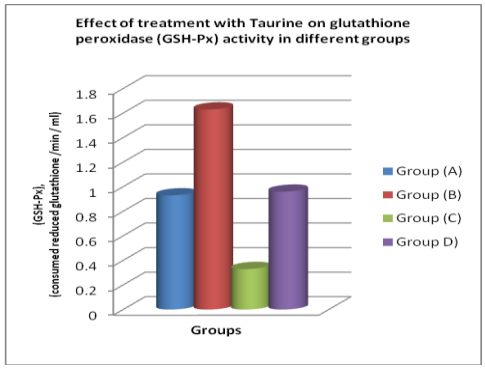
I



J



K



L

Fig. 1. Effect of taurine on changes of lipids profiles (A-D), galactose (E), glucose (F), glycogen (G), insulin (H) and antioxidants (I-L) in normal, hypergalactosemic and taurine-treated hypergalactosemic albino rats. Rats were given taurine in water for 21 days. Plasma levels of cholesterol, TG, LDL, HDL, galactose, glucose, glycogen, insulin and antioxidants (catalase, SOD,GSH-Px activities and GSH level) were measured for the 4 groups. Values are means ± S.E (SE: Standard Error)

Disscution

Concerning to galactose level in hypergalactosemic rats the recorded data demonstrated in (Table 1) showed significant increase in serum galactose concentrations in experimental hypergalactosemic rats allover the periods of the experiments. These results were nearly similar to those reported by (Ronald L. Engerman., and Timothy S Kern, 1984) who observed that, in animals offered the galactose-rich diet, blood galactose concentrations were found to vary from hour to hour throughout the day, from values near 0 after an overnight fast, up to values of about 150-250 mg/dl later in the day, after the galactose-rich diet had been eaten.

Table 1. Effect of treatment with Taurine on serum galactose in Treated and Untreated Hypergalactosemic Rats (mg/dl)

grups / duration	Group (A)	Group (B)	Group (C)	Group (D)
Two weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	10 ± 2.0^{a}	5 ± 3.0^{b}
Four weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	75 ± 21^{a}	50 ± 15^{b}
Six weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	130 ± 26^{a}	96 ± 22^{b}
Eight weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	180 ± 23^{a}	129 ± 34^{b}
Ten weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	195 ± 27^{a}	120 ± 32^{b}
Twelve weeks	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	275 ± 28^{a}	132 ± 26^{b}

Data are represented as mean ± SE

Mean values with different superscript letters in the same row are significantly different at $P \leq 0.05$.

To clarify the relationship between glucose utilization and hyperglycemia as a consequence of galactose feeding, this study showed significant increase in serum glucose concentrations, glycated hemoglobin HbAic and fructosamine in experimental hypergalactosemic rats allover the periods of the experiments. These results were in agreement with (Ramana *et al.,* 2006) who reported that, Blood glucose levels in hypergalactosemic rats were significantly higher than in control rats ($p<0.01$).

Moreover, (Mary Otsyula *et al*., 2003) who reported that, Galactose-fed animals have elevated levels of blood hexose and As expected, 3 months of galactose feeding resulted in significant elevations in glycated hemoglobin, and cessation of experimental galactosemia had a marginal, but statistically significant, effect on glycated hemoglobin values. Furthermore, (Peter F. Kador *et al.,* 2002) who reported that, the levels of Hb A1C increased in dogs fed a galactose diet and decreased in the control dogs.

Experimental galactosemia, which activates the polyol pathway, has been used extensively to explore the pathogenesis of diabetic complications. The galactose-fed dog is an animal model that both histologically and clinically demonstrates retinal vascular changes associated with diabetic retinopathy.

Treatment with Taurine in normal rats significantly decreased serum blood glucose concentration after 21 days of treatment. Meanwhile, Treatment with Taurine significantly reduced elevated serum glucose level in experimental hypergalactosemic rats allover the

periods of the experiments. These results are nearly similar to those reported by (KatarzynaWiniarska *et al.*, 2009) who showed that, three weeks of taurine administration to diabetic rabbits resulted in 30% decrease in serum glucose level and the normalisation of diabetes-elevated rate of renal gluconeogenesis.

On contrary to diabetes which believed to cause vascular dysfunction via a common biochemical mechanism, Galactose-fed animals have elevated levels of blood hexose, but do not develop other sequelae of insulin deficiency, such as alterations in lipids and protein metabolism (Renu A. Kowluru and PrashantKoppolu. 2002).

Table 2. Effect of treatment with Taurine on serum Glucose in Treated and Untreated Hypergalactosemic Rats (mg/dl)

grups / duration	Group (A)	Group (B)	Group (C)	Group (D)
Two weeks	100 ± 20^{b}	97 ± 18^{b}	150 ± 19^{a}	155 ± 21^{a}
Four weeks	93 ± 23^{b}	102 ± 20^{b}	178 ± 21^{a}	180 ± 15^{a}
Six weeks	97 ± 16^{b}	95 ± 15^{b}	190 ± 26^{a}	193 ± 22^{a}
Eight weeks	110 ± 24^{c}	103 ± 10^{c}	215 ± 23^{a}	129 ± 24^{b}
Ten weeks	102 ± 18^{c}	95 ± 19^{c}	235 ± 27^{a}	120 ± 23^{b}
Twelve weeks	104 ± 29^{c}	98 ± 16^{c}	275 ± 28^{a}	122 ± 26^{b}

Data are represented as mean ± SE

Mean values with different superscript letters in the same row are significantly different at $P \leq 0.05$.

In our study, we had found that hypergalactosemia (Tables 3) in the short term (3 months) revealed that, a non-significant increase in serum total cholesterol, LDL and triacylglycerols TG concentrations. In addition our results revealed a non-significant decrease in HDL concentration in experimentally hypergalactosemic rats.

These results of atherosclerosis complications like higher in TG, LDL-C and decrease in HDL levels were predominantly due to reduced lipolysis of triglyceride-rich lipoproteins (Goldfard and Passas 2002). The recorded dyslipidemia observed in experimental diabetes were attributed mainly to the decreased activity of lipoprotein lipase which was an insulin sensitive enzyme demonstrated significant alteration in diabetics. This enzyme could bind to glycosaminoglycans at the luminal side of capillary endothelium hydrolyzing TG and liberates free fatty acids joined with excessive uptake (Semenkovich and Heinecke 1997).

In diabetes glycoxidation could be an important pathway for accelerated LDL oxidation through the formation of the reactive oxygen species. So, glycoxidation induced significant damage to lipoproteins (Brownlee, M. 1996). In this respect, the characteristic lipid abnormalities in diabetes include higher level of TC, TG, LDL-C, VLDL-C and HDL-C.

Treatment with Taurine in experimental hypergalactosemic rats non-significantly reduced serum LDL, total cholesterol and significantly reduced serum TG concentration. In addition, treatment with taurine non-significantly increased HDL concentration in rats after 21 days of drug administration. These results are nearly similar to those reported by (John

Lombardini and Julius Militante 2006) reported that, administration of taurine to diabetic rats decrease the concentration of serum total cholesterol,TG and LDL. Also, (Sun Heeet al., 2009) who reported that, Serum total cholesterol (TC) levels of the taurine group were significantly reduced compared to those of the other groups. Therefore, these results suggest a possible effect of PTP1B inhibitors and taurine on blood total cholesterol in the obese adolescent. Also, (Tawfek and Taha 2006) reported that, Diabetic rats supplemented with antioxidants (taurine) showed decrease in serum total lipid. Also, (Choi 2006) reported that, The purpose of this study was to investigate the effect of dietary taurine supplementation on plasma and liver lipid content in ovariectomized (OVX) rats. The concentrations of plasma total cholesterol, LDL and triglycerides were lower in the taurine treated groups. In addition, (Mi-Ja Choi 2006) reported that, the plasma concentration of HDL cholesterol (HDL-C) significantly increased while total cholesterol, TG and LDL showed significantly decreased in the rats fed TSD (taurine diet) compared to those fed control diet.

Table 3. Effect of Taurine on several physiological and Metabolic Parameters in Rats with Experimentally hypergalactosemic rats

Groups	Group (A)	Group (B)	Group (C)	Group (D)
Survived animals,(%)	100	100	70	80
Bodyweight,(g)	200 ± 15	200 ± 15	175 ± 10	189 ± 18
HbA1C , (%)	2.0 ± 0.1^{a}	1.2 ± 0.2^{b}	3.8 ± 0.1^{a}	1.9 ± 0.35^{b}
Fructosamine, (ml/dl)	40.58±0.32^{b}	24.12± 0.12^{c}	128.8 ± 1.03^{a}	17.76 ± 0.34^{c}

Data are represented as mean ± SE

Mean values with different superscript letters in the same row are significantly different at $P \leq 0.05$.

Table 4. Effect of treatment with Taurineon serum cholesterol, TG, HDL, LDL, Insulin, Glycogen in Liver and Total protein concentrations in treated and untreated hypergalactosemic Rats

Groups / Parameters	Group (A)	Group (B)	Group (C)	Group (D)
Cholesterol (ml/dl)	77 ± 3.33^{a}	76 ± 6.53^{a}	89.3 ± 4.23^{a}	81.6 ± 3.13^{a}
Triglyceride (ml/dl)	124.4 ± 11.86^{a}	110.74 ± 9.6^{a}	133 ± 6.91	87.6 ± 9.36^{b}
HDL (ml/dl)	36.8 ± 5.49^{a}	34.33± 4.46^{a}	25.2 ± 4.15^{a}	35.5 ± 2.9^{a}
LDL (ml/dl)	15.6 ± 2.59^{b}	12.4± 2.82^{b}	25.5 ± 1.31^{a}	19.3 ± 1.2^{b}
Insulin (ml/dl)	14.07 ± 0.65^{c}	22.24 ± 0.61^{a}	12.37 ± 0.35^{d}	19.63 ± 0.58^{b}
Total Protein(mg/dl)	6.84 ± 0.44^{b,c}	9.26 ± 0.27^{a}	5.84± 0.39^{c}	7.84 ± 0.33^{b}
Glycogen in Liver (µg/mg)	16.73 ± 0.98^{b}	19.66 ± 0.74^{a}	12.10± 0.28^{c}	16.47 ± 0.35^{b}

Data are represented as mean ± SE

Mean values with different superscript letters in the same row are significantly different at $P \leq 0.05$.

Also, Treatment with Taurine in experimental hypergalactosemic rats significantly decreased glycogen concentration in liver and significantly increased insulin concentration. These results were nearly similar to (Nagakatsu Harada 2004) who reported that, the treatment of otsuka long-evanstakushima fatty (OLETF) rats with taurine increased muscle glycogen content in the OLETF rats.Also, (Nandhini*et al.*, 2007) reported that, Taurine administration improved insulin sensitivity and controlled hyperglycemia and hyperinsulinemia in fructose-fed rats. Taurine treatment also restored the glucose metabolizing enzyme activities in fructose-fed rats.

Regarding to antioxidants the presented data in tables (4) revealed a significant decrease in GSH level, GSH-px, catalase and SOD activity in hypergalactosemic group when compared to control group.

Table 5. Effect of Taurine on Catalase , Superoxide Dismutase , Glutathione Peroxidase activities and Reduced Glutathione content in treated and untreated hypergalactosemic rat

Groups / parameters	Group (A)	Group (B)	Group (C)	Group (D)
Catalase (u/l)	0.78 ± 0.03^{b}	1.02 ± 1.81^{a}	0.40 ± 0.74^{c}	0.87 ± 3.52^{b}
(SOD) (u/l)	4.18 ± 0.69^{b}	5.09 ± 0.54^{a}	2.83 ± 0.47^{c}	4.03 ± 0.78^{b}
(GSH) (ml/dl)	145.18 ± 7.16^{b}	157.39 ± 4.21^{a}	124.13 ± 5.14^{c}	141.88 ± 0.21^{b}
(GSH-Px) (CRG/min / ml)	0.93 ± 0.39^{b}	1.63 ± 0.94^{a}	0.33 ± 0.51^{c}	0.96 ± 1.29^{a}

Data are represented as mean ± SE

Mean values with different superscript letters in the same row are significantly different at $P \leq 0.05$.

These results were in agreement with (Mary Otsyula *et al.,* 2003) who reported that, the effect of 60 days of streptozotocin-induced diabetes and 50% galactose diet is a decrease in the enzymatic activities of catalase (cat) in liver, kidney, and heart in the hypergalactosemic rats. Also, (Robert M. Strothera *et al.,* 2001) reported that, the Effects of 30 days of streptozotocin-induced diabetes, insulin-treated diabetes, and 50% galactose diet on enzyme activities in liver, kidney, and heart is Hepatic catalase activity levels in both diabetic and galactosemic rats were significantly decreased when compared to normal. Hepatic and renal levels of GSSG were significantly diminished compared to normal in both diabetic and galactosemic rats.

Treatment with Taurine in normal rats and in experimental hypergalactosemic rats significantly increases GSH content, GSH-px, CAT. And SOD activities after 21 days of drug administration. These results were nearly similar to those reported by (Yu J and Kim AK 2009) they reported that, Taurine increased the activities of superoxide dismutase, glutathione peroxidase and Catalase compared to those of the control group. Likewise, (Henry*et al.,* 1974) reported that, taurine is a unique antioxidant and has beneficial effects on glutathione redox state metabolism. Also, taurine-induced increase in the activities of catalase and the enzymes of glutathione metabolism was of importance for antioxidative action of this amino acid.

Conclusion

It can be concluded that, taurine plays an important role in experimental hypergalactosemia, because it shows preventive action against its secondary complications due to: hypoglycaemic, antioxidativeand Hypolipidemic action also, It's important effect on insulin.

References

1. Berry, G.T., Segal, S., and Gitzelmann, R. (2006): Disorders of Galactose Metabolism. In: Fernandes J, Saudubray M, van den Berghe G, Walter JH. *Inborn Metabolic Diseases - Diagnosis and Treatment*. 4. New York, NY: Springer-Verlag, Inc; :chap 7.
2. Brownlee, M. (1996):Advanced glycation end products: in diabetic complications. Curr. Opin. endocrinol. Diabetes. 3: 291 — 297.
3. Choi, M.J., Kim, J.H., Chang, K.J. (2006): "The Effect Of Dietary Taurine Supplementation On Plasma And Liver Lipid Concentrations And Free Amino Acid Concentrations In Rats Fed A High-Cholesterol Diet".*Advances In Experimental Medicine And Biology*. Advances In Experimental Medicine And Biology 583: 235–42.
4. Fridovich-Keil, J. Walter. (2008): Galactosemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D. The Metabolic and Molecular Bases of Inherited Disease. 8th ed. New York, NY: McGraw-Hill Medical Publishing Division; 2008:72.
5. Garden, A.S., Davidson, D.C. (2000): Recommendations for the management of galactosaemia. *Arch Dis Child*. Mar 2000;82(3):266.
6. Goldfard, A.S., and Passas, L. (2002): Prediction of renal insufficiency in pima Indians with nephropathy of type-2 diabetes mellitus. Am. J. Kidney Dis.; 40 (2): 252-264.
7. Gubbels, C.S., Land, J.A., Rubio-Gozalbo, M.E. (2008): Fertility and impact of pregnancies on the mother and child in classic galactosemia. ObstetGynecolSurv. May 2008;63(5):334-43.
8. Henry, R.J., Cannon, D.C., and Winkelman, J.W. (1974): Clinical chemistry: Principles and technics. *Hagerstown, Maryland: Harper and Row. pp*. 1106.
9. Huxtable, R.J. (1992): "Physiological Actions OfTaurine". *Physiol Rev* 72 (1): 101–63
10. Jacobson, A.M., Ryan, C.M., Cleary, P.A., Waberski, B.H., Weinger, K., Musen, G. (2011):Biomedical risk factors for decreased cognitive functioning in type 1 diabetes: an 18 year follow-up of the Diabetes Control and Complications Trial (DCCT) cohort. *Diabetologia*. Feb 2011;54(2):245-55.
11. John Lombardini., and Julius Militante. (2006):Effects of Taurine Supplementation on Cholesterol Levels with Potential Ramification in Atherosclerosis. Advances in Experimental Medicine and Biology, 2006, Volume 583, Part 3., 251-254, DOI:10.1007/978-0-387-33504-9_27
12. KatarzynaWiniarska., Konrad Szymanski., PatrykGorniak., Marta Dudziak., Jadwiga Bryla. (2009):"Hypoglycaemic, antioxidative and nephroprotective effects of taurine in alloxan diabetic rabbits", Biochimie 91 (2009) 261e270.
13. Mary Otsyula., Matthew S. King., Tonya G. Ketcham., Ruth A. Sanders., And John B. Watkins III., International Journal Of Toxicology. (2003): 22: 423 (Oxidative Stress In Rats After 60 Days Of Hypergalactosemia Or Hyperglycemia).
14. Mi-Ja Choi.,Jung-Hee Kim., Kyung Chang. (2006):The Effect of Dietary Taurine Supplementation on Plasma and Liver Lipid Concentrations and Free Amino Acid Concentrations in Rats Fed a High-Cholesterol Diet.
15. Nagakatsu Harada.,Chika Ninomiya.,Yoshie Osako.,Masaki Morishima.,Kazuaki Mawatari., Akira Takahashi., and Yutaka Nakaya. (2004):Taurine Alters Respiratory Gas Exchange and Nutrient Metabolism in Type 2 Diabetic Rats.
16. Nandhini, A.T.A., Thirunavukkarasu, V., Anuradha, C.V. (2007):Stimulation of glucose utilization and inhibition of protein glycation and AGE products by taurine, Acta. Physiol. Scand. 181 (2004) 297e303.
17. PardeepSidhu., Garg, M.L., Dhawan, D.K. (2004):Protective role of zinc in nickel induced hepatotoxicity in rats.
18. Peter F. Kador., Yukio Takahashi., Yoshio Akagi., Heike Neuenschwander. (2002):Effect of Galactose Diet Removal on the Progression of Retinal Vessel Changes in Galactose-Fed Dogs.
19. quizlet.com/_bpmwy, Detail the role of sugars in cataract formation(Combo with MTC Module 2: Diseases and Drugs).
20. Ramana, B.V., Kumar, V.V., Krishna., P.N.R., ActaDiabetol. (2006): effect of quercetin on galactose-induced hyperglycaemia oxidative stress in hepatic and neuronal tissues of wistar rats.

21. Renu A. Kowluru., and PrashantKoppolu.(2002): Termination of Experimental Galactosemia in Rats, and Progression of Retinal Metabolic Abnormalities.
22. Robert M. Strothera., Tonya G. Thomasa., Mary Otsyul., Ruth A. Sander, John B. Watkins. OPA (Overseas Publishers Association). (2001):characterization of oxidative stress in various tissues of diabetic and galactose-fed rats.
23. Ronald L Engerman., and Timothy S Kern. (1984): Experimental Galactosemia Produces Diabetic-like Retinopathy.
24. Segal S., (1995):Galactosemia unsolved. *Eur J Pediatr*. 1995;154(7 Suppl 2):S97-102.
25. Semenkovich, C.F, and Heinecke, J.W. (1997):Perpectives in diabetes: the mystery of diabetes and atherosclerosis: time for anew plot. Diabetes; Vol. 46.
26. Snedecor, G.W., and Cochran, W.G. (1989): Statistical Methods, 8th ed. Ames: Lowa State University Press, 1989, P 97.
27. Sun Hee Cheong., Hyeongjin Cho., Kyung Ja Chang. (2009):Effect of PTP1B Inhibitors and Taurine on Blood Lipid Profiles in Adolescent Obesity.
28. Tawfek, N.S. and Taha, K.G. (2006): Effect OfTaurine, Selenium And Azathioprine Supplements On Blood Glucose, Lipid Peroxidation And Lipid Profile In Experimental Diabetes Mellitus.
29. Thirunavukkarasu, V., Anita Nandhini, A.T. and Arunadha, C.Y. (2004): Effects of alpha lipoic acids in rats fed a high-fructose diet. Experimental Diabetes Research. 5 (3): 195-200.
30. Yu J., Kim A.K. (2009): Effect of taurine on antioxidant enzyme system in B16F10 melanoma cells.

BIOCHEMICAL STUDIES ON LIPOIC ACID AS ANTIOXIDANT

Omayma A.R. Abou Zaid[1], Dowidar M F [2] and Dina M I [3]
[1, 3]Biochemistery Department, Faculty of Vet. Med. Moshtohor, Benha University, Egypt;[2]Biochemistery Department, Faculty of Veterinary Medicine, Zagazig University, Egypt;omayma.ragababozaid55@gmail.com

Abstract

This study was carried out to spot light on the effective role of Lipoic Acid as a powerful antioxidant in delaying aging process and its possible defense role by reducing the oxidative damage of free radicals. Experiments were applied in 60 rats which divided in to: young-30 rats (4-month-old) and aged-30 rats (24-month-old). The animals were divided into four groups and each group consisted of 15 animals: Group I, young control rats; Group II, young rats supplemented with lipoic acid (40 mg/kg for 30 days); Group III, aged control rats; Group IV, aged rats supplemented with lipoicacid(40 mg/kg for 30 days). Liver samples were obtained to estimate the activity of some hepatic antioxidant enzymes as (SOD, CAT, GSHPX and GSHR).The results revealed that Lipoic Acid induce a significant increase of hepatic antioxidant enzymes. Generally, administration of Lipoic Acid caused significant increase in activity of antioxidant enzymes in young and aged rats in comparison with control groups.

Keywords: *Lipoic Acid; antioxidants; oxidative stress; aging process.*

Introduction

The free radical theory of aging hypothesizes that oxygen-derived free radicals are responsible for the age-related damage at the cellular and tissue levels. In a normal situation, a balanced-equilibrium exists among oxidants, antioxidants and biomolecules (Domenico *et al.,* 2007).Co-supplementation of lipoic acid has a beneficial effect in reversing the age-related abnormalities seen in aging. This effect was associated with the decrease in free radical production and rise in antioxidant levels by lipoic acid, thereby lowering oxidative stress (Sethumadhavan *et al.*, 2005).

Oxidative stress is one of the factors responsible for the damaging effect of free oxygen radicals in cells and tissue (Bechman and Koppenol1996). Alpha-lipoic acid has been called a "universal antioxidant" because it is both water- and fat-soluble, and thus can penetrate tissues composed mainly of fat, such as the nervous system, as well as those made mainly of water, such as the heart, to protect them from free-radical damage. Alpha-lipoic acid also helps the body use other antioxidants, such as vitamin E, vitamin C, and glutathione, more efficiently (Podda *et al.,* 1994).The antioxidant defenses consist of low molecular mass antioxidants such as vitamin C and vitamin E and enzymes e.g. SOD, CAT and GSH-px, their function is to act as a coordinated and balanced system to protect tissues and body fluids from damage by ROS/RNS/RCS whether produced physiological or as response to inflammation, infection or disease (Evans and Halliwell, 2001)).Aging significantly decreased levels of the antioxidant enzymes as super oxide dismutase (SOD) and concurrent treatment with alpha lipoic acid significantly reversed the oxidative effects related to aging (Joachim *et al.,* 2011).

Catalase works closely with superoxide dismutase to prevent free radical damage to the body. SOD converts the dangerous superoxide radical to hydrogen peroxide, which catalase converts to harmless water and oxygen. Catalases are some of the most efficient enzymes found in cells; each catalase molecule can convert millions of hydrogen peroxide molecules every second **(**Kula *et al., 2000***).**

Oxygen free radicals have been proposed to be involved in the process of aging. Catalase (CAT) are important for antioxidative defense, where the activity of catalase and its mRNA level which investigated in hepatic tissues increased with age and exhibited higher levels at 6 and 12 months but decreased thereafter, that due to the increase of oxidative potential and the loss of proper antioxidant defense in the rats appear to be highly involved in the aging process. But after supplementation with lipoic acid it showed a highly significantly increasing in both the activity and gene expression of catalase enzyme **(**Huey-Jen *et al.*, 2000).

The activities of GPx and GR were found to be decreased in aged rats when compared with young rats. Supplementation of lipoic acid to aged rats significantly increased the GSH levels thereby increasing the activity of GPx, GR, and G6PDH in liver of aged rats. In conclusion, so suggests that supplementation of lipoic acid to aged rats improves the glutathione redox system **(**Kumaran *et al.*, 2001*).*

Glutathione peroxidase (GSH-px) catalyzes the reduction of hydrogen peroxide to water and oxygen at the expense of glutathione (GSH). Therefore the increase in GSH-px activity indicates that more oxidized glutathione (GSSH) is reduced to GSH. This recycling of glutathione is hypothesized to be due to ALA (Joachim *et al.*, 2011).

Materials and methods

A- Material

Animals**:** Sixty male albino rats were used in the present experiment and divided in to four groups according to age:

Group (A): (4-month-old) young control (n=15).

Group (B): (4-month-old) young rats supplemented with lipoic acid (n=15).

Group (C): (24-month-old) aged control (n=15).

Group (D): (24-month-old) aged rats supplemented with lipoic acid (n=15).

Animal management and housing:

Animals were housed in cages at 24±2ºC, under a 12:12h light –dark cycle, and provided with free access of food and water.

Animals from all groups were kept under similar environmental conditions of temperature, illumination, acoustic noise, and ventilation, and received the same diet during the course of the experiment. Food and water were kept in special open containers in cages. Cleaning and changing water and food was done for all animals twice daily.

Dosage:

Lipoic acid (40 mg/kg body weight/day) dissolved in physiological saline were administrated orally for 30 days. Control animals received physiological saline alone.

Sampling

A part used for preparation of tissue homogenate with 0.9% saline using electrical homogenizer, centrifuged at 3000 r.p.m for 15 minutes, the resulting supernatant were collected and used for estimation of antioxidant enzymes activities as Super oxide dismutase, catalase, glutathione peroxidase and glutathione reductase**(**Sidhu*et al.*, 2004**).** Livers from animals were preserved at -20ºC until performing the investigations.

B- Methods

1. Determination of superoxide dismutase activity in liver homogenate.

Hepatic superoxide dismutase was determined spectrophotometerically using ready-made kits (Nishikimi*et al*., 1972).

2. Determination of catalase activity in liver homogenate.

Hepatic catalase was determined spectrophotometerically using ready-made kits (Aebi1984).

3. Determination of glutathione peroxidase activity in liver homogenate.

Hepatic glutathione peroxidase was determined spectrophotometerically using ready-made kits (Paglia and Valentine 1967).

4. Determination of glutathione reductase activity in liver homogenate.

Hepatic glutathione reductase was determined spectrophotometerically using ready-made kits (Goldberg and Spooner 1983).

Statistical analysis

The results are expressed as mean±standard deviation (SD). Differences between groups were assessed by one-way ANOVA analysis using the SPSS software package for windows.

Results

Table 1. Effect of lipoic acid on Superoxide dismutase (SOD) activity in young and aged rat liver (U/gm tissue)

SOD activity (U/gm tissue) in rats liver				
Group	**Number**	**Rang**		**Mean±S.E**
		Mini.	**Max.**	
Control (young)	15	2.66	3.15	2.90±0.24
Lipoic Acid (young)	15	3.40	3.69	3.54±0.14
Control (Aged)	15	1.58	2.41	1.99±0.41
Lipoic Acid (Aged)	15	2.21	2.81	2.51±0.30

Means within the same row carrying different superscripts are significant at (p ≤0.05).

From the table it is noticed that the hepatic superoxide dismutase (SOD) enzyme activity was significantly increased in young rats that administrated Lipoic Acid compared with the young control group and other groups, also there was a significantly increased in aged rats that administrated Lipoic Acid compared with the aged control group, which indicates that the administration of Lipoic Acid to rats leads to increase of hepatic Superoxide dismutase SOD (Table 1& fig.1).

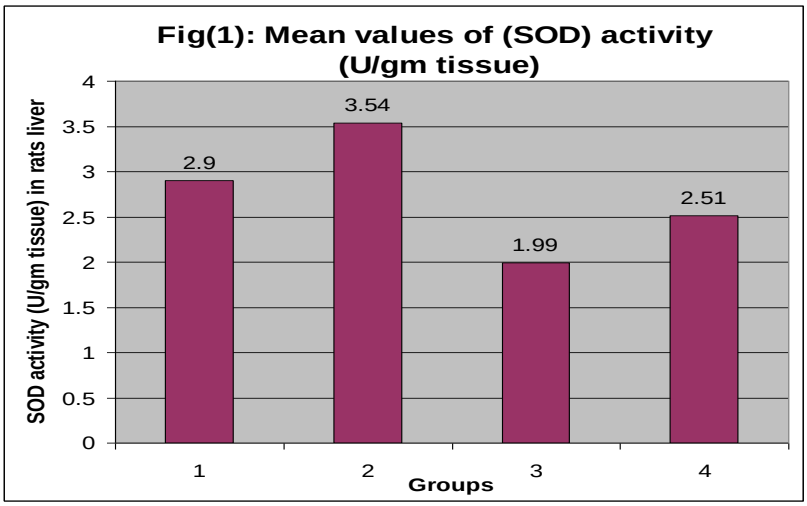


Fig. 1. Effect of lipoic acid on Superoxide dismutase (SOD) activity in young and aged rat liver (U/gm tissue)

From the table it is noticed that the hepatic Catalase (CAT) enzyme activity was significantly increased in young rats that administrated Lipoic Acid compared with the young control group and other groups, also there was a significantly increased in aged rats that administrated Lipoic Acid compared with the aged control group, which indicates that the administration of Lipoic Acid to rats leads to increase of hepatic Catalase CAT (Table 2& fig.2).

Table 2. Effect of lipoic acid on Catalase (CAT) activity in young and aged rat liver (U/g tissue)

CAT activity (U/g tissue) in rats liver				
Group	**Number**	**Rang**		**Mean±S.E**
		Mini.	**Max.**	
Control (young)	15	0.77	0.84	0.80±0.035
Lipoic Acid (young)	15	0.84	0.96	0.90±0.06
Control (Aged)	15	0.65	0.74	0.69±0.045
Lipoic Acid (Aged)	15	0.74	0.82	0.78±0.04

Means within the same row carrying different superscripts are significant at ($p \leq 0.05$).

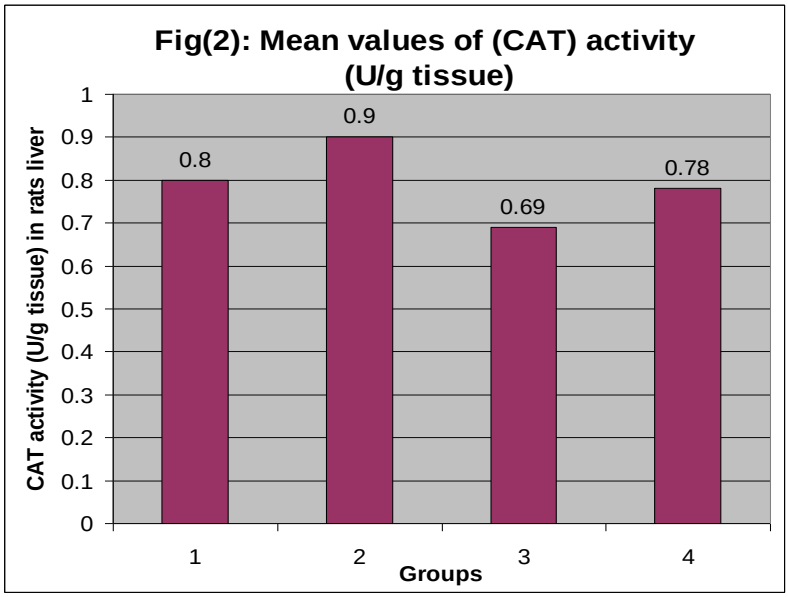


Fig. 2. Effect of lipoic acid on Catalase (CAT) activity in young and aged rat liver (U/g tissue)

From the table it is noticed that the hepatic Glutathione Peroxidase (GPX)enzyme activity was significantly increased in young rats that administrated Lipoic Acid compared with the young control group and other groups, also there was a significantly increased in aged rats that administrated Lipoic Acid compared with the aged control group, which indicates that the administration of Lipoic Acid to rats leads to increase of hepatic Glutathione Peroxidase GPX (Table 3& fig.3).

Table 3. Effect of lipoic acid on Glutathione Peroxidase (GPX) activity in young and aged rat liver (mU/ml)

GPX activity (mU/ml) in rats liver				
Group	**Number**	**Rang**		**Mean±S.E**
		Mini.	**Max.**	
Control (young)	15	77.8	116.72	97.26±19.46
Lipoic Acid (young)	15	116.72	175.08	145.9±29.18
Control (Aged)	15	19.45	58.36	38.90±19.45
Lipoic Acid (Aged)	15	58.36	97.26	77.81±19.45

Means within the same row carrying different superscripts are significant at ($p \leq 0.05$).

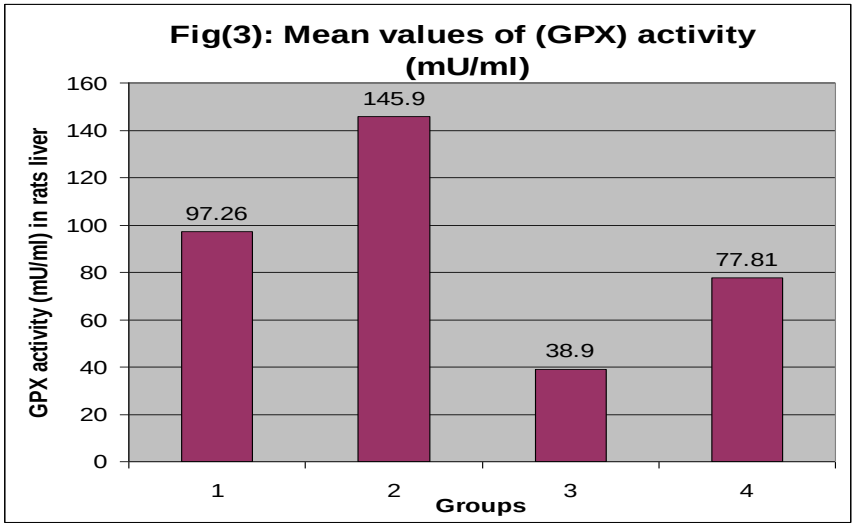


Fig. 3. Effect of lipoic acid on Glutathione Peroxidase (GPX) activity in young and aged rat liver (mU/ml)

From the table it is noticed that the hepatic Glutathione Reductase (GRD)enzyme activity was significantly increased in young rats that administrated Lipoic Acid compared with the young control group and other groups, also there was a significantly increased in aged rats that administrated Lipoic Acid compared with the aged control group, which indicates that the administration of Lipoic Acid to rats leads to increase of hepatic Glutathione Reductase GRD (Table 4& fig.4).

Table 4. Effect of lipoic acid on Glutathione Reductase (GRD) activity in young and aged rat liver (U/L)

GRD activity (U/L) in rats liver				
Group	**Number**	**Rang**		**Mean±S.E**
		Mini.	**Max.**	
Control (young)	15	60.28	76.36	68.32±8.04
Lipoic Acid (young)	15	80.38	92.40	86.39±6.01
Control (Aged)	15	32.15	56.26	44.20±12.05
Lipoic Acid (Aged)	15	52.24	64.34	58.29±6.05

Means within the same row carrying different superscripts are significant at ($p \leq 0.05$)

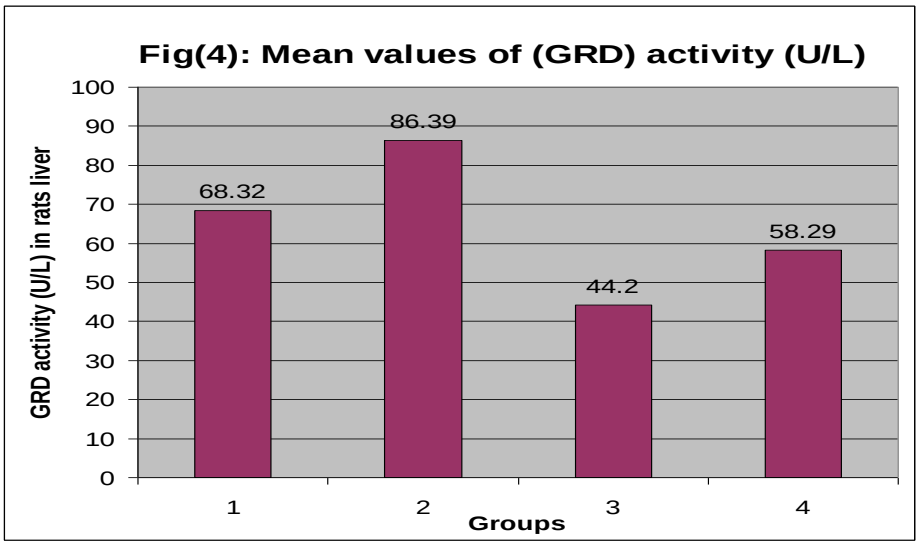


Fig. 4. Effect of lipoic acid on Glutathione Reductase (GRD) activity in young and aged rat liver (U/L)

Discussion

Hepatic antioxidant enzymes (Superoxide dismutase, Catalase, Glutathione peroxidase and Glutathione reductase) activities were significantly increased after administration of Lipoic Acid for 30 days in young and aged rats in comparison with control groups.

Administration of DL-α-lipoic acid, a thiol antioxidant to the aged rats, led to a time-dependent reduction in hydroxyl radicals and elevation in the activities/level of glutathione systems. Hence it can be suggested that lipoate, a dithiol prevents the oxidation of reduced glutathione and protects its related enzymes from peroxidative damage (Savitha and Panneerselvam2006**)**. During aging, mitochondria decay rats of oxidant production increase and oxidative damage to important biomolcules increase and may in part be responsible for aging as well as age-associated degenerative diseases such as cancer and atherosclerosis. It is important to understand whether the cellular distribution and bioavailability of key antioxidants have become altered with age **(**Jens*et al*., 1998**).**By aging which results in increasing in free radical activity in living organisms which leads to form of excessive amounts of active oxygen forms. They induce damage to DNA, change enzymes activity, gene expression, and affect membrane structure and function (Sawada and Carlson1987)**)**.Moreover, DL-α-lipoic acid treated aged rats showed a decrease in the level of lipid peroxides and an increase in the antioxidant status. The results of this study provide evidence that DL-α-lipoic acid treatment can improve antioxidants during aging and minimize the age-associated disorders in which free radicals are the major cause **(**Palaniyappan*et al*., 2001**).**Oxygen is an essential component of living organisms. The generation of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen is inevitable in aerobic metabolism of the body. Reactive oxygen species cause lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression. The body protects itself from the potential damages of

reactive oxygen species. Its first line of defense is superoxide dismutase, glutathione peroxidase, and catalase.

Scientists have indicated that antioxidant as lipoic acid supplied from daily diets quench the reactive oxygen species or are required as cofactors for antioxidant enzymes. Lipoic acid plays significant roles in the prevention of a number of age-related diseases and is essential for healthy aging **(**Lee*et al*., 2004**).**The antioxidant enzymes are those including glutathione redox cycle (glutathione peroxidase, glutathione reductase) as well as SOD and catalase. All these enzymes have been regarded as the primary defense system against oxidative stress; they exert their work by eliminating reactive oxygen species and other hydroperoxides during normal cellular metabolism **(**Fardy and Silverman1995**), (**McDonough 1999**).**In endogenous antioxidant systems SOD is widely distributed and plays a critical role within mammalian organism. SOD has a pivotal role against damaging effect from superoxide radical**.**Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are important enzymes in the body. SOD catalyses the conversion of superoxide free radical to hydrogen peroxide and water. GPx continues where SOD leaves off by catalyzing the reduction of hydrogen peroxide to water at the expense of glutathione. Previous studies have shown that aging reduces the activity of SOD and GPx rendering the body more vulnerable to oxidative damage**.** A decrease in SOD activity results in accumulation of superoxide anion radicals in blood and liver. And we can conclude that a decrease in SOD activity may be due to aging effect.

Oxidative stress is a possible aging-accelerating factor. During the aging process, tissues are damaged to some extent due to the oxidative processes primarily caused by reactive oxygen species. In particular, superoxide anion radicals are believed to be the major cause for such oxidative damages of living tissues. Among various antioxidative mechanisms in the body, SOD is thought to be one of the major enzymes which protects against tissue damage caused by the potentially cytotoxic reactivates of radicals. It is therefore possible that the decreases in SOD activities with age may further accelerate the aging process **(**Gülinnaz*et al.*,1998**)**. Hydrogen superoxide is then eliminated with CAT. As hydrogen superoxide is a product of SOD study it is also a strong inhibitor of this enzyme. As CAT was the first antioxidant. Enzyme to be characterized and catalyses the two stages conversion of hydrogen peroxide to water and oxygen and sharing this function with GSH-px**.**

Glutathione is the most abundant non thiol protein in the mammalian cells **(**. Irvine 1996**).**It plays a vital role in annihilating O_2 toxicity by interrupting the reaction lead to formation O_2^- in its reduced form it metabolizes H_2O_2 and OH^-**(**Agarwal and Prabakaran 2005**).**As glutathione plays an important role in intracellular protection against the toxic compounds, ROS and other free radicals also glutathione protects liver microsomes against ROS**.**The activity of antioxidant enzyme superoxide dismutase (SOD) was highly significantly increased in hepatic tissue of young and aged rats that administrated lipoic acid when compared with the young and aged control groups. Super oxide dismutase activity in liver was increased in aged rats after treatment with α-lipoic acid, which has been shown to have substantial antioxidant properties **(**Maritima*et al*., 2003**).**Superoxide dismutase (SOD) activity was determined in liver at two different age groups (4 months; 24 months) and it was founded that, the activity of superoxide dismutase (SOD) decrease significantly for the aged liver, but after administration of lipoic acid, the activity of SOD increased significantly. The results indicate that, the liver antioxidative defense decrease with age but its reversible after treatment with lipoic acid as effective antioxidant (Francine and Jean1989**)**. Due to the fact that superoxide dismutase (SOD) has an important role in free radical detoxification of the

liver, the age-related decrease in the activity of these enzymes might predispose this tissue to increased free radical damage, but after treatment with lipoic acid it noticed that, there was a significantly increased in activity of SOD enzyme. The activity of antioxidant enzyme catalase (CAT) was highly significantly increased in hepatic tissue of young rats that administrated lipoic acid when compared with the young control group also there was a significantly increased in aged rats hepatic tissues after supplemented on lipoic acid when compared with control aged group. The decrease in enzymatic activity of catalase (CAT) which related to aging decreasing the genetic expression of this enzyme. On the other hand, the activity of this enzyme was brought back to normal levels with co-administration of ALA.The activity antioxidant enzyme glutathione peroxidase (GSH-px)was highly significantly increased in hepatic tissue of young and aged rats that administrated lipoic acid when compared with the control groups. There was a significant reduction in the activity levels of antioxidants as glutathione peroxidase (GSH-px) in both middle-aged and aged rats which is reversible with co-supplementation of lipoid acid which improved the antioxidant status by increasing the activity and gene expression of glutathione peroxidase (GSH-px).

Several studies provided evidence that α-lipoic acid supplementation decreases oxidative stress and restores reduced levels of other antioxidants *in vivo*. However, there is also evidence indicating that α-lipoic acid and dihydrolipoic acid may exert prooxidant properties *in vitro*. α-Lipoic acid and dihydrolipoic acid were shown to promote the production of glutathione peroxidase (GSH-px) in rat liver mitochondria (Hadi*et al*., 2002**)**.

The activity of antioxidant enzyme glutathione reductase (GSH-R)was highly significantly increased in hepatic tissue of young rats that administrated lipoic acid when compared with the young control group also there was a significantly increased in aged rats hepatic tissues after supplemented on lipoic acid when compared with control aged group.In aged rats, activities of glutathione reductase (GSH-R) and the level of glutathione were low, whereas the level of hydroxyl radical was higher than in the young ones. Administration of DL-α-lipoic acid, a thiol antioxidant to the aged rats, led to a time-dependent reduction in hydroxyl radicals and elevation in the activities/level of glutathione systems and antioxidant enzymes as glutathione reductase (GSH-R). Hence it can be suggested that lipoate, a dithiol prevents the oxidation of reduced glutathione and protects its related enzymes from peroxidative damage **(**Arivazhagan*et al.,* 2001**)**.

References

1. Domenico, F., Giuseppe, C., Maria, R. and Matteo, C. (2007): Effect of antioxidant supplementation on aging process. *Clin.Interv.Aging. 23: 377-387.*
2. Sethumadhavan, S., Jayavelu, T., Muthuswamy, A. and Chinnakannu, P. (2005): Oxidative stress on mitochondrial antioxidant defense system in the aging process: Role of dl-α-lipoic acid. *Med. Biol. J. 355: 173-180.*
3. Bechman, J.S. and Koppenol W.H. (1996): Nitric oxide, superoxide, and peroxy nitrite: *The good, the bad and the ulgy. Am. J. Physiol., 271:C1424-C1437.*
4. Podda, M., Tritschler, H.J. and Ulrich, H. (1994): Alpha-lipoic acid supplementation prevents symptoms of vitamin E deficiency. *Biochem.Biophys. Res. Commun. 204: 98-104.*
5. Evans, P. and Halliwell, B. (2001): Micronutrients: oxidant / antioxidant status. *British j. of Nutrition 85 (2): 567-574.*
6. Joachim, P., Joon, H., Srikumar, C. and Nagaraja, H. (2011): Hepatoprotective effects of alpha lipoic acid on aging-induced oxidative stress in the rat liver. *Cell & Bioscience. 12: 1186-1197.*
7. Kula, B., Sobczak, A. and Kuska, R. (2000): Effect of catalase on free radical processes in rat liver and kidney. *J. occup. Health, 19: 99-105.*

8. Huey-Jen, T., Wang, P. and Shyang, W. (2000): Age-Associated changes of superoxide dismutase and catalase activities in the rat liver. *J. Biomed. Sci. 7:466-474.*
9. Kumaran, S., Savitha, S., Anusuya, D.M. and Panneerselvam, C. (2001): DL-alpha-lipoic acid reverses the age-related deficit in glutathione redox state in skeletal muscle and liver tissues. *U.S. National Lib.Of Med. 2398:2412.*
10. Sidhu, M.L., Gary and Dhawan, D.K. (2004): Protective effects of zinc on oxidative stress enzymes in liver of protein deficient rats *Nutr. Hosp. XIX, 6: 341-347.*
11. Nishikimi, M., Roa, N.A. and Yogi, K. (1972): Biochem. Bioph. Res. Common. 46: 849-854.
12. Aebi, H. (1984): Methods Enzymol. 105: 121-126, Fossati, P., et al. (1980): *Clin. Chem. 26: 227-231.*
13. Paglia, D.E. and Valentine, W.N. (1967): *J. Lab. Clin. Med. 70: 158-169.*
14. Goldberg, D.M. & Spooner, R.J. (1983): in methods of Enzymatic Analysis (Bergmeyen, H.V. Ed.) *3rd edn. Verlog.Chemie. Deerfield beach, Fl. 3: 258-265.*
15. Savitha, S. and Panneerselvam, C. (2006): Mitochondrial membrane damage during aging process in rat liver: Potential efficacy of α lipoic acid. *FASEB J. 13: 349-355.*
16. Jens, L., Tory, M.H., Vladimir, V. and Bruce, N.A. (1998): Age-associated decline in ascorbic acid concentration, recycling, and biosynthesis in rat hepatocytes-reversal with (R)-α-lipoic acid supplementation. *FASEB J. 12: 1183-1189.*
17. Sawada, M. and Carlson (1987): Changes in superoxide radical and lipid peroxide formation in the brain, heart and liver during the lifetime of the rat. *Mech. Aging Dev. 41: 125-137.*
18. Palaniyappan, A., Kadirvel, R. and Chinnakkannu, P. (2001): Effect of DL-α-lipoic acid on mitochondrial enzymes in aged rats. *Chemico-Bio. Int. 138: 189-198.*
19. Lee, J., Koo, N. and Min, D.B. (2004): Reactive oxygen species, Aging and AntioxidativeNutraceuticals. *WILEY Libr. 3: 21-33.*
20. Fardy, C.H. and Silverman, M. (1995): Free radical, antioxidant, and nutrition. *Nutrition Oct. 18 (10):872.*
21. McDonough, K.H. (1999): The role of alcohol in the oxidant-antioxidant balance in heart. *Frontiers in Bioscience. 4: D 601-6.*
22. Gülinnaz, A., Eser, Y., Biltan, E. and Fatma, Z. (1998): Age- related alternations in superoxide dismutase and catalase activites in rat liver. *Tr.J. Med. Sci. 28: 491-494.*
23. Irvine, D.S. (1996): Glutathione as a treatment for male infertility. *Rev. Reprod., 1:6-128.*
24. Agarwal, A. and Prabakaran, S.A. (2005): Mechanism, measurement, and preventation of oxidative stress in male reproductive physiology. *Indian Journal of Experimental Biology. 43:963-974.*
25. Maritima, A.C., Sandersb, R.A. and Watkins, J.B. (2003): Effects of α-lipoic acid on biomarkers of oxidative stress in aged rats. *J. Biol. Chem.14: 288-294.*
26. Francine, C. and Jean, V. (1989): Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation in the major organs of the aging rats. *Free Radic. Bio.And Med. 7: 59-63.*
27. Hadi, M., Lester, P. and Nils, L. (2002): Antioxidant and prooxidantactivites of α-lipoic acid and dihydrolipoic acid. *Toxic.And Appl. Pharm. 182: 84-90.*
28. Arivazhagan, P., Ramanathan, K. and Panneerselvam, C. (2001): Effect of DL-α-lipoic acid on glutathione metabolic enzymes in aged rats. *FASEB J. 37: 81-87.*

ASSOCIATION OF INFLAMMATORY, ENDOTHELIAL DYSFUNCTION MARKERS AND CARDIOVASCULAR RISK FACTORS IN OVARIECTOMIZED STZ-DIABETIC RATS

Omayma ,A., Ragab Abo-zaid[1] ,Atef Goda Hussain[2]. and Sahar E. Ahmed[3].
Biochemistry Department, Faculty of Vet. Med. Moshtohor, Benha University, Egypt.
[1,3]Department of Biochemistry,faculty of Veterinary Medicine, Benha University and
[2]Department of Biochemistry, Faculty of Medicine, Zagazig University

Abstract

The present study was carried out on eighty female albino rats . They were randomly divided into four equal groups ;the first group sham operated control group, the second group was subjected to induction of diabetes type 2, the third group was rendered to bilateral ovariectomy and the fourth group was subject to bileral ovariectomy with type 2 diabetes induction. Blood sample were collected from all animals group after 5 weeks from the onset of experiment. All sera were subjected for the investigation of the following parameters including: total cholesterol, triacylglecerols, high density lipoprotein (HDL), low density lipoprotein (LDL), nitric oxide (NO), high sensitive c-Reactive Protein (hs-CRP), and tumor necrosis factor-α (TNF-α). The obtained results indicated that inflammation, unfavorable lipid profile in form of significant increase of total cholesterol, triacylglecerols, and low density lipoprotein (LDL). Meanwhile, serum nitric oxide in diabetic rats as well as those of ovarictomy were significantly different from those of control .However ,serum nitric oxide and, high density lipoprotein (HDL) concentrations in the ovarictomized STZ-Diabetic rats were significantly decreased when compared with sham operated group.

Kay word: *inflammation, endothelial dysfunction, cardiovascular risk, diabetes, and ovarictomy.*

Cardiovascular Risk Factors In Ovariectomized STZ-Diabetic Rats

Introduction

The incidence of cardiovascular disease differs significantly between men and women, in part because of differences in risk factors and hormones **(1)**. Human life was prolonged by 30 years in the past century, with the result that about 40% of a woman's life falls within the postmenopausal period **(2).** There is now a large body of evidence suggesting that, the decline in ovarian function with menopause is associated with spontaneous increases in proinflammatory cytokines. The cytokines that have obtained the most attention are interleukin-1 (IL-1), (IL-6), and TNF-β. The exact mechanisms by which estrogen interferes with cytokine activity are still incompletely known but may potentially include interactions of the estrogen (ER) with other transcription factors, modulation of nitric oxide activity, antioxidative effects, plasma membrane actions, and changes in immune cell function **(3).** The menopausal consequences, both early and remote, in the form of cardiovascular disease, osteoporosis and neoplastic disease are most pronounced in women suffering from one of the most common diseases, i.e., diabetes mellitus. These patients are problematic for physicians**[2].** Many postmenopausal women live with diabetes mellitus; however, little information is available about how the changes that occur around the time of menopause might uniquely affect management of diabetes mellitus in this population **(4).** postmenopausal diabetic patients encountered the reality of increased atherogenic lipid profile **(5)**, as well as redox imbalance **(6)**, and thereby increased cardiovascular risk factors. Accordingly this study was undertaken to evaluate the role of inflammatory markers on endothelial dysfunction hence cardiovascular risk factor in estrogen deficiency in diabetes via determination of some inflammatory markers such as: Serum Tumor Necrosis Factor-alpha.(TNF-α), high sensitive

C- Reactive Protein (hs-CRP). Also, determination of serum Nitric oxide as a marker of endothelial dysfunction and serum lipid profile were also investigated.

Material and methods

Animals:

Eighty Female albino Wistar rats of 30-45 days and weighted (200-250) g were used in the experimental study. Rats were obtained from the Laboratory Animals Research Center, Faculty of Veterinary Medicine, Moshtohor, Benha University. Animals were housed in separate metal cages, fresh and clean drinking water was supplied ad-libtium. Rats were kept at a constant environmental and nutritional condition throughout the period of experiment. The animals were left for 15 days for acclimatization before the beginning of the experiment.

Experimental design:

Rats were divided into four equal groups as follows:

Group 1: Sham-operated control rats (Sham): (n =20)

Rats in this group were subjected to all surgical procedures without ovariectomy

Group 2: Sham-operated streptozotocin diabetic rats (STZ): (n =20)

Rats in this group were subjected to type 2 diabetes induction [(7)], and all surgical procedures without ovariectomy

Group 3: Ovariectomized rats (OVX): (n =20)

Rats in this group were subjected to bilateral ovariectomy. tweenty days after the operation, they received a single dose of i.p. injection of citrate buffer (1 ml/kg) from body weight.

Group 4: Ovariectomized STZ diabetic rats (OVX–STZ): (n =20)

Rats in this group were subjected to bilateral ovariectomy. Twenty days after the operation, they renderd to diabetes.

Ovariectomy:

Bilateral ovariectomy [(9)] was performed under anesthetic condition with a single mid-ventral incision using inhaled ether. Following surgery, animals were kept in a resting phase for about 20 days to enable them to recover completely from surgical stress and to allow the circulating sex steroid levels to diminish [(8)].

Diabetes induction:

To enhance type 2 diabetes rats were rendered diabetic by following the method used earlier[(7)]. For this purpose, streptozotocin and nicotinamide were dissolved in citrate buffer (pH 4.5) and normal saline, respectively. Nicotinamide was injected (120 mg/kg, i.p.) 15 min. before the injection of Streptozotocin (60 mg/kg, i.p.).

Blood Sample Collection:

Blood samples for serum separation were collected from all animals groups after overnight fasting from eye plexus. The samples were centrifugated at 3000 rpm, separated in sterile tubes by using sterile pipette and stored at -20 °C until used for determination serum lipid profile (Triglycrols, total cholesterol, LDL-c, HDL-c), Nitric Oxide and inflammatory markers (hs-CRP and TNF-α).

Statistical analysis:

All data were expressed as mean ± SD and statistical significance was evaluated using One Way ANOVA followed by Tukey's Multiple Comparisons test using Graph Pad Prism Version 5.0 for Windows (Graph Pad Software, San Diego, CA, USA). The mean difference is significant at the 0.05 level.

Results and discussion

In general the combination of female sex steroid hormone deficiency with insulin hormone deficiency in ovariectomized diabetic rats resulted in worsening of many metabolic aspects as compared to rats exposed to any of them alone. Unfriendly lipid profile persisted and was aggravated following combination of ovariectomy to diabetes where serum triglycerides, total cholesterol and LDL-c showed significant increase as compared to control, ovariectomized and diabetic rats while serum HDL-c was significantly decreased compared to controls, ovariectomized and diabetic (table 1). This result was similar to a recent study [(10)] who showed that an increment in lipid profile cannot only lead to increasing insulin resistance and exaggerated type 2 diabetic complications but also predispose PMW and POF/surgical menopause individuals to cardiovascular disorders. The prominent effects of estradiol deficiency on decreasing the number of LDL-c receptors and elevating hepatic lipase activity **[(11,12)]**, were added to the deleterious effects of insulin deficiency on altering the activity of lipoprotein lipase, increasing VLDL-c, triglycerides and LDL-c due to defective clearance of these particles from circulation together with acceleration of clearance of HDL-c, all of which resulted in a significant elevation of these hazardous triglycerides, total cholesterol and LDL-c and lowering of HDL-c in ovariectomized diabetic rats as compared to controls and even to rats exposed to either ovariectomy or diabetes **[(13)]**.

Table 1. Changes in serum lipid profile in control and experimental groups

Animal group	TC (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	AI
Sham operated	83±8.27	49.5±4.8	47.5±2.6	25.6±8.9	1.6±0.23
Diabetic	120.6±10.29[a]	111.3±9.9[a]	26.6±2.08[a]	71.7±11.1[a]	4.6±0.9[a]
Ovarictomiezed	111.6±4.8[a,d]	101.3±8.01[a,b]	28±1.6[a,d]	63.3±4.9[a,d]	4±0.4[a,d]
Ovarictomiezed STZ-diabetic	133±4.1[a,b,c]	131.7±4.7[a,b,c]	28.8±3.9[a,b,c]	84.8±10.9[a,b,c]	6.3±1.5[a,b,c]

P is statistically significant if (P≤0.05). ([a]) significant against control group.([b]) Significant ovarictomized rats. ([c]) Significant against diabetes. TG (Triglyceride), ([d]) non significant against diabetes. TC (Total Cholesterol), HDL-C (high density lipoprotein-cholesterol); LDL-C (low density lipoprotein- cholesterol). Atherogenic index (AI).

In the present study hs-CRP was showed a significant increased in ovarictomiezed STZ-diabetic group compared to sham operated and significant increase compared to diabetic and ovarictomized groups. Also serum TNF-α revealed a significant increased when compared to sham operated group and a significant elevation when compared to diabetic and ovarictomized groups (table 2). This finding was similar to the results obtained by other study showed that Induced diabetes and ovariectomy significantly increase both TNF-α and IL-1 b levels and it was demonstrated that diabetes mellitus (DM), combined with ovariectomy,

augments the levels of oxidant and pro-inflammatory cytokines in the lung, liver, and heart[14].

Table 2. Tumor necrosis factor-α (TNF-α) and high sensitive c-Reactive protein (hs-CRP) in diabetic , ovarictomized and ovariectomized STZ- diabetic rats and their control group

Animal groups	NO (µmol/L)	TNF-α (pg/ml)	Hs-CRP (mg/L)
Sham	20.72 ±1.06	32.6±4.7	0.95 ±0.33
Diabetic	17.32 ±0.6[a]	201.2±16.01[a]	5.52 ±2.8[a]
OVarectomized	17.63 ±1.27[a]	178.3±17.4[a,b]	4.3 ±1.47[a]
Ovarectomized STZ-diabetic	15.7 ±0.78[a,b,c]	273.2±20.4[a,b,c]	8.82 ±3.8[a,b,c]

Data were expressed as mean±SD. Standerd deviation (SD). (a) Significant when compared to sham operated control group, (b) Significant when compared to diabetic control group and (c) Significant when compared to ovarectomized group ;P is statistically significant if (P≤0.05).

The recorded results may be also attributed to an insulin resistance state may facilitate hepatic hs-CRP production because insulin has anti-inflammatory effects and resistance to this effect would then lead to increased synthesis of CRP [15]. It is also possible that mildly impaired glucose status with-out clinical diagnosis may elicit oxidative stress and production of free fatty acids that may raise levels of hsCRP [16], these effects added to the deleterious effects of estrogen deficiency which has also been shown to enhance the sensitivity of cells such as monocytes, osteoblasts, and endothelial cells to these cytokines by upregulating cytokine receptor expression [3]. The role of hs-CRP in the pathogenesis of atherosclerosis is receiving increased attention because of the effect of hs-CRP on the production of the chemokine monocyte chemo-attractant protein-1, which is abolished by fenofibrate but not by aspirin [17], CRP promotes monocyte chemoattractant protein 1–mediated chemotaxis through upregulating CC chemokine receptor 2 expression in human monocytes [18]. Each CRP molecule consists of 5 identical protomers; each protomer has 2 calcium ions that bind to LDL to form a complex. This complex induces the formation of foam cells on the endothelial cell wall, attracting monocytes. Thus, a high level of CRP is both a marker and a cause of atherosclerotic lesions [19]. There are also in (table 2) a significant decreased in serum nitric oxide in ovarictomized-diabetic rats compared to sham operated control group, diabetic and ovarictomized groups. The results of the present study further support the concept that, the endothelial dysfunction observed in the insulin-resistant states of obesity and type 2 diabetes has been attributed to decreased nitric oxide (NO) availability, likely related to both impaired production and increased consumption. Endothelial function reflects an imbalance of both vasodilating and vasoconstricting factors [20,21]. Endogenous endothelin-1 activity is increased in obesity and type 2 diabetes, and several studies suggest that this factor may itself reduce the bioavailability of NO, through impairment of NO generation by the vascular endothelium as well as via direct vasoconstrictor effects on the vascular endothelium [20], but, other study

related it to the positive vascular effects of estrogen which have been attributed primarily to estrogen upregulation of endothelium-derived nitric oxide (NO) [22]. Also, in this group there are negatively significant correlation between serum nitric oxide and TG and LDL-c but, there was positively correlation between nitric oxide and HDL-c. The recorded correlation may be also attributed to that hypercholesterolemia was reported to impaired endothelial function, manifested as an attenuation of endothelium-dependent relaxation prior to the formation of atherosclerosis [23].

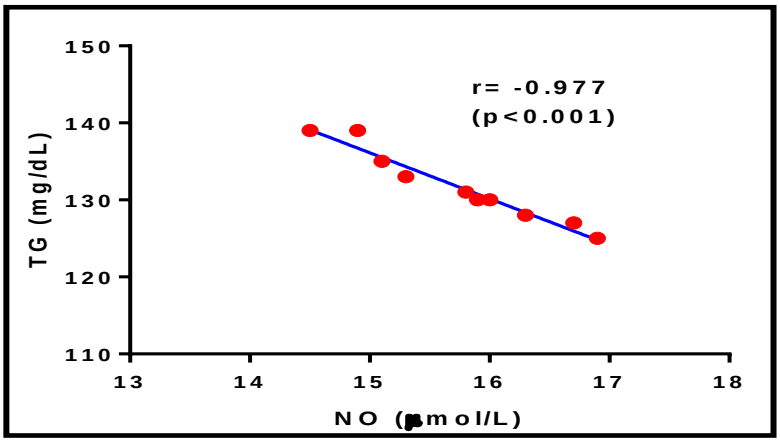


Fig. 1. Correlation coefficient (r) between serum nitric oxide and triacylglecerols in ovariectomized STZ-diabetic group

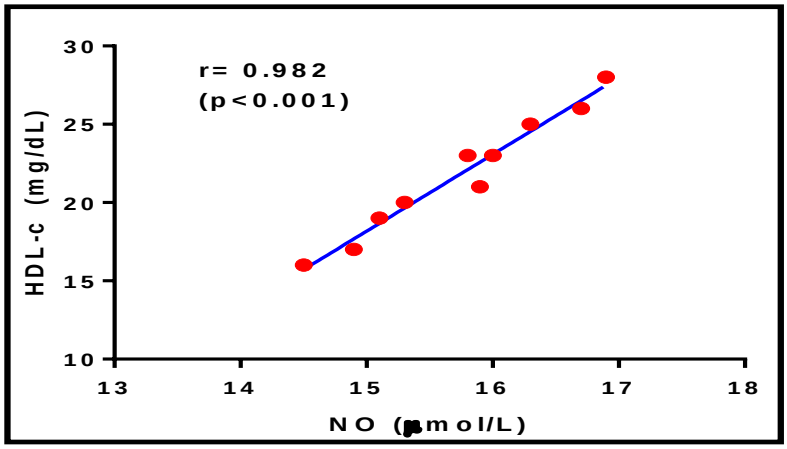


Fig. 2. Correlation coefficient (r) between serum nitric oxide and HDL-c in ovariectomized STZ-diabetic group

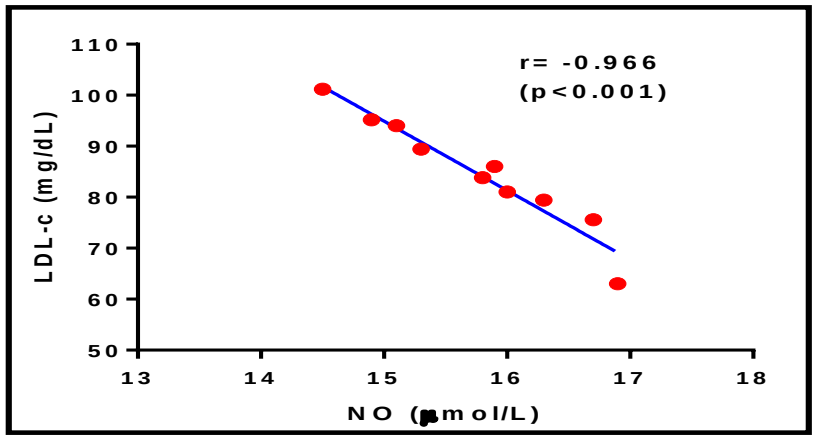


Fig. 3. Correlation coefficient (r) between serum nitric oxide and LDL-c in ovariectomized STZ-diabetic group

The additive effect of estrogen and insulin deficiencies could explain the significant rise in blood glucose following streptozotocin injection in ovariectomized rats that was even significantly higher than diabetic rats [(24)]. Not only, but also a highly significant elevation of inflammatory markers, endothelial dysfunction, and dyslipedimia. Ovariectomy and diabetes teamed up for worsening dyslipidemia, where serum triglycerides, total cholesterol and LDL-c were significantly higher than sham-operated, ovariectomized and diabetic groups and the atherogenic index was significantly higher than control and ovariectomized groups while serum HDL was significantly lower than sham-control group. These data are confirmed by the significant negative correlation between each of plasma estradiol and insulin with triglycerides, total cholesterol, LDL-c and atherogenic index and significant positive correlation with plasma HDL-c [(24)]. The complications of Diabetes Mellitus include cardiovascular disease (CVD) and it has been found that CVD is due in part to low grade systemic inflammation [(25)]**.** In general, there is accumulating evidence that inflammation is an important risk factor in cardiovascular disease (CVD). Elevated levels of the inflammatory marker high-sensitivity C-reactive protein (hs-CRP) are associated with increased risk for CVD and diabetes mellitus. Adding hs-CRP to the definition of the metabolic syndrome has been shown to improve the prediction of CVD. Elevated hs-CRP levels may also be predictive of development of the metabolic syndrome [(26)]. Overall, inflammation is an important risk factor in cardiovascular disease (CVD), Dyslipidemia together with hypertension and diabetes is major modifiable risk factor for atherosclerotic disease and the subsequent development of cardiovascular events [(27,28).] Endothelial dysfunction, which is a condition that has been strongly associated with dyslipidemia, plays a key role in the development and progression of atherosclerosis [(29)]. The reduced availability of nitric oxide (NO) resulting from both a decreased synthesis and/or an enhanced degradation by reactive oxygen species seems to be the major cause of endothelial dysfunction documented in subjects with cardiovascular risk factors including dyslipidemia [(30)]. It is also well accepted that atherosclerosis can be considered a chronic vascular inflammatory disease [(31)]. Inflammatory cytokines are responsible for activation of endothelial cells, a condition characterized by the expression of endothelial cell surface adhesion molecules such as

vascular cell adhesion molecule-1 (sVCAM-1) and p-Selectin, that favor the attachment of circulating monocytes to the endothelium[32]. Similarly, C-reactive protein (CRP), which is a well-described inflammatory marker, has been shown to be an independent predictors of future cardiovascular events in both high-risk and healthy subjects **[33,34].** Moreover, increased circulating cytokines including tumor necrosis factor alpha (TNF α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) have also been associated with cardiovascular events **[35].** In view of the aforementioned data, diabetic rats showed marked hyperglycemia, dyslipidemia, elevated inflammation and significant endothelial dysfunction. Ovariectomized rats showed increased inflammation, unfavorable lipid profile and increased endothelial dysfunction. Combination of these two metabolic threats in ovariectomized diabetic rats was associated with dyslipidemia , inflammation and endothelial dysfunction than did ovariectomy or diabetes alone. Also, a significant correlation was established between serum nitric oxide with lipid profile and inflammatory markers in this study this is can report that, there are correlation of dislipidemia, inflammatory, endothelial dysfunction markers and cardiovascular risk factor in ovarictomized STZ- diabetic rats. It can be concluded that estrogen deficiency with diabetes worsen the metabolic consequences of either disorder alone. These data suggest that insulin and estrogen deficiency may act as a mixed hazards, and increase cardiovascular risk factors. Therefore it must be careful and under physician supervision to avoid more risk and guard against its effects.

Reference

1. 1-Barrett-Connor, E., 1997: Sex differences in coronary heart disease: why are women so superior? The 1995 Ancel Keys Lecture. *Circulation* 95: 252-64.
2. 2-Jedrzejuk, D., and Milewicz, A., 2005: Consequences of menopause in women with diabetes mellitus a clinical problem. *Gynecol Endocrinol*. 21:280-286.
3. 3-Pfelilschifter, J., Koditz, R., Pfohl M., and Schatz, H., 2002: Changes in Proinflammatory Cytokine Activity after Menopause. *Endocrine Reviews* 23:90 –119.
4. 4-Szmuilowicz, E.D., Stuenkel, C.A. and Seely, E.W., 2009: Influence of menopause on diabetes and diabetes risk. *Nature Reviews Endocrinology* **5**: 553-558.
5. 5-Masding M.G., Stears A.J ., Burdge G.C., Wootton S.A., and Sandeman D.D., 2003: Premenopausal advantages in postprandial lipid metabolism are lost in women with type 2 diabetes. *Diabetes Care* 26: 3243-3249.
6. 6-Zhang, L.F., Zalewski, A., Liu, Y., Mazurek, T., Cowan, S., Martin, J.L., Hofmann, S.M., Vlassara, H. and Shi, Y., 2003: Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries. *Circulation* 108:472-478.
7. 7-Shirwaikar, A, Rajendran, K., Dinesh, C., and Bodla, R., 2004: Antidiabetic activity of aqueous leaf extract of Annona squamosa in streptozotocin-nicotinamide type 2 diabetic rats. J. Ethnopharmacol., 91 : 171-175.
8. 8-Singh, P.K., Baxi, D.B., Mukherjee, R., Potnis, K.H., Ramachandran, A.V., 2010: Supplementation with a polyherbal extract and melatonin together with exercise effectively reverses diabetic glycaemic status and carbohydrate metabolism and insulin level. *Int J Biol Med Res* 1:54-60.
9. 9-Ingie, M., and Griffith, JQ., 1942: The rat in laboratory investigation, second edition, Lippincott. J.B. Company.
10. 10-Baxi, D., Singh, P., Vachhrajani, K., Ramachandran A., 2011: Diabetic glucose dyshomeostasis and dyshomeostasis and dyslipidemia in estrogen deficient rats: Melatonin replacement therapy in alleviating the symptoms supplementation more potent than estrogen. *Diabetologia Croatica* 40-1.
11. 11-Distefano, E., Marino, M., Gillette J.A., Hanstein, B., Pallottini, V., and Brüni. J., 2002: Role of tyrosine kinase signaling in estrogen-induced LDL receptor gene expression in HepG2 cells. *Biochim Biophys Acta.*1580:145-149
12. 12-Carr, M.C., 2003: The emergence of the metabolic syndrome with menopause. *J Clinical Endocrinology and Metabolism* 88: 2404-2411.
13. 13-Andallu, B., Kumar, AVV. and Varadacharyulu, NC., 2009: Lipid abnormalities in streptozotocin-diabetes: Amelioration by Morus indicaL. cv Suguna leaves. *Int J Diabetes Dev. Ctries* 29: 123-128.

14. 14-Albayrak A., Uyanik M., Odabasoglu , Halici Z., et al., (2011): The Effects of Diabetes and/or Polymicrobial Sepsis on the Status of Antioxidant Enzymes and Pro-Inflammatory Cytokines on Heart, Liver, and Lung of Ovariectomized Rats. *Journal of Surgical Research* 169: 67–75.
15. 15-Bloomgarden, ZT., 2005: Inflammation, atherosclerosis, and aspects of insulin action. *Diabetes-Care*. 28:2312-2319.
16. 16-Liu, S., Tinker, L., Song, y., and Rifai, N., et al., 2007: A Prospective Study of Inflammatory Cytokines and Diabetes Mellitus in a Multiethnic Cohort of Postmenopausal Women *Arch Intern Med*. 167:1676-1685.
17. 17-Pasceri, V., Cheng, JS., Willerson, JT., Yeh, ET., 2001. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation*.103: 2531-2534.
18. 18-Han, KH., Hong, KH., Park, JH., Ko J, Kang, DH., Choi, KJ., Hong, MK., Park, SW., Park, SJ., 2004: C-reactive protein promotes monocyte chemoattractant protein-1–mediated chemotaxis through upregulating CC chemokine receptor 2 expression in human monocytes. *Circulation* 109:2566-2671.
19. 19- Wu, T., Ou, H., Chou, C., Hsiao, S., Lin, C., and Kao, P.,2007: Decrease in Inflammatory Cardiovascular Risk Markers in Hyperlipidemic Diabetic Patients Treated with Fenofibrate. *Annals of Clinical & Laboratory Science*. 37: 159-164.
20. 20-Mather, KJ., Lteif, A., Steinberg, HO., Baron, AD., 2004: Interactions between endothelin and nitric oxide in the regulation of vascular tone in obesity and diabetes. *Diabetes* 53:2060-2066.
21. 21-Barzilay, J. I. and Freedland, E. S., 2003: Inflammation and its relationship to insulin resistance, type 2 diabetes mellitus, and endothelial dysfunction. *Metab Synd Rel. Dis* 1:55-67.
22. 22-Tostes, RC., Nigro, D., Fortes, ZB., Carvalho, MH., 2003: Effects of estrogen on the vascular system. *Braz J Med Biol Res*. 36:1143–1158.
23. 23-Ghaneimah, A., Nafady, A. Mandour, M., and Seif El-din, N., 1999: Endothelial dysfunction in hypercholesterolimic rats: Effect of benzafibrate on endothelialum-drived substances. *The Egyptian Journal of Biochemistry*. 17: 23-36.
24. 24-El-Dakak, S., 2010: Metabolic Consequences of Estrogen Deficiency in Experimental Diabetes. M.D. Faculty of Medicine Ain Shams University.
25. 25-Haffner, S., Greenberg, A., Weston, W., Chen, H., Williams, K., Freed, M., 2002: Effect of Rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes. *American Heart Association*. 106: 679-684.
26. 26-Haffner, S., 2006: The Metabolic Syndrome: Inf lammation, Diabetes Mellitus, and Cardiovascular Disease. *Am J Cardiol* 97 3A–11A
27. 27- L ´opez-Jaramillo, P., Casas, J. P., Bautista, L., Serrano, N. C., And Morillo, C.A., (2001): " An integrated proposal to explain the epidemic of cardiovascular disease in a developing country: from socioeconomic factors to free radicals," *Cardiolog y*, 96: 1–6.
28. 28- Heras, N. de las, Cediel, E., Oubina, M. P., et al., 2003: "Comparison between the e ffects of mixed dyslipidaemia and hypercholes-terolaemia on endothelial function, atherosclerotic lesions and fibrinolysis in rabbits," *Clinical Science* 104:357–365.
29. 29-L ´opez-Farr ´e, A., Farr ´e, J., S ´anchez, de Miguel, L., Romero, J., Gonz´alez-Fern ´andez, F., and Casado, S., 1998: "Endothelial dysfunction: a global response," *Revista Espanola de Cardiologia* 51:18–22.
30. 30-Mason, R. P., 2006: "Nitric oxide mechanisms in the pathogenesis of global risk," *Journal of Clinical Hypertension*. 8: 31–38.
31. 31-Libby, P., 2002: Inflammation in atherosclerosis. *Nature* 420:868 – 874.
32. 32-Hansson, G. K., Robertson, A.-K. L., and oderberg-Naucl´er, C., 2006: "Inflammation and atherosclerosis," Annual Review of Pathology.1:297–329.
33. 33-Ridker, P. M., Buring, J. E., Rifai, N., and Cook, N. R., 2007: "Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: the reynolds risk score," *Journal of the American Medical Association*. 297:611–619.
34. 34-Sanchez-Recalde, A., and Kaski, J. C., 2001: "Diabetes mellitus, inflammation and coronary atherosclerosis: current and future perspectives," *Revista E spanolade Cardiologia* 54: 751–763.
35. 35-Sukhija, R., I. Fahdi, L. Garza, et al., 2007"Inflammatory markers, angiographic severity of coronary artery disease, and patient outcome," *The American Journal of Cardiology* 99: 879–884.

INVESTIGATIONS ON THE HISTOLOGICAL STRUCTURE OF THE PECTORALIS SUPERFICIALIS MUSCLE, IN COBB-500 COMMERCIAL MEAT-TYPE HYBRID HEN, SLAUGHTERED AT DIFFERENT AGES

V.Teusan*; Anca Prelipcean (Teusan)*
*U.S.A.M.V. IASI
vasile.teusan@yahoo.com

Abstract

From 30 young chickens, males and females, of COBB-500, sacrificed at 35, 42 and 49 days we collected histological samples from the surface of the Pectoral superficial muscle (PS). These samples were processed using a paraffin sectioning technique, yielding 50 slides that have been studied with a MC_3 photonic microscope. In the microscopic field, using cytological and histometrical techniques, we measured the thickness of muscle cells and primary muscle fascicles (PMF). Based on these data we calculated: the average thickness, the perimeter and cross-sectional surface of PMF, the number of myocytes and proportion of the two main categories of this muscle tissue. The followingresults were obtained: average thickness of PMF from PS muscle of males was of 238.5-240.25μ, for the age of 35 days; of 320.25-341μ, for the age of 42 days and of 316.14-280.99μ, for the age of 49 days. The average thickness of females PMF is with 0.74% higher than in males, at the age of 35 days and more than 6.48% at the age of 42 days. As for the appearance, PMFs are close clotted, which is evidenced by the format index values (1.613/1-2.20/1), as regarding the number of myocytes contained, it was of 36.40-59.10 being higher in females with 20.86-24.45% than in males. PMF contain 52.60-65.50% muscular tissue and 34.5-47.40% connective tissue, in males and 49.13-53.75% muscular tissue and 46.25-50.87 connective tissue, in females. For all studied parameters and all slaughter ages the differences between males and females are statistically insignificant. Exceptions are the differences in the proportion of muscular and connective tissue at the age of 42 days, which are statistically significant ($\hat{F} > F\alpha$; $W_{0.001}$<tested differences).

Key words: *COBB-500; Superficial pectoral; PMF; tissue categories.*

Introduction

At present poultry meat, which is characterized by a superior dietetico-nutritional value, is obtained by an increase of specialised hybrids with outstanding bio-economic performances [13].

For the species *Gallus domesticus*, the hybrids most commonly and most efficient used are: ROSS-308; COBB-500; SHAVER-STARBRO and others. In spite of the fact that their performances are well known and also the nutritional and microclimat requirements of these hybrids, the quality of the meat obtainned from these birds it is not well-known yet.

The quality of poultry meat is apreciated using some parameters such as the following: chemical composition, biological value, technological and physical features, the histological structure of muscles that make up carcasses, etc. Regarding the histological structure of COBB-500 meat there have already been made a series of research [5]; [7]; [8]; [9]; [10], which target the thickness of muscle fibers and fascicles from some pectoral, thigh and calf muscles of male and female chickens (of the hybrid mentioned above) slaughtered at different age stages. Such research should be continued and extended to other histological parameters and other muscles. It is also the case of the paper here in which we studied 10 histological parameters, for males and females at three slaughter ages (35, 42 and 49 days).

Material and methods

The biological material was represented by 30 chickens from meat-type hybrid COBB-500, 15 males and 15 females, were sacrificed at the ages of 35, 42 and 49 days when they had the body weight: 1406-1614g, 2168 - 2350-2296g and 2378g, based on gender [7]. After slaughter from those carcases we took histological samples of muscle Pectoralis superficialis (PS) [3], [4], [11], [12], which were processed by paraffin sectioning technique [2], [14]. Thus we obtained 60 histological slides with cross-sections through the middle area of PS muscle, coloured with hematoxylin-eosin dichromate (HE). These blades have been studied using a binocular photon microscope MC3 type, adjusted and calibrated in advance. Calibration was done with the following associations: OC10 x OB6; OC10 x OB10 and OC10 x OB20, for which three values were calculated on micrometer used in our study namely: 15.011µ; 9.011µ and 4.441µ [2]. On the microscopic field were found, photographed and studied the best images representing cross-sections through muscle PS. Using an ocular micrometer and a micrometer scale we measured (in the microscopic field) the large diameter and small diameter of myocytes and of muscle fascicles (PMF) and we counted the striated muscle fibers within PMF [8], [9], [10]. The calculations we determined were: the average diameter, perimeter and cross-sectional area of the PMF. Also we dteremined the index profile and the index size of these muscle fibers and the proportion of muscle and connective tissue in the histological formations. We used the following mathematical relations:

(1)For the mean diameter of PMF: $D\bar{x}=\frac{LD+SD}{2}$, where: $D\bar{x}$=mean diameter of PMF (µ); LD=large diameter (µ); SD=small diameter of PMF (µ).

(2)For PMF perimeter: $P_{PMF}=\frac{LD+SD}{2}x\pi$, where: P_{PMF}=PMF perimeter; π=3.141592656.

(3)For the format index (FI): FI=LD/SD, where: FI=format index (x/1).

(4)For the profile index (PI): $PI=\frac{SD\ x\ 100}{LD}$, where: PI=profile index (%).

(5)For the cross-section surface (Css): $Css=\frac{LD\ x\ SD}{4}$ x π, where: Css=cross-section surface of PMF (μ^2).

(6)For the proportion of muscular tissue (Pmt): $Pmt=\frac{Nmf\ x\ Cssmf\ x\ 100}{Css\ PMF}$, where: Pmt=proportion of muscular tissue; Nmf=number of muscular fibers (myocytes) of PMF; Cssmf=cross-section surface of muscular fibers (μ^2); Css PMF=cross-section surface of PMF (μ^2).

(7) For the proportion of connective tissue: Ptc=100-Pmt, where: Ptc=proportion of connective tissue.

All data obtained from micrometer measurements and calculations in the microscopic field were tabulated and then analyzed and interpreted statistically. The general statistical estimators used were: the average and standard deviation (error) of mean; strandard deviation; variance and coefficient of variation and finally Fischer ($\hat{F}$) and Tukey (W) values – to test and determine the statistical significance of the differences between the two sexes and also between the three slaughter ages, for all parameters studied. For this study we used ASF (ANOVA SINGLE FACTOR) algorithm included within Microsoft Excel software.

Results and discussions

The avian meat-type hybrid COBB-500 has pectoral muscles that represent 68-71% of the weight of this carcass cutting area (breast with skin and bones). On the chest there are

two superficial pectoral muscles (left and right) and two deep pectoral muscles (left and right).

In these muscles predominate the striated muscle tissue that consists of white fibers (myocytes), but there are several other types of connective tissue (loose, tendon, fascia, and fat). In the myocytes from superficial pectoral muscle the average thickness is of 28.253µ, 35 days, for 42 days 34.729 µ and 37.443 µ to 49 days. These values were found in the male chickens (table 1).

Table 1. The average thickness and myocyte cross-sectional surface of the superficial pectoral muscle in hybrid COBB-500 chickens, depending on age and sex

Specification			Slaughter age and sex of the chickens											
Muscle	Studied parameters of myocytes	MU	35 days				42 days				49 days			
			♂ (M)	♀ (F)	Mean of sexes	±% F/M*	♂ (M)	♀ (F)	Mean of sexes	±% F/M*	♂ (M)	♀ (F)	Mean of sexes	±% F/M*
Pectoralis superficialis	Mean diameter of muscle fibers	µ	28.253	24.752	26.502	-12.39	34.729	29.403	32.066	-15.34	37.443	32.472	34.957	-13.28
	Muscle fibers surface in cross-section	μ^2	605.457	483.801	544.629	-20.09	930.579	681.309	805.944	-26.79	1101.254	843.319	972.286	-23.42

For females, the muscle myocytes studied (PS) have a mean thickness of 24.752 µ at the age of 35 days, of 29.403 µ, at the age of 42 days and of 32.472 µ, at hte age of 49 days (table 1). It is observed that myocytes thickness increases with age in both sexes and is lower in females than in males with 12.39-15.34% (table 1).

Table 2. Statistical estimators for some structural parameters of primary (PMF)* from Pectoralis superficialis muscle of the avian hybrid COBB-500 males, depending on the slaughtering age

Specification			MU	n	Statistical indicators calculated			Limits of variation	
MF	Age	Studied characters			$\bar{x}\pm s_{\bar{x}}$	s	V(%)	Min	Max
Primay muscle fascicle	35 days	Large diameter	µ	30	298.5±9.896	54.205	18.16	225.0	450.0
		Small diameter	µ	30	178.5±8.964	49.099	27.51	105.0	300.0

		PMF Perimeter	µ	30	238.5±7.205	39.461	16.55	172.5	315.0
		Profile index	µ	30	749.27±22.63	123.970	16.55	541.92	989.60
		Format index	x/1	30	1.773±0.096/1	0.528	29.80	1.100/1	3.000/1
		Profile index	%	30	60.96±3.00	16.461	27.00	33.33	90.91
		Number of m.f.**	n	30	36.40±2.15	11.784	32.37	16.0	61.0
		Cross-sect. surface	μ^2	30	42181.7±72734	14975.70	35.50	19792.03	77754.42
		MT proportion	%	30	52.60±1.18	6.475	12.31	39.66	66.24
		CT proportion	%	30	47.40±1.18	6.475	13.66	33.76	60.34
Primay muscle fascicle	42 days	Large diameter	µ	30	409.5±29.733	162.854	39.77	225.0	1020.0
		Small diameter	µ	30	231.0±7.80	42.718	18.49	135.0	315.0
		PMF Perimeter	µ	30	320.25±14.625	80.106	25.01	217.5	600.0
		Profile index	µ	30	1006.095±45.95	251.660	25.01	683.296	1884.956
		Format index	x/1	30	1.867±0.181/1	0.9895	53.00	1.071/1	5.667/1
		Profile index	%	30	63.35±3.93	21.532	33.99	17.65	93.33
		Number of m.f.**	n	30	48.90±3.17	17.377	35.54	23.0	82.0
		Cross-sect. surface	μ^2	30	73277.6±5490	26860.43	36.66	37110.063	144199.10
		MT proportion	%	30	62.61±1.52	8.311	13.27	46.81	79.41
		CT proportion	%	30	37.39±1.52	8.312	22.23	20.59	53.19

Primay muscle fascicle	49 days	Large diameter	µ	30	423.81±722.104	121.068	28.57	207.253	810.99
		Small diameter	µ	30	208.45±410.695	58.577	28.10	99.121	333.407
		PMF Perimeter	µ	30	316.13±612.153	66.566	21.06	189.230	518.132
		Profile index	µ	30	993.17±38.18	209.123	21.06	594.485	1627.761
		Format index	x/1	30	2.201±0.158	0.865	39.31	1.045/1	3.600/1
		Profile index	%	30	53.04±3.87	21.210	39.99	27.78	95.65
		Number of m.f.**	n	30	40.43±2.73	14.954	36.99	15.0	76.0
		Cross-sect. surface	μ^2	30	68874.6±64806	26322.658	38.22	24998.96	143488.92
		MT proportion	%	30	65.50±1.99	10.887	16.62	42.81	91.05
		CT proportion	%	30	34.50±1.99	10.887	31.56	8.95	57.19

*PMF=primaru muscle fascicle (ordin I); **m.f.=muscle fascicle

Muscle fibers (myocytes) are grouped and associated forming primary muscle fascicles (PMF). The PMF of muscle PS have an average thickness of 238.5-240.25 µ at the age of 35 days, 320.25-341 µ at the age of 42 days and 316.136-280.993 µ, at the age of 49 days (tables 2, 3, 4). The difference between the sexes is a minor, at 35 days (+0.73%) and more pronounced at 42 and 49 days (+6.48% -11.12%) (table 4). The slaughter age affected the average thickness of the PMF, so that it is less than 27.6% at 35 days (as average of both sexes) and with less than 9.7% at 49 days compared to 42 days, which is 330.625 µ (as average of both sexes) (table 5). Similarly are evolving the values representing the PMF area (table 4 and 5). In appearance, the PMF are quite flat (slotted), which is shown by the format index values (1.613/1-2.201/1) and those of the profile index (53.04-69.51%), and there were no significant differences based on sex or by age of slaughter of chickens (tables 4, 5).

Regarding the number of myocytes from a PMF, our data show that the females, that have these muscle cells very fine, that is thinner (24.752-29.403-32.472 µ) and their number is higher compared to males. Thus, at the age of 35 days, we found an average of 36.40 myocytes / PMF, for males and 45.30 myocytes / PMF females.

The difference between the sexes is 24.45% for females (table 4). At the age of 42 days the number of myocytes is 48.9/PMF in males and 59.1/PMF, in females, the difference in this case is of 20.86%, more for females (table 4).

At the age of 49 days, the number of myocytes is 40.43/PMF, for males, 36.43/PMF for females, the difference between the sexes is now 9.89%, with a disadvantage for females and obvious for males too (table 4).

Comparing the three ages of slaughter of these chickens, through the number of myocytes from a PMF, we can see that it has grown from 35 to 42 days by 12.5 muscle fibers (34.34%), for the males sex and by 13.8 muscle fibers (30.46%) for females (table 4). If we consider the average of the two sexes, the number of myocytes from a PMF increased by 32.19% between days 35-42 of the chickens studied.

After the age of 42 days, a decrease in the number of myocytes has been observed, and it reached 38.43 mf / PMF, as the average of the two sexes (table 5) at 49 days, and decreased by 28.83% as compared to the age of 42 days (table 5).

Table 3. Statistical estimators for some structural parameters of primary (PMF)* from Pectoralis superficialis muscle of the avian hybrid COBB-500 females, depending on the slaughtering age

Specification			MU	N	Statistical indicators calculated			Limits of variation	
MF	Age	Studied characters			$\bar{x}\pm s_{\bar{x}}$	s	V (%)	Min	Max
Primay muscle fascicle	35 days	Large diameter	µ	30	293.5±19.966	109.357	37.26	135.0	585.0
		Small diameter	µ	30	187.0±7.837	42.932	22.95	120.0	270.0
		PMF Perimeter	µ	30	240.25±11.527	63.134	26.28	127.5	382.5
		Profile index	µ	30	754.768±36.212	198.342	26.28	400.553	1202.66
		Format index	x/1	30	1.613±0.114/1	0.625	38.73	1.000/1	3.250/1
		Profile index	%	30	69.51±3.83	20.962	30.16	30.77	100.00
		Number of m.f.**	n	30	45.3±3.76	20.576	45.42	13.0	96.0
		Cross-sect. surface	μ^2	30	43919.46±359	19666.265	44.78	12723.45	86590.15
		MT proportion	%	30	49.99±0.57	3.115	6.23	44.64	56.16
		CT proportion	%	30	50.01±0.57	3.115	6.23	43.84	55.36
Primay muscle fascicle	42 days	Large diameter	µ	30	433.5±21.688	118.788	27.40	240.0	885.0
		Small diameter	µ	30	248.5±8.377	45.885	18.46	150.0	345.0
		PMF Perimeter	µ	30	341.0±10.239	56.081	16.45	225.0	532.5

		Profile index	µ	30	1071.283±32.167	176.184	16.45	706.858	1672.898
		Format index	x/1	30	1.846±0.154/1	0.843	45.64	1.087/1	4.917/1
		Profile index	%	30	61.32±3.29	18.046	29.43	20.34	92.00
		Number of m.f.**	n	30	59.1±2.17	11.891	20.12	32.0	78.00
		Cross-sect. surface	μ^2	30	81556.727±4438	24308.22	29.81	39584.07	125113.93
		MT proportion	%	30	49.13±0.92	5.017	10.21	41.31	59.12
		CT proportion	%	30	50.87±0.92	5.017	9.86	40.88	58.69
Primay muscle fascicle	49 days	Large diameter	µ	30	354.132±15.221	83.368	23.54	225.275	540.660
		Small diameter	µ	30	207.854±8.14	44.582	21.45	135.165	324.396
		PMF Perimeter	µ	30	280.993±8.445	46.258	16.46	189.231	369.451
		Profile index	µ	30	882.766±26.532	145.325	16.46	594.487	1160.665
		Format index	x/1	30	1.788±0.115/1	0.6319	35.34	1.087/1	3.750/1
		Profile index	%	30	61.72±3.32	18.171	29.44	26.67	92.00
		Number of m.f.**	n	30	36.43±1.89	10.381	28.50	17.0	64.0
		Cross-sect. surface	μ^2	30	57667.67±3094	17590.731	30.50	26529.51	105607.85
		MT proportion	%	30	53.75±0.80	4.3656	8.12	47.87	63.58
		CT proportion	%	30	46.25±0.80	4.3656	9.44	36.42	52.13

*PMF=primaru muscle fascicle (ordin I); **m.f.=muscle fascicle

Table 4. Myocyte thickness and profile of the superficial pectoral muscle in avian hybrid COBB-500, according to sex and age at slaughter

Striated muscle	Parameters studied in PMF	MU	Slaughter age and sex of the studied chikens								
			35 days			42 days			49 days		
			Male (M)	Female (F)	±% F/M*	Male (M)	Female (F)	±% F/M*	Male (M)	Female (F)	±% F/M*
Pectoralis superficialis	Large diameter	µ	298.50	293.50	-1.675	409.50	433.50	+5.86	423.817	354.132	-16.44
	Small diameter	µ	178.50	187.00	+4.76	231.00	248.50	+7.58	208.454	207.854	-0.29
	Average	µ	238.50	240.25	+0.73	320.25	341.00	+6.48	316.136	280.993	-11.12

	diameter										
	PMF Perimeter	μ	749.270	754.768	+074	1006.095	1071.283	+6.48	993.17	882.766	-11.12
	Format index	x/1	1.713/1	1.613/1	-5.84	1.867/1	1.846/1	-1.12	2.201/1	1.788/1	-18.76
	Profile index	%	60.96	69.51	+8.55	63.35	61.32	-2.03	53.04	61.72	+8.68
	Number of m.f.**	N	36.40	45.30	+24.45	48.90	59.10	+20.86	40.43	36.43	-9.89
	Cross-sect. surface	μ^2	42181.77	43919.46	+4.12	73277.65	81556.727	+11.30	68874.66	57667.67	-16.27
	MT proportion	%	52.60	49.99	-2.61pp**	62.61	49.13	-13.48	65.50	53.75	-11.75
	CT proportion	%	47.40	50.01	+2.61pp**	37.39	50.87	+13.48	34.50	46.25	+11.75

*Percentage comparison between females and males; **pp=percentage points.

Primary muscle fascicles (PMF) of the superficial pectoral muscle (SPM), have on their cross-section an oval shape and a surface of this section of 42.182-43.919 μ^2, at the age of 35 days, 73.278-81.557 μ^2 at the age of 42 days and 68.875-57.668 μ^2 to 49 days, with differences between the sexes (table 4) and between the three slaughter ages (table 5).

Table 5. Myocyte thickness and profile of the superficial pectoral muscle in avian hybrid COBB-500, depending on the age of slaughter

Studied muscle	Parameters studied in the myocytes	Slaughter age and sex of the studied chikens			
		35 days			
		Male	Female	Gender average	
		±% A1/A2	±% A1/A2	Average values	±% (pp) A1/A2
Pectoralis superficialis	Large diameter(μ)	-27.11	-32.30	296.00	-29.77
	Small diameter (μ)	-22.73	-24.75	182.75	-23.77
	Average diameter - D$\bar{x}$ (μ)	-25.53	-29.55	239.375	-27.60
	PMF ** perimeter (μ)	-25.53	-29.54	752.019	-27.60
	Profile index - PI (x/1)	-8.25	-12.62	1.693/1	-8.81
	Format index - FI (%)	-2.39	+8.19	65.235	+2.9pp***
	Number of myocytes (n)	-25.56	-23.35	40.85	-24.35
	Cross-section surface (μ^2)	-42.44	-46.15	43050.615	-44.39
	Muscular tissue - MT proportion (%)	-10.01pp***	+0.86	51.295	-4.575pp***

	Connective tissue - CT proportion (%)	+10.01pp***	-0.86	48.705	+4.575pp***
		42 days*			
	Large diameter(μ)	100	100	421.50	100
	Small diameter (μ)	100	100	239.75	100
	Average diameter - $D\bar{x}$ (μ)	100	100	330.625	100
	PMF ** perimeter (μ)	100	100	1038.689	100
	Profile index - PI (x/1)	100	100	1.8565/1	100
	Format index - FI (%)	100	100	62.335	100
	Number of myocytes (n)	100	100	54.0	100
	Cross-section surface (μ^2)	100	100	77417.188	100
	Muscular tissue - MT proportion (%)	100	100	55.87	100
	Connective tissue - CT proportion (%)	100	100	44.13	100
		49 days			
	Large diameter(μ)	+3.50	-18.31	388.974	-7.72
	Small diameter (μ)	-9.76	-16.36	208.154	-13.18
	Average diameter - $D\bar{x}$ (μ)	-1.28	-17.60	298.565	-9.70
	PMF ** perimeter (μ)	-1.29	-17.60	937.968	-9.60
	Profile index - PI (x/1)	+17.89	-3.14	1.9945/1	+7.43
	Format index - FI (%)	-10.31	+0.4pp***	57.38	-4.955pp***
	Number of myocytes (n)	-17.32	-38.36	38.43	-28.83
	Cross-section surface (μ^2)	-6.01	-29.29	63271.165	-18.27
	Muscular tissue - MT proportion (%)	+2.89pp***	+4.62pp***	59.625	+3.755pp***
	Connective tissue - CT proportion (%)	+2.89pp***	-4.62pp***	40.375	-3.755pp***

*Technological slaughter age of chikens taken as standard; **absolute values; ***relative values (±)(pp)

Regarding the proportion of striated muscle tissue (MT) and connective tissue (CT) the data we obtained show values for MT of 52.6% for males and 49.99% for females at the age of 35 days, gender difference being of 2.61 percentage points (pp) (table 4). At the age of 42 days from the PMF, MT had a weight of 62.61%, in males and of 49.13% in females, the gender difference in this case was of 13.48 percentage (table 4). At the age of 49 days, the proportion of MT from PMF of muscle PS was 65.50%, in males and 53.75%, in females, the difference between the sexes was, in this case, of 11.75 pp (table 4). If we compare the proportion of MT, as the average of the two sexes, at the technological age of slaughter of 42

days (55.87%) with the age of 35 and 49 days respectively, we find that it increases by 4.575 percentage points in the range of 35-42 days, and 3.755 percentage in the range of 42-49 days (table 5).

For the studied parameters the differences between the sexes and between the three slaughter ages were tested in terms of their statistical significance and the results of these calculations are presented in tables 6 and 7.

Table 6. Statistical significance of differences between the sexes regarding the structural parameters of primay muscle fascicles from superficial pectoral muscle of COBB-500 meat-type hybrid

Slaughter age	Studied parameters in the muscular fibers	Diferences between the compared sex averages	Tukey values (w=0.01)	Statistical significance	At 1; 58DF, for:			
					p	p≤0.05	p≤0.01	p≤0.001
					Fα	4.008	7.103	12.034
35 days	Large diameter (µ)	M-F*=5.000	59.341	n.s.	$\hat{F}$	0.0503455		
	Small diameter (µ)	M-F=8.500	31.707	n.s.	$\hat{F}$	0.509638		
	Average diameter (µ)	M-F=1.750	36.198	n.s.	$\hat{F}$	0.016575		
	PMF ** perimeter (µ)	M-F=5.498	113.718	n.s.	$\hat{F}$	0.016575		
	Profile index (x/1)	M-F=0.100	0.3978	n.s.	$\hat{F}$	1.15317		
	Format index (%)	M-F=8.55	12.958	n.s.	$\hat{F}$	3.083657		
	Number of myocytes (n)	M-F=8.90	11.528	n.s.	$\hat{F}$	4.226350		
	Cross-section surface (μ^2)	M-F=1737.69	12018.0	n.s.	$\hat{F}$	0.1484112		
	Muscular tissue proportion	M-F=2.61	3.4935	n.s.	$\hat{F}$	3.94295		
	Connective tissue proportion	M-F=2.61	3.4932	n.s.	$\hat{F}$	3.94656		
42 days	Large diameter (µ)	M-F=24.00	98.003	n.s.	$\hat{F}$	0.42528		
	Small diameter (µ)	M-F=17.50	30.480	n.s.	$\hat{F}$	2.33763		
	Average diameter (µ)	M-F=20.75	47.542	n.s.	$\hat{F}$	1.35085		
	PMF ** perimeter (µ)	M-F=65.188	149.358	n.s.	$\hat{F}$	1.350846		
	Profile index (x/1)	M-F=0.021	0.632	n.s.	$\hat{F}$	0.007815		

	Format index (%)	M-F=2.03	13.659	n.s.	$\hat{F}$	0.156936
	Number of myocytes (n)	M-F=10.20	10.237	n.s.	$\hat{F}$	7.039899
	Cross-section surface (μ^2)	M-F=8279.077	17612.997	n.s.	$\hat{F}$	1.5673446
	Muscular tissue proportion	M-F=13.48	4.720	***	$\hat{F}$	57.82804
	Connective tissue proportion	M-F=13.48	4.720	***	$\hat{F}$	57.82805
49 days	Large diameter (μ)	M-F=69.685	71.4678	n.s.	$\hat{F}$	6.742007
	Small diameter (μ)	M-F=0.600	35.789	n.s.	$\hat{F}$	0.0019979
	Average diameter (μ)	M-F=35.143	39.411	n.s.	$\hat{F}$	5.638626
	PMF ** perimeter (μ)	M-F=110.404	123.813	n.s.	$\hat{F}$	5.638639
	Profile index (x/1)	M-F=0.413	0.512	n.s.	$\hat{F}$	4.44437
	Format index (%)	M-F=8.68	13.5788	n.s.	$\hat{F}$	2.895871
	Number of myocytes (n)	M-F=4.00	8.851	n.s.	$\hat{F}$	1.44837
	Cross-section surface (μ^2)	M-F=11206.99	15392.425	n.s.	$\hat{F}$	3.766014389
	Muscular tissue proportion	M-F=11.75	5.703	n.s.	$\hat{F}$	30.111237
	Connective tissue proportion	M-F=11.75	5.703	***	$\hat{F}$	30.111237

*M=male; F=female

Thus, most of the differences between the males and females were found to be statistically significant (at 35 days, 42 days and 49 days of slaughter) (table 6). Exceptions were for the differences regarding the proportion of MT and CT (between males and females) at the age of 42 days and 49 days (CT) (table 6).

Regarding the differences between the three ages, the situation is different and varied, finding statistically significant differences and both distinct and highly statistically significant differences. And this applies to both males and females (table 7).

Table 7. Statistical significance of differences between the three slaughter ages of chickens, regarding the structural parameters of primay muscle fascicles (PMF) from superficial pectoral muscle of COBB-500 meat-type hybrid

Chiken sex	Studied parameters of the muscular fibers	Diferences between the compared slaughter ages	Tukey values (w=0.01)	Statistical significance	At 2; 87 DF, for:			
					p	p≤0.05	p≤0.01	p≤0.001
					Fα	3.1140	4.8945	7.5575
Male (M)	Large diameter (μ)	V_1-V_2=111.00	93.9631	***	F	9.5985		
		V_1-V_3=125.317		***				
		V_2-V_3=14.317		n.s.				
	Small diameter (μ)	V_1-V_2=52.50	39.1703	***	F	8.1427		
		V_1-V_3=29.954		n.s.				
		V_2-V_3=22.546		n.s.				
	Average diameter (μ)	V_1-V_2=81.75	49.8259	***	F	15.38954		
		V_1-V_3=77.636		***				
		V_2-V_3=4.114		n.s.				
	PMF ** perimeter (μ)	V_1-V_2=256.825	155.9158	***	F	228.50383		
		V_1-V_3=243.90		***				
		V_2-V_3=12.925		n.s.				
	Profile index (x/1)	V_1-V_2=0.154	0.6337	n.s.	F	2.262455		
		V_1-V_3=0.488		n.s.				
		V_2-V_3=0.334		n.s.				
	Format index (%)	V_1-V_2=2.39	15.3963	n.s.	F	2.2146985		
		V_1-V_3=7.92		n.s.				
		V_2-V_3=10.31		n.s.				
	Number of myocytes (n)	V_1-V_2=12.50	11.5316	***	F	5.51281		
		V_1-V_3=4.03		n.s.				
		V_2-V_3=8.47		n.s.				
	Cross-section surface (μ^2)	V_1-V_2=31095.88	18109.041	***	F			
		V_1-V_3=26692.89		***				

		V_2-V_3=4402.99		n.s.		15.55119188
	Muscular tissue proportion (%)	V_1-V_2=10.01	6.7777	***	F	17.97314
		V_1-V_3=12.90		***		
		V_2-V_3=2.89		n.s.		
	Connective tissue proportion (%)	V_1-V_2=10.01	6.7776	***	F	17.9707
		V_1-V_3=12.90		***		
		V_2-V_3=2.89		n.s.		
Female (F)	Large diameter (µ)	V_1-V_2=140.0	81.2907	***	F	13.43537
		V_1-V_3=60.632		n.s.		
		V_2-V_3=79.368		n.s.		
	Small diameter (µ)	V_1-V_2=61.50	34.4651	***	F	14.832754
		V_1-V_3=20.854		n.s.		
		V_2-V_3=40.646		***		
	Average diameter (µ)	V_1-V_2=100.75	43.07385	***	F	24.935275
		V_1-V_3=40.743		n.s.		
		V_2-V_3=60.007		***		
	PMF ** perimeter (µ)	V_1-V_2=316.515	135.3205	***	F	24.935266
		V_1-V_3=127.998		n.s.		
		V_2-V_3=188.517		***		
	Profile index (x/1)	V_1-V_2=0.233	0.547592	n.s.	F	0.916424
		V_1-V_3=0.175		n.s.		
		V_2-V_3=0.058		n.s.		
	Format index (%)	V_1-V_2=8.19	14.80492	n.s.	F	1.7533745
		V_1-V_3=7.79		n.s.		
		V_2-V_3=0.40		n.s.		
	Number of myocytes (n)	V_1-V_2=13.80	11.6017	***	F	17.4593439
		V_1-V_3=8.87		n.s.		

		V_2-V_3=22.67		***		
	Cross-section surface (μ^2)	V_1-V_2=37637.267	16174.159	***	F	25.36653536
		V_1-V_3=13748.21		n.s.		
		V_2-V_3=23889.057		***		
	Muscular tissue proportion (%)	V_1-V_2=0.86	3.28529	n.s.	F	10.072585
		V_1-V_3=3.76		***		
		V_2-V_3=4.62		n.s.		
	Connective tissue proportion (%)	V_1-V_2=0.86	3.28529	n.s.	F	10.072593
		V_1-V_3=3.76		***		
		V_2-V_3=4.62		***		

*The chikens slaughter ages were: V_1=35 days; V_2=42 days; V_3=49 days;
**pp=procentual points

Conclusions

1. The average thickness of PMF from PS muscle has values of 238.5-240.25 μ, for the age of 35 days, of 320.25-341 μ, for the age of 42 days and of 316.14-280.99 μ, at 49 days, depending on the sex, and more higher in females than in males, with 0.73% at 35 days and 6.48% at 42 days. At 49 days the average thickness of PMF is reduced by 11.12% in females than in males.
2. The perimeter and the area of PMF cross-section evolves like their average thickness, both regarding the gender and the slaughter age of studied chickens.
3. PMF has an oval appearance, with values of FI from 1.613 / 1 to 2.201 / 1.
4. The number of myocytes from PMF is higher in females than in males, at 35 and 42 days (20.86-24.45%), but is lower at 49 days (9.89%). This is correlated with the smoothness of the myocytes, which is more pronounced in females than in males.
5. The proportion of muscle tissue in the PMF increases with age in both males and females and has values of 52.6-62.61-65.5% for the male chickens and 49.13-49.99-53.75% for the female chickens.
6. The differences between males and females, regarding the proportion of the MT, are of 2.61 percentage points for the slaughter age of 35 days, 13.48 pp. for the age of 42 days, and to 11.75 percentage points for the age of 49 days.
7. Most of the differences between males and females at all three slaughter ages studied were found to be statistically insignificant.
8. The differences between the three investigated slaughter age, sex, both male and female were found to be statistically significant for 53.33% of the studied parameters and distinct and highly statistically significant, for 46.67% of the studied parameters.

Reference

1) Cotea C.V. – 2001 – *Biologie celulară; Histologie; Embriologie generală*, Editura Tehnopress, Iaşi.
2) Cotea C.V. – 2012 – *Histologie şi Embriologie generală şi specială*, Editura Tehnopress, Iaşi.
3) Gheţie V., Chiţescu Şt., Coţofan V., Hillebrand A. – 1976 – *Atlas de Anatomie a păsărilor domestice*, Editura Academieei RSR, pg. 89 – 127.
4) Nickel R., Schummer R., Seiferle E. – 1977 – Anatomy of the domestic birds, Editura Verlog Paul Parey, Berlin, Hamburg.
5) Radu-Rusu R.M., Teuşan V., şi colab. – 2008 – Histometric assessment of the myocytes within some skeletal muscles issued from COBB-500 commercial broilers, Book of prooceedings of 1st Mediteranean Summit of World Poultrz Science Association, Chalkidiki, Greece, pg. 1033 – 1039.
6) Sandu GH – 1995 – *Metode experimentale în Zootehnie*, Editura Coral-Sanivet, Bucureşti.
7) Teuşan V, Radu-Rusu R.M., Teuşan Anca – 2009 – *Investigations on the histological structure of the superficial pectoral muscle in COBB-500 commercial meat-type hibrid hen*, Agricultural Research Stations from Moldavia, ISSN0,79-5837, vol XLII, No 4 (140), pg. 75 – 83.
8) Teuşan V., Anca (Teuşan) Prelipcean, A.A. Prelipcean – 2011 – *Investigations on the histological structure of the gastrocnemius muscles in COBB-500 commercial meat-type hibrid hen*, Lucrări Ştiinţifice U.S.A.M.V.Iaşi, Seria Medicină-Veterinară, vol. 54 (13) nr. 4, ISSN 1454-7406, pg. 248-260.
9) Teuşan V., Anca Prelipcean (Teuşan) A.A. Prelipcean – 2012 – *Research on the thickness and profile of superficial pectoral muscles myocytes of meat type hybrid COBB-500, slaughtered at different ages*, Lucrări Ştiinţifice U.S.A.M.V.Iaşi, Seria Zootehnie, vol. 57 (17), ISSN 1454-7368, pg. 651-657.
10) Teuşan V., Anca Prelipcean (Teuşan) A.A. Prelipcean – 2012 – *Research regarding the surface in transversal section and density of superficial pectoral muscle myocytes of meat-type hybrid COBB-500, slaughtered at different ages*, Lucrări Ştiinţifice U.S.A.M.V.Iaşi, Seria Medicină-Veterinară, vol. 55, ISSN 1454-7368.
11) Tudor Despina, Constantinescu Gh., M., Constantinescu Ileana, Cornilă N. – 2002 – *Nomina Anatomica Veterinaria*, Ediţie bilingvă, Editura Vergiliu, Bucureşti.
12) Tudor Despina, Constantinescu Gh., M., Constantinescu Ileana, Cornilă N. – 2010 – *Nomina Histologica şi Embriologica Veterinaria*, Ediţie bilingvă, Editura Vergiliu, Bucureşti.
13) *** - *COBB-500 – Management Guide* – http://www.cobb-vantress.com
14) *** - *Tehnica histopatologică* – Editura de Stat-RSR, pentru literatura ştiinţifică

RESEARCH ON THE THICKNESS AND PROFILE OF PROFOUND PECTORAL MUSCLE MYOCYTES OF MEAT TYPE HYBRID COBB-500, SLAUGHTERED AT DIFFERENT AGES

V. Teusan*; Anca Prelipcean (Teusan)*
***U.S.A.M.V. IASI**
vasile.teusan@yahoo.com

Abstract

We collected histological samples of deep pectoral muscle (PP) from 30 individuals belonging to the meat type avian hybrid COBB-500, males and females, which were sacrificed at the ages of 35, 42 and 49 days. These samples were processed by paraffin sectioning technique, yielding 60 slides that have been studied with a photonic binocular type of microscope- MC_3. The microscopic field was measured by using a micrometer objective to determine the large and small diameters of 1350 myocytes (muscle fibers). We also determined the average diameter and the profile and format indices of the 1350 myocytes. All data obtained from measurements and calculations were statistically processed and interpreted. The following results were obtained: at the age of 35 days, the average thickness of the muscle myocytes was 25.355±0.318μ, for males and 22.925±0.271μ, for females (v=17.74-18.82%)(table 1); at the age of 42 days the PP muscle myocytes had an average thickness of 30.734±0.417μ, for males and 26.056±0.315μ, for females; at the age of 49 days the average diameter of myocytes was 33.288±0.335μ, for males and 29.68±0.38μ, for females. The appearance of myocytes was cilindroid because both profile and format indices had similar values for the two sexes and the three slaughter ages (1.304/1-1.378/1)(75.50-79.66%). The differences between males and females at all 3 slaughter ages and the differences between the ages of the two sexes, for the mean diameter were found to be very significant statistically.

Key words: *COBB-500; deep pectoral, myocytes, thickness, profile.*

Introduction

Since it has a very high trofico-biological value, meat is situated on one of the top spots on the list of foods of animal origin intended for human consumption. It is obtained Animal Husbandry by considerably economic, scientific and technical efforts from species, breeds and hybrids that are becoming more and more efficient. At the same time consumers want to have healthy and safe products that will not jeopardize their biological integrity or affect their health. Poultry meat, especially chicken meat, which is produced today in prepeonderent, specializing in hybrids, is increasingly appreciated and requested by consumers and food industry profile.

Of all the avian hybrids specialized for meat production, the one that it has been used more frequently is the common hybrid COBB-500, which is known for its bio-economic performance [13].

However in terms of meat quality obtained from this hybrid, there are plenty unknown date and uncertainties.

Thus, the structure of the pectoral muscles (superficial and deep), the thickness, density and their component myocytes profile is little or no at all known [7], [10]. It is known that these histological parameters of somatic muscles affects all physical, chemical, organoleptic and technological properties of the meat from the carcasses of slaughtered animals including birds [5].

Our work attempts to bring new data on the thickness and profile of the deep pectoral muscle myocytes of COBB-500 meat-type hybrid sacrificed at ages of 35, 42 and 49 days.

Material and methods

Conducting this research required the use of a specific diversified material that included 30 individuals belonging to meat-type hybrid COBB-500 of both sexes and different ages; specific anatomical and histological tools, various equipment, reagents and dyes. This hybrid chickens were sacrificed at the ages of 35, 42 and 49 days, when they had a body weight of 1406-1614 grams (35 days), of 2168-2296 grams (42 days) and of 2350 to 2378 grams (49 days). Lower values belong to the females and the largest to males [7], [10]. From carcasses that were obtained at slaughter some histological samples were taken from the middle of the deep pectoral muscle (*Pectoralis profundus*) (PP) [1] [3] [4], [11], which were processed by the paraffin sectioning technique [2], [14]. We obtained 60 histological slides having on them cross-sections prepared from the medial area of the deep pectoral muscle colored with hematoxylin and eosin (HE). The slides prepared were studied using a photonic binocular microscope MC_3 type, equipped with cyto and histometric devices (micrometers eyepieces and objectives), which has been previously adjusted and calibrated to 3 ocular associations (OC) and objective (OB). These associations were: OC10XOB6; OC10XOB10; OC10xOB20, and micrometer values (MV) were calculated: 15.00 μ, 9.011 μ and 4.441 μ.

In the microscopic field were found most successful images and we have measured the large and small diameters of muscle cells, performing a total of 1350 x 2 = 2700 measurements.

The values measured (using micrometer divisions) were amplified with some micrometric values (MV) of the MC_3 microscope, with which measurements were made. We used the following mathematical relation:

(1) LD (μ) = n x MV, where: LD=large diameter (μ); n=number of micrometric divisions; MV=micrometric value (μ);

(2) SD (μ) = n x MV, where: SD=small diameter (μ);

(3) $D\bar{x} = \frac{LD+SD}{2}$, where: $D\bar{x}$=average diameter (μ);

(4) $PI = \frac{LD}{SD}$, where: PI=profile index of myocytes (x/1);

(5) $FI\ (\%) = \frac{SD\ x\ 100}{LD}$, where: FI=format index of myocytes (%).

All data obtained from measurements and calculations in the microscopic field were tabulated and then analyzed and interpreted statistically.

We calculated the usual statistical estimators (statistic mean and standard error of mean, standard deviation, variance and coefficient of variation), also Fisher and Tukey (W), test values ($\hat{F}$), the latter to test the statistical significance of differences between the sexes and between the two slaughter ages, for the characters studied.

All statistical calculations were made using ANOVA Single Factor algorithm, which has been included within Microsoft Office - Excel [6]. For all studied traits were made comparisons between males and females and between the three ages of slaughtering and the data are represented graphically and in tables for most of these characters.

Results and discussions

Birds have an important pectoral muscle in carcass weight. For commercial hybrid chickens for meat COBB-500, research (T.Fl. Apostol, 2009) have shown that chest (muscle, bone and skin) are from 28.82 to 29.64% of carcass weight and weight of chest (the region of

cutting of carcasses), pectoral muscles, ie the two pectoral muscles, superficial and deep, represent 67.54 to 71.51%.

Of the total weight of the pectoral muscle, the avian hybrid COBB-500 deep pectoral muscle represents about 15 - 20% [10].

From the structural point of view deep pectoral muscles consist of striated muscle tissue and several kinds of connective tissue (loose, fatty, fibrous) plus blood, nerves and epithelium, the latter being in small quantities. Therefore, it is mostly striated muscle tissue, which has as a morpho-functional unit the myocyte (rabdocite) or muscle fiber [2]. The shape of these cells is cilindroid with ovoid extremities and their thickness is very variable being influenced, as the research conducted to date [5], [7], [8], [9], [10], by sex and age.

Thus, our research confirms these statements, so they will be discussed in the following.

The male chickens, slaughtered at the age of 35 days had cilindroid deep pectoral muscle myocytes (PM) which is shown by the average specific indices, namely the profile index PI = 1.378 ± 0.017 / 1 and FI, the format index = 79.66 ± 0.99% (v = 18.82 to 18.96%) (table 1).

The large diameter of these myocytes has an average value of 29.115 ± 0.378 μ (v = 19.96%), the small diameter of these cells is 21.596 ± 0.314 μ (v = 21.84%) and the average diameter for the 225 fibers measured in the muscles, is 25.355 ± 0.31 μ (v = 18.82%) (table 1). The female chickens, sacrificed at the age of 35 days, have myocytes with a large diameter of 25,843 ± 0,295 μ, a small diameter of 20.008 ± 0.308 μ and an average diameter of 22,925 ± 0,271 (v = 17.12%; 23.11%, 17.74%) (table 1). As for their appearance, these muscle fibers are all cilindroide so that PI = 1.333 ± 0.018 / 1 and FI = 77.83 ± 0.92% (v = 17.65 to 20.46%) (table 1).

The male chickens slaughtered at 42 days (which is the technological age that this avian hybrid it is slaughtered) has PM myocytes with a large diameter of 34.703 ± 0.486 μ (v = 21.01%), a small diameter of 26 783 ± 0.41 μ (v = 22.97%) and an average diameter of 30.743 ± 0.417 μ (v = 20.36%) (table 1). And here the muscle fibers (myocytes) retains the cilindroid look (PI = 1.320 ± 0.016 / 1, FI = 77.91 ± 0.83%) (table 1).

Table 1. Statistical estimators for myocyte thickness and profile of the deep pectoral muscle in the avian hybrid COBB-500, depending on age and sex

Specification			MU	N	Statistical indicators calculated			Limits of variation	
Slaughter age	Chicken sex	Parameters studied in the muscle fibers			$\bar{x}\pm s_{\bar{x}}$	s	V(%)	Min	Max
35 days	Male	Large diameter	μ	255	29.115±0.387	5.8100	19.96	13.323	42.190
		Small diameter	μ	255	21.596±0.314	4.7157	21.84	8.882	31.087
		Average diameter	μ	255	25.355±0.318	4.7707	18.82	11.546	35.528
		Profile index	x/1	255	1.378±0.017/1	0.26124	18.96	1.000/1	2.500/1
		Format index	%	255	79.66±0.999	14.9875	18.82	40.00	100.00

	Female	Large diameter	µ	255	25.843±0.295	4.4236	17.12	13.323	33.307
		Small diameter	µ	255	20.008±0.308	4.6230	23.11	8.882	31.087
		Average diameter	µ	255	22.925±0.271	4.0661	17.74	12.212	31.087
		Profile index	x/1	255	1.331±0.018/1	0.2724	20.46	1.000/1	2.500/1
		Format index	%	255	77.83±0.92	13.7395	17.65	40.00	100.00
42 days	Male	Large diameter	µ	255	34.703±0.486	7.2915	21.01	15.543	48.851
		Small diameter	µ	255	26.783±0.41	6.1532	22.97	12.435	39.969
		Average diameter	µ	255	30.743±0.417	6.2591	20.36	14.433	42.189
		Profile index	x/1	255	1.320±0.016/1	0.2375	17.99	1.000/1	2.500/1
		Format index	%	255	77.91±0.834	12.5120	16.06	40.00	100.00
	Female	Large diameter	µ	255	29.628±0.38	5.70166	19.24	13.323	39.969
		Small diameter	µ	255	22.483±0.313	4.6937	20.88	8.882	33.307
		Average diameter	µ	255	26.056±0.315	4.7259	18.14	11.768	35.528
		Profile index	x/1	255	1.343±0.016/1	0.2415	17.98	1.000/1	2.400/1
		Format index	%	255	76.68±0.85	12.7590	16.64	41.67	100.00
49 days	Male	Large diameter	µ	255	37.423±0.405	6.0689	16.22	22.205	53.292
		Small diameter	µ	255	29.154±0.35	5.2489	18.00	17.764	48.851
		Average diameter	µ	255	33.288±0.335	5.0257	15.10	22.204	51.072
		Profile index	x/1	255	1.304±0.015/1	0.2185	16.76	1.000/1	2.111/1
		Format index	%	255	78.67±0.82	12.2304	15.55	47.368	100.00
	Female	Large diameter	µ	255	33.951±0.446	6.6922	19.71	15.543	48.851
		Small diameter	µ	255	25.408±0.373	5.6025	22.05	13.323	39.969
		Average diameter	µ	255	29.68±0.38	5.6965	19.19	14.433	42.189
		Profile index	x/1	255	1.358±0.014/1	0.2169	15.97	1.000/1	2.000/1
		Format index	%	255	75.50±0.79	11.8517	15.70	50.00	100.00

For the females slaughtered at the same technological age of 42 days, the muscle myocytes studied (PM) had a large diameter of 29.628 ± 0.38 µ (v = 19.24%), a small diameter of 22.483 ± 0.313 µ (v = 20.88%) and an average diameter of 26.056 ± 0.315 µ (v = 18.14%). The average values found for PI = 1.343 ± 0.016 / 1 and FI = 76.68 ± 0.85% (table 1) emphasizes the cilindroid format of these striated muscle cells.

At slaughter age of 49 days, which is 16.67% higher than the technological age, males deep pectoral muscle myocytes have a large diameter of 37.423 ± 0.405 μ. The small diameter of such myocytes was 29.154 ± 0.35 μ, and the average was 33.288 ± 0.335 μ (v = 16.22%, 18%, 15.10%) (table 1). The values of format and profile indices (FI = 78.67%) (PI = 1.304/1) in this case confirms that these cilindroide myocytes are very similar to those observed at other ages. In females slaughtered at an age of 49 days, the thickness of myocytes is 33.951 ± 0.446 μ, for the large diameter, values of 25.408 ± 0.373 μ for the small diameter and of 29.68 ± 0.38 μ, for the average diameter (v = 19.71%, 22.05%, 19.19%) (table 1). Here, their appearance is cilindroid (table 1). Comparing the males with females at all three studied traits and slaughter ages, we find the existence of significant differences that are calculated and presented in table 2.

Table 2. Myocyte thickness and profile of the deep pectoral muscle in avian hybrid COBB-500, according to sex and age at slaughter

Striated muscle	Parameters studied in the myocytes	MU	Slaughter age and sex of the studied chikens								
			35 days			42 days			49 days		
			Male (M)	Female (F)	±% F/M*	Male (M)	Female (F)	±% F/M*	Male (M)	Female (F)	±% F/M*
Deep Pectoralis	Large diameter	μ	29.115	25.843	-11.24	34.703	29.628	-14.62	37.423	33.951	-9.28
	Small diameter	μ	21.596	20.008	-7.35	26.783	22.483	-16.05	29.154	25.408	-12.85
	Average diameter	μ	25.355	22.925	-9.58	30.743	26.056	-15.25	33.288	29.680	-10.84
	Profile index	x/1	1.378/1	1.331/1	-3.41	1.320/1	1.343/1	+1.74	1.304/1	1.358/1	+4.14
	Format index	%	79.66	77.83	-1.83pp**	77.91	76.68	-1.23pp**	78.67	75.50	-3.17pp**

*Percentage comparison between females and males; **pp=percentage points

Table 3. Myocyte thickness and profile of the deep pectoral muscle in avian hybrid COBB-500, depending on the age of slaughter

Studied muscle	Parameters studied in the myocytes	Slaughter age and sex of the studied chikens																	
		35 days						42 days*						49 days					
		Male		Female		Gender average		Male		Female		Gender average		Male		Female		Gender average	
		A.v.**	R.v.***	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.	A.v.	R.v.

Deep Pectoralis	LD (μ)	29.115	-16.10	25.843	-12.77	27.479	-14.57	34.703	100.0	29.628	100.0	32.165	100.0	37.423	+7.84	33.951	+14.59	35.687	+10.95
	SD(μ)	21.596	-19.37	20.008	-11.01	20.802	-15.55	26.783	100.0	22.483	100.0	24.633	100.0	29.154	+8.85	25.408	+13.01	27.281	+10.75
	D(μ)	25.355	-17.33	22.925	-12.02	24.140	-15.00	30.743	100.0	26.056	100.0	28.400	100.0	33.288	+8.28	29.680	+13.91	31.484	+10 86
	PI(x/1)	1.378/1	+4.39	1.331/1	-0.89	1.354/1	+1.73	1.320/1	100.0	1.343/1	100.0	1.331/1	100.0	1.304/1	-1.21	1.358/1	+1.12	1.331/1	-
	FI (%)	-	79.66	-	77.83	-	78.74	-	77.91	-	76.68	-	77.29	-	78.67	-	75.50	-	77.09

*Technological slaughter age of chikens taken as standard; **absolute values; ***relative values (±)(pp)

Thus, at the pretechnological age of 35 days, the thickness of the muscle myocytes of PM was lower in females than in males, the percentage differences were of 11.24% for large diameter, 7.35% for small diameter and 9.58% for the average diameter (table 2).

When the slaughter was done at the technological age of 42 days the differences between males and females were accentuated, so they are now 14.62% for large diameter of 16.05% for small diameter and of 15.25% for the average diameter (table 2).

Table 4. Statistical significance of differences between the sexes, the thickness and profile of the deep pectoral muscle myocytes of COBB-500 hybrid

Slaughter age	Studied parameters in the muscular fibers	Diferences between the compared sex averages	Tukey values (w=0.01)	Statistical significance	At 1; 448DF, for:			
					p	p≤0.05	p≤0.01	p≤0.001
					Fα	3.840	6.640	10.830
35 days	Large diameter (μ)	M-F*=3.272	1.2530	***	$\hat{F}$	45.16685		
	Small diameter (μ)	M-F=1.588	1.1332	***	$\hat{F}$	13.00958		
	Average diameter (μ)	M-F=2.430	1.0756	***	$\hat{F}$	33.80718		
	Profile index (x/1)	M-F=0.047	0.06476	n.s.	$\hat{F}$	3.47812		
	Format index (%)	M-F=1.83	3.2747	n.s.	$\hat{F}$	4.963450		
42 days	Large diameter (μ)	M-F=5.075	1.588265	***	$\hat{F}$	67.62376		
	Small diameter (μ)	M-F=4.300	1.32796	***	$\hat{F}$	69.46060		
	Average diameter (μ)	M-F=4.687	1.345767	***	$\hat{F}$	80.3640455		
	Profile index (x/1)	M-F=0.023	0.05813	n.s.	$\hat{F}$	1.01596		
	Format index (%)	M-F=1.23	3.06637	n.s.	$\hat{F}$	1.074818		

49 days	Large diameter (µ)	M-F=3.472	1.55021	***	$\hat{F}$	33.229289
	Small diameter (µ)	M-F=3.746	1.31733	***	$\hat{F}$	53.554548
	Average diameter (µ)	M-F=3.608	1.3035	***	$\hat{F}$	50.774187
	Profile index (x/1)	M-F=0.054	0.05283	**	$\hat{F}$	6.83645
	Format index (%)	M-F=3.170	2.92233	**	$\hat{F}$	7.815444

*M=male; F=female

Finally, when the slaughter was done at an older age ie 49 days, the thickness of the muscle myocytes studied (PM), again recorded differences between females and males, which are now 9.28% for large diameter, 12.85% for the small diameter and 10.84%, for the average diameter (table 2). These differences found between females and males have been statistically tested which showed the fact that they are very significant ($\hat{F}$>Fα) (D>$W_{0,01}$) for all the 3 diameters studied (large, small and average) and for all the three slaughter age stages (table 4). The differences regarding PI and FI at the ages of 35 and 42 days are statistically insignificant ($\hat{F}$<Fα) (D<$W_{0,01}$) (table 4).

The differences between PI and FI at the age of 49 days are significantly distinct ($\hat{F}$>$F\alpha_{0,01}$) (D>$W_{0,01}$),(table 4). When comparison was made between the three slaughter ages, taking as standard the technological age of 42 days and was calculated the average of the two sexes were also found significant differences in all parameters studied. Thus, if we consider a the mean of sexes for the large diameter of the PM myocyte, it is µ 27.479 µ at 35 days, with 14.57% lower than the same at 42 days (29.628 µ) (table 3).

If we consider the average of the sexes, for the small diameter of the myocytes, which is 20.802 µ, at 35 days, and 24.633 µ at 42 days, the difference being in this case by 15.55% (table 3).

Finally when we talk about the average diameter of myocytes we have a mean value of sexes of 24.140 µ at 35 days and one of 28.400 µ for the age of 42 days. In this case the difference is 15.00% (table 3).

Table 5. Statistical significance of differences between the three slaughter ages, regarding the thickness and profile of the deep pectoral muscle myocytes of COBB-500 hybrid

Chiken sex	Studied parameters of the muscular fibers	Diferences between the compared slaughter ages	Tukey values (w=0.01)	Statistical significance	At 2; 672DF, for:			
					p	p≤0.05	p≤0.01	p≤0.001
					Fα	2.990	4.600	6.910
Male (M)	Large diameter (µ)	V_1-V_2=5.588	1.764113	***	$\hat{F}$	97.85871705		
		V_1-V_3=8.308		***				
		V_2-V_3=2.720		***				
	Small diameter (µ)	V_1-V_2=5.187	1.484654	***	$\hat{F}$	115.06056		

		V_1-V_3=7.558		***		
		V_2-V_3=2.371		***		
	Average diameter (µ)	V_1-V_2=5.388	1.480764	***	$\hat{F}$	127.00784
		V_1-V_3=7.933		***		
		V_2-V_3=2.545		***		
	Profile index (x/1)	V_1-V_2=0.058	0.06585	n.s.	$\hat{F}$	5.885075
		V_1-V_3=0.074		**		
		V_2-V_3=0.016		n.s.		
	Format index (%)	V_1-V_2=1.750	3.47997	n.s.	$\hat{F}$	5.269192
		V_1-V_3=0.990		n.s.		
		V_2-V_3=0.760		n.s.		
Female (F)	Large diameter (µ)	V_1-V_2=3.785	1.560724	***	$\hat{F}$	114.694327
		V_1-V_3=8.108		***		
		V_2-V_3=4.323		***		
	Small diameter (µ)	V_1-V_2=2.475	1.37142	***	$\hat{F}$	65.95105
		V_1-V_3=5.400		***		
		V_2-V_3=2.925		***		
	Average diameter (µ)	V_1-V_2=3.131	1.33920	***	$\hat{F}$	108.131181
		V_1-V_3=6.755		***		
		V_2-V_3=3.624		***		
	Profile index (x/1)	V_1-V_2=0.012	0.0672	n.s.	$\hat{F}$	0.674148
		V_1-V_3=0.027		n.s.		
		V_2-V_3=0.015		n.s.		
	Format index (%)	V_1-V_2=10150	3.517556	n.s.	$\hat{F}$	1.871344
		V_1-V_3=2.330		n.s.		
		V_2-V_3=1.180		n.s.		

*The chikens slaughter ages were: V_1=35 days; V_2=42 days; V_3=49 days;
**pp=procentual points

Regarding the values of PI and FI, as an average of the two sexes (1.354/1, 78.74%) at 35 days and at 42 days (1.331/1, 77.29%), they are significantly close , which proves that this character, the profile and format indices of these myocytes, it is not influenced by age (table 3). At the age of 49 days, the average of the two sexes, for the large diameter of myocytes was of 35.687 μ value that is higher than the one of the age of 42 days, with 10.95% and is higher by 29.87 % compared to the 35 days (table 3).

For the small diameter of the muscle cells, the average gender for the age of 49 days, is 27.281 μ, that is 10.75% greater than the age of 42 days, and 31.15% greater than that of the age 35 days (table 3). For the average diameter of myocytes, gender average at the age of 49 days is 31.484 μ, this value being 10.86% higher than that found at the age of 42 days, and 30.42% greater than that of the age of 35 days (table 3).

Regarding the values for PI and FI, the average of sexes at the age of 49 days (1.331/1, 77.09%) are very close to those found at the ages of 35 and 42 days, which confirms that character is not influenced by age (table 3).

The differences found between the three slaughter ages for the characters studied in the myocytes (in PM) were tested for their statistical significance, for both sexes, male and female (table 5). Thus, for the males, the differences between A1 (35 days) and A2 (42 days) and the A2 and A3 (49 days), for the profile index (PI) is not statistically significant (n.s.) ($\hat{F}$<Fα) (D<$W_{0,01}$) (table 5).

The differences between the three ages for the format index (FI) are also statistically significant ($\hat{F}$<Fα) (D<$W_{0,01}$). All the other differences tested for the thickness of myocytes are statistically significant ($\hat{F}$>Fα) (D>$W_{0,01}$) (table 5). To females we found to be statistically insignificant all the differences between ages, both for the profile index and the format index ($\hat{F}$<Fα) (D<$W_{0,01}$) (tabelul 5).

The other differences tested, for large, small and average diameter of myocytes were found to be very statistically significant ($\hat{F}$>Fα) (D>$W_{0,01}$) (tabelul 5).

Conclusions

1) At the age of 35 days, the average diameter of the deep pectoral muscle myocytes was: 25.355 μ for males to 22.925μ for females and 24.140 as the average of both sexes.
2) At the age of 42 days, the average diameter of the deep pectoral muscle myocytes was: 30.743 μ, for males, of 26.056μ, for females and 28.400 μ as the average of both sexes.
3) At the age of 49 days, the average diameter of the deep pectoral muscle myocytes was: 33.288 μ, for males, of 29.680μ, for females and 31.484 μ as the average of both sexes.
4) The average diameter of myocytes in the deep pectoral muscle is lower in females than in males with: 9.58%, at the age of 35 days, with 15.25% at the age of 42 days and 10.84% , at the age of 49 days.
5) In all three slaughter ages studied (35, 42, 49 days), the differences between males and females, regarding the thickness of the deep pectoral muscle myocytes are highly statistically significant.

6) The profile of deep pectoral muscle myocytes, in both males and females, is low cilindroid, tested differences being mostly statistically insignificant.
7) The myocytes thickness from the middle area of deep pectoral muscle, for avian meat hybrid COBB-500, increases with age, both in males (25.355µ-30.743µ-33.288µ) and females (22,925µ-26,056µ-29,680 and also the average of both sexes (24.140µ-28.400µ-31.484µ).
8) The increase in the thickness of the muscle myocytes studied (PM) is 17.65% at the age of 42 days, and 30.42% at the age of 49 days, compared to the age of 35 days.

References

1. COŢOFAN V. şi colab. – 2000 – *Anatomia animalelor domestice*, vol I, Editura Orizonturi Universitare, Timişoara, pg. 263 – 270.
2. COTEA C.V. – 2001 – 2012 – *Biologie celulară; Histologie; Embriologie generală*, Editura Tehnopress, Iaşi.
3. GHEŢIE V., CHIŢESCU ŞT., COŢOFAN V., HILLEBRAND A. – 1976 – *Atlas de Anatomie a păsărilor domestice*, Editura Academieei RSR, pg. 89 – 127.
4. NICKEL R., SCHUMMER R., SEIFERLE E. – *Anatomy of domestic birds*, Editura Varlog Paul Parey, Berlin Hamburg.
5. RADU-RUSU R.M., TEUŞAN V. şi colab. – 2008 – *Histometric assessment of the myocytes within some skeletal muscles issued from COBB-500 commercial broilers*, Book of prooceedings of 1st Mediteranean Summit of World Poultrz Science Association, Chalkidiki, Greece, pg. 1033 – 1039.
6. SANDU GH – 1995 – *Metode experimentale în Zootehnie*, Editura Coral-Sanivet, Bucureşti.
7. TEUŞAN V, RADU-RUSU R.M., TEUŞAN ANCA – 2009 – *Investigations on the histological structure of the superficial pectoral muscle in COBB-500 commercial meat-type hibrid hen*, Agricultural Research Stations from Moldavia, ISSN0,79-5837, vol XLII, No 4 (140), pg. 75 – 83.
8. TEUŞAN V., A.A. PRELIPCEAN, ANCA PRELIPCEAN – 2011 – *Cercetări privind grosimea şi densitatea miocitelor din muşchii gemeni ai gambei, la hibridul comercial de găină pentru carne – COBB-500*, Agricultural Research Stations from Moldavia, ISSN0,79-5837, vol XLII, No 3 (147/2011), pg. 89 – 102.
9. TEUŞAN V., ANCA (TEUŞAN) PRELIPCEAN, A.A. PRELIPCEAN – 2011 – *Investigations on the histological structure of the gastrocnemius muscles in COBB-500 commercial meat-type hibrid hen*, Lucrări Ştiinţifice U.S.A.M.V.Iaşi, Seria Medicină-Veterinară, vol. 54 (13) nr. 4, ISSN 1454-7406, pg. 248-260.
10. TEUŞAN V., ANCA PRELIPCEAN (TEUŞAN) A.A. PRELIPCEAN – 2012 – *Research on the thickness and profile of superficial pectoral muscles myocytes of meat type hybrid COBB-500, slaughtered at different ages*, Lucrări Ştiinţifice U.S.A.M.V.Iaşi, Seria Zootehnie, vol. 57 (17), ISSN 1454-7368, pg. 651-657.
11. TUDOR DESPINA, CONSTANTINESCU GH., M., CONSTANTINESCU ILEANA, CORNILĂ N. – 2010 – *Nomina Histologica şi Embriologica Veterinaria*, Ediţie bilingvă, Editura Vergiliu, Bucureşti.
12. *** - *COBB-500 – Management Guide* – http://www.cobb-vantress.com
13. *** - *Tehnica histopatologică* – Editura de Stat-RSR, pentru literatura ştiinţifică

THE IMPORTANCE OF EUROPEAN FUNDS IN VETERINARY MEDICINE

Cătălin Florin Pandelaş, Alexandru Bogdan Viţălaru
University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Faculty of Veterinary Medicine, 8, Aleea Sadoveanu, 700489 – Iasi, Romania**,**
cpandelas@yahoo.com; alexandrumv@yahoo.com

Abstract

This article aims to discuss the issue of financing the veterinary medicine activities and the consequences of these funds on human beings, not only on animals. European Union gives importance to animal health and veterinary medicine,as we can observe from the amount of money reserved for this policy area: 541.09 Million of euros. In fact the main problem that Romania is facing is the low absorption rate level of EU funds, so, through this articlewe intend to demonstrate the importance of attracting not only allocating European funds in veterinary medicine. The third axis of EAFRD (European Agricultural Fund for Rural Development)- through the 312 Measure is one of the few funds that facilitates the transformation and modernization of agriculture, forestry and veterinary medicine. EAFRD maintains the quality of the rural environment in Romania, which may ensure an adequate economic and social living standard.

Key Words:*312Measure. EAFRD, EU. Structural Funds, Veterinary medicine*

Introduction

Veterinary medicine is for sure a human health activity. All veterinary scientists activities affect human health either directly through biomedical research or indirectly by addressing domestic animal,wildlife, or through activities related to environmental health. Moreover, veterinary scientists have the responsibility to protect human health and well-being by ensuring food security and safety, protecting environments and ecosystems.This is the reason why attracting funds and research, are both very important in this domain.

Materials and methods

The third Axis of European Agricultural Fund for Rural Development (hereinafter the EAFRD) through the 312 Measure, is the one that finances activities related to veterinary medicine and agriculture. Operations to which it contributes must be compatible with the other Community policies and comply with all Community legislation. Each Member State should prepare its rural development national strategy plan constituting the reference framework for the preparation of the rural development programmes. Member States and the Commission should report on the monitoring of the national and Community strategy. In Romania, the National Strategy Plan is centred on three key challenges:

- facilitate the transformation and modernization of the dualistic structure of agricultureand forestry,
-contribute to growth and income convergence in rural areas (where possible), while ensuring the living conditions and environmental protection of these areas.
- to maintain and enhance the quality of the rural environment in Romania.
- manage and facilitate the movement of labour out of agriculture into other sectors thatcan ensure a adequate economic and social living standard.

The strategy will require investment across the four axes of the NRDP:

(a) Axis 1 –Improving the competitiveness of the agriculture and forestry sector (43.95% ofEAFRD allocation for the 4 axes).

(b) Axis 2 – Improving the environment and the countryside (26.05% of EAFRD allocation for the 4 axes).
(c) Axis 3 – The quality of life inrural areas and the diversification of the rural economy (27.40% of EAFRD allocation for the4 axes).
(d) Axis 4 – LEADER (2.6% of EAFRD allocation for the 4 axes).

The support granted through Axis 3, the one that includes 312 Measure, aims to encourage the diversification of the rural economy and through this to improve the quality of life in the rural environment.

There are three strategic objectives being identified for Axis 3, under the NSP: maintenance and development of economic activities through the increase of number of jobs; increasing the attractiveness of rural areas; developing the abilities and raising the awareness of local stakeholders regarding the importance of local governance. Regarding Axis 3,we will focus on Measure 312, because it is the one that ensures funds for veterinary medicine. Named "Support for the Creation and Development of Micro-enterprises" – "Improving the Quality of Life in Rural Areas and Diversification of the Rural Economy" this measure has as general objective the durable development of rural economy by encouraging non-agricultural activities for the purpose of increasing the number of jobs and additional incomes.

Specific objectives of Measure 312 refer to: creating and maintaining jobs in the rural area; increasing the added value in non-agricultural activities; creating and diversifying services provided by micro-enterprises for the rural population.

Total allotted funds for Measure 312,from EAFRD, amount to 395,147,628 Euros out of which: Romanian Government's contribution–20% and European Union's contribution – 80%. For creation and development of micro-enterprises, over Euro 383 million are available, non-reimbursable funds that may be accessed by 2013. The non-reimbursable funds may be fully used before 2013, given that an extremely high interest has been shown for this type of investments.

Regarding the budget, Council Decision 2009/470/EC of 25 May 2009 on expenditure in the veterinary field brings together all Community financial measures for the eradication and monitoring of animal diseases which involve compulsory Community budget expenditure and confers upon the Commission the task of taking the necessary applicatory measures. These specific measures relate to: emergency measures; campaigns against different disease; measures for the protection of animals; technical and scientific measures. Also, by the end of 2016 is announced to be designed specific legislation regarding animal health. Although theoretically many rules are settled on the EU budget, in fact, a more detailed analysis on the amount allocated to Health and Consumer Policy (category that includes funds allocated to veterinary medicine) shows us a big difference between commitments (541.65 million) and payments (91.76 million) made to this policy, the reason for this large difference is the low absorbption rate of funds. Being the second poorest country in the EU, Romania continues to fail with the absorption of EU funds. At around 7.4% it is the state with the lowest absorption rate in the European Union. What are the main reasons for the failure to absorb the EU's structural funds? Analysts point to corruption, a lack of motivation and information, inadequate administrative capacity and major gaps in understanding how EU institutions work.

Another important notice in veterinary medicine it's the establishment of a new "Animal Health Strategy" for the European Union (2007-2013) "Prevention is better than cure", and regarding this decision it is belived that animal health is a concern for all European

citizens. This concern stems from the public health and food safety aspects of animal health but also from the economic costs that animal disease outbreaks can trigger and the animal welfare considerations, including the implications of disease control.

Conclusions

Fast strides made in terms of legislation and bureaucracy are not enough, It would be more effective if EU would allocate more money for research and development in this field.In this way,it will be much easier to control animal disease and also will decrease investments made in consumer protection.

Therefore it would be desirable a higher access level of EU funds . There is a quoatesaying :" God gives you but he is never stuffing it into your bag". This quote it's available in Romania when it comes about rural areas where information is not always easily accessible.

References

1. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32009D0470:EN:T
2. http://bookshop.europa.eu/en/the-value-of-eu-agricultural-landscapepbLFNA24868/
3. http://ec.europa.eu/food/animal/index_en.htm
4. http://ec.europa.eu/agriculture/cap-funding/beneficiaries/shared/index_en.htm
5. http://ec.europa.eu/budget/biblio/documents/2013/2013_en.cfm

RECOGNITION OF ACADEMIC DIPLOMAS IN VETERINARY MEDICINE (FOR EUROPEAN AND NON-EUROPEAN AREA)

Cătălin Florin Pandelaş
University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad",
Faculty of Veterinary Medicine, Iasi, Romania
cpandelas@yahoo.com

Abstract

This article aims to debate recognition issues in veterinary medicine. In Romania, Resolution no. 860 of 3 June 2004 concerning veterinary qualification recognition and regulation of certain aspects of practicing as veterinarian, is the one that brings together the methodology and applicable legislation in this area.Throughout this article we will study the recognition of diplomas inside of EU and non-European areas. Regarding Non-European, the studied areas are: North America, Canada, North Africa, United Kingdom, New Zealand and India. Each veterinary College from all over the world requires different types of accreditation to accomplish the degrees recognition, therefore it is recommended to research on recognition of the diploma before leaving the original country.

Key words:*Veterinary Medicine, Degree, EU*

Introduction

The recognition of a diploma after graduating a university, is a key criteria when it comes to choose a career. Why it is considered so important diploma recognition inside the European Union and non-European area? Firstly because it provides global mobility and secondly because it gives greater credibility and trust on knowledge acquired over one's studies . After all a diploma accreditation is a procedure whereby an accrediting body officially recognizes and certifies in writing that one person is able to carry out specific activities. We deduce from the previous definition the importance of recognizing a diploma, and this procedure is more important within medicine medicine because life is at stake in human beings and animals.

Materials and methods

Recognition of academic degrees inside of European Community. There is no automatic recognition of academic diplomas inside the European Union. Graduates may therefore need to go through a national procedure to get their academic degree or diploma recognised in another EU country. Individual governments of EU countries remain responsible for their education systems and are free to apply their own rules, including whether or not to recognise academic qualifications obtained elsewhere. Consulting consulting law degree accreditation regarding veterinary medicine

Recognition of academic degrees for Non-European Areas. In **New Zealand** for example to practice in as a veterinarian, one should take part of the Veterinary Council of the country (VCNZ), and hold a current practicing certificate. Also, for new member of the council, it is established if he is eligible for registration without examination, or registration by examination (or neither). All applicants must satisfy the Council that they can communicate in and understand English to an appropriate standard for practising as a veterinarian in New Zealand. The Council will require a letter of good standing from the previous registration authority and will require doctors to make statements about their recency of practice. Regarding general Registrations, without examination there are recognized veterinary graduates who meet

minimum recency of practice and English language requirements and who hold undergraduate veterinary degrees from schools which have been accredited by a recognised authority or who hold a pass in a recognised registration examination or assessment programme. For a list of the schools, degrees and examination/assessment programmes recognised by VCNZ, it is required to consult a local Gazette, and if the graduated institution is on the list then the doctor or graduate is entitled to apply for registration without examination.

In the **United Kingdom of Great Britain and Northern Ireland** academic recognition of qualifications obtained in Romania can be done in two ways: directly - by the institution / organization to which the application is made or through intermediary organizations like - National Academic Recognition Centre in the United Kingdom (UK NARIC).

Recognition of academic degrees in **Canada and North America**.There has been a significant increase in the immigration of foreign-trained veterinarians to Canada. Most of the foreign-trained veterinarians are graduates of veterinary medical schools that are not accredited by the Council of Education of the American Veterinary Medical Association. Therefore, they are required to complete the North American Veterinary Licensing Examination and Clinical Proficiency Examination, and to demonstrate proficiency in English in order to obtain a Certificate of Qualification to become eligible to practise as a veterinarian in Canada. Because of considerable variation in training standards among veterinary colleges, these rigorous and demanding examinations are important and are designed to ensure that foreign-trained veterinarians are as qualified as graduates of accredited veterinary medical schools in North America.

Until very recently, there were no structured provisions in Canada for foreign-trained veterinarians to upgrade their skills, especially their clinical skills. In contrast, many veterinary medical schools in the United States offered 1-year evaluated clinical training programs, which foreign trained veterinarians could complete in lieu of the Clinical Proficiency Examination in order to become licensed to practise in the USA. [It is another matter that after nearly 20 years this program is no longer being recognized by the Educational Commission for Foreign Veterinary Graduates (ECFVG)]. However, the Canadian system [the National Examining Board (NEB)] neither recognized this program nor created any comparable opportunities for foreign-trained veterinarians to upgrade their skills. Other than a very short-lived pilot program led by Dr. Alastair Summerlee at the Ontario Veterinary College in the 1990s, Canadian veterinary schools have not shown any leadership in this area. Notwithstanding lack of training opportunities, foreign-trained veterinarians have found a way to prepare and complete the required series of examinations to become successful veterinarians in Canada.

Now there are growing numbers of licensed foreign-trained veterinarians in Canada, especially in Ontario and British Columbia. Based on anecdotal evidence, there are conflicts between the regulatory bodies and the licensed foreign trained veterinarians, resulting in many lawsuits. There are many issues, among them complaints by foreign-trained veterinarians that they are being unfairly targeted for disciplinary actions or the imposition of more rigorous English proficiency standards. The English standards accepted by the NEB are similar to those used by the ECFVG, as well as by most of the Canadian universities for accepting students into graduate programs. The British Columbia Veterinary Medical Association has decided to impose a higher standard in spoken English,which is perceived to be discriminatory by foreign-trained veterinarians whose first language is not English, and this issue has become a bone of contention. Such situations can potentially create a heightened degree of mistrust between foreign-trained veterinarians and the regulatory bodies, which is not good for the profession.

Hundreds of foreign-trained veterinarians are currently registered with the NEB to write licensing examinations and join the ranks of practising veterinarians in Canada. There is a critical need to develop a cohesive and meaningful policy to integrate foreign-trained veterinarians into the mainstream of the Canadian veterinary profession. There needs to be widespread consultation on developing programs in partnership with Canadian veterinary schools and the veterinary medical associations, as well as the provincial and federal governments, to provide training opportunities, as needed, in veterinary medicine, and in related areas, such as communications (oral and written), culture, and standards of veterinary practice in Canada. Such a consultation, resulting in the development of training opportunities, will help us move beyond the current series of examinations for foreign-trained veterinarians!

In the **United States**, requirements for licensure are set by individual state regulatory boards. The North American Veterinary Licensing Exam (NAVLE) and any additional state exams must be taken by a graduate to become eligible for state licensure. The NAVLE, which is administered by the National Board of Veterinary Medical Examiners (NBVME), fulfills a core requirement for licensure to practice veterinary medicine in all jurisdictions in the United States and Canada. Mexico does not require NAVLE. In addition to the NAVLE, state regulatory boards will have other licensure requirements, which may include state-specific examinations.

Applicants who graduated from a non-AVME/COE accredited college must also have a certification of eligibility, which can come from one of two sources: the Educational Commission for Foreign Veterinary Graduates (ECFVG) Certification Program or the Program for the Assessment of Veterinary Education Equivalence (PAVE).

Recognition of academic degrees in **South Africa**. Persons with qualifications from any other institution throughout the world are required to pass an examination prior to being registered in South Africa to practice the profession of a veterinary nurse in South Africa. This requirement must not be construed in any way as a comment on the standard or quality of other educational training but is purely related to the fact that no agreement of reciprocity exists between this Council and other bodies concerned.

Such persons shall submit an application to the Council for permission to enroll for the registration examination. Applications for permission to write must be received by this office on or before **1 April.** If permission is granted, the applicant will be required to sit the examination during September of the same year in Pretoria or a venue to be advised. Please note that an examination is required unless the Council expressly decides otherwise. All applicants are required to submit certified copies of all educational certificates together with their application and produce original copies of the said certificates for scrutiny on the first day of the examination and prior to sitting the South African Veterinary Council registration examination.

Persons registered with other registering authorities prior, are required to submit with their application a certificate of good standing issued within the previous three months by the relevant registering authorities. The Veterinary Council is the regulatory authority governing the registration of qualified persons and the conduct of the profession.

Results and discussions

Admissions practices, requirements and difficulty vary widely among veterinary schools, and from nation to nation. Generally speaking, gaining admission to a veterinary school is highly competitive, due to the small number of places available

Conclusions

Not all nations accredit veterinary schools, but all developed countries and most newly industrialized and developing countries do. Few failed states have any accreditation system, however. In the United States, the American Veterinary Medical Association (AVMA) Council on Education (COE) accredits veterinary schools. Accreditation systems and standards vary widely, however, Australia, New Zealand and the United Kingdom all have vet programs that hold similar standards as those in the United States and Canada. The European Union is developing a common accreditation standard, but as of 2008 accreditation was most often provided by the European Association of Establishments for Veterinary Education (EAEVE). Most AVMA-accredited institutions in Australia, Canada, Ireland, New Zealand, the United Kingdom, and the United States share a common, online application system, known as the Veterinary Medical College Application Service (VMCAS). Many colleges belonging to VMCAS have additional, individualized application requirements as well, and admissions standards are quite high. Admissions standards in Europe, South America, Asia, and Africa also vary widely. Many veterinary schools limit admission to students from their area, state or country. For example, 25 of the 28 veterinary schools in the US are public universities, and by law may set aside relatively few places for out-of-state residents.

Other countries have similar schemes. For example, in India, federal law requires that each veterinary college set aside 15 percent of its places for students coming from other parts of India. The Veterinary Council of India (a body of the federal government), conducts the All India Common Entrance Examination, and the top scorers on the exam are placed throughout India.

In conclusion, each veterinary College from all over the world requires different types of accreditation to accomplish the degrees recognition. It's not easy but it's going to worth the effort, for those who want to start practicing abroad.

References

http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32009D0470:EN:T
http://www.vetcouncil.org.nz/gainReg.php
http://www.savc.co.za/
(http://www.aavsb.org/PAVE/PAVE Home.aspx).

HISTOLOGICAL, MORPHOMETRIC AND ULTRASTRUCTURE OF THE BUFFALO'S ADRENAL GLAND

Hossam Fouad Attia [1] and Gamal Hasan Abdel-Rahman[2]
Histology and cytology department, faculty of veterinary medicine. Benha university[1] and Zoology department, faculty of science, Assuit University[2]. Taif University, Faculty of applied medical science[1] and faculty of science[2]

Abstract

This study was done on 14 buffalo's adrenal glands. The adrenal gland of buffalo consisted of outer cortex and inner medulla. It was surrounded by thick CT capsule. The thickness of the capsule varied according to the age of the animals. It was thin (242.6 ± 12.1 µm) in young and thick (265.3 ± 13.2 µm) in adult animals. Thin septa extended into the parenchyma of the gland. The cortex consisted of three distinct zones, zona glomuralis (Arcuata) which was (385.5 ± 13.9 µm) in young animals while (415.7 ± 14.6 µm) in adult animals. The columnar cells of the arcuata contained numerous round mitochondria and smooth endoplasmic reticulum , while few rough endoplasmic reticulum. Zona fasiculata, which was the widest zone of the gland (865.6 ± 17.5 µm) in young animals while (912.8 ± 18.3 µm) in adult animals. The cuboidal cells of the zona fasiculata showed numerous round and oval mitochondria , small round electron-lucent lipid droplets , well developed Golgi complex and smooth endoplasmic reticulum. Zona reticularis which was (432.2 ± 14.3 µm) in young animals while (471.3 ± 15.8 µm) in adult animals. The cells contained round mitochondria, Golgi complex and less vacuole.The medulla was consisted of outer part that separated from the cortex by CT capsule. The thickness of the medulla was (457.8 ± 15.2 µm) in young animals while (482.5 ± 16.6 µm) in adult animals .It consisted of large columnar cells with dense eosinophilic cytoplasm and large oval basophilic nuclei. These cells showed small, round electron-dense oval granules with variable densities and size. Mitochondria are present but less numerous than that of the fasicuolata cells .It considered adrenaline secreting cells. The inner part of medulla consisted of polyhedral cells, small in size and stained less eosinophilic than the outer cells.It considered nor adrenaline secreting cells. The nor-adrenaline cells contain bigger irregularly shaped electron-dense, oval granules with an intensely electron-dense core.

Key words: *Adrenal gland-Buffaloes-Ultrastructure-Morphometric*

Introduction

The adrenal gland consists of cortex and medulla. The adrenal cortex is composed of three concentric layers: the zona glomerulosa, the zona fasciculata, and the zona reticularis, all of which present different morphological and functional properties. Zona glomerulosa is specialized in the production of aldosterone while zona fasciculata/reticularis synthesize cortisol in humans and bovine, and corticosterone in rodents (Rainey 1999).

The adrenal gland is a dynamic organ that requires constant stimuli from pituitary derived proopiomelanocortin (POMC) peptides to maintain its tonic state since either hypo-physectomy or dexamethasone treatment results in rapid adrenal atrophy (Bicknell, 2002).
Suprarenal gland cortex synthesizes and secretes steroid hormones involved in several important functions, such as hydrosaline homeostasis, body metabolism and reaction to internal or external stress stimuli. This process is principally controlled by the Hypothalamus–Pituitary–Adrenal axis (HPA) via corticotropic hormones, i.e. by adrenocorticotropin hormone (ACTH) and by corticotrophin releasing hormone (CRH), which, in addition to stimulating pituitary ACTH secretion, is also known to have some direct effect on the adrenal gland (Willenberg et al., 2000). Aside from angiotensin II (Ang II), adrenocorticotropin hormone (ACTH) is the most potent stimulus of aldosterone secretion by glomerulosa cells and of corticosterone by fasciculata cells (Gallo-Payet and Payet 2003). ACTH acts not only on the immediate, transcription-independent stimulation of adrenal

steroid synthesis and release, but also increases the expression of a number of genes including those involved in steroidogenesis (Sewer and Waterman 2003). The heart, kidneys and adrenal glands are interconnected through rennin-angiotensin-aldosterone mechanism and play an important role in the blood pressure regulation (Saavedra and Trimmermans, 1994).

The current study, therefore aimed primary to investigate the histological, morphometric and ultrastructure of the buffalo's adrenal glands.

Material and Methods

Animals and tissues

14 buffaloes (Bubalus bubalis) adrenal glands were collected from Toukh abattoir in kalyobia governorates, Egypt. Their ages were ranged from 1-12 years. The samples were preserved in Bouin's fluids, dehydrated in ascending grades of ethanol, cleared in xylene and cut at 3µm for further processing (Bancroft, Cook , Stirling and Turner,1994).

Morphometric study

Four slides of each age from each individual (8 sections per slide) were measured. For each section, 10 measurements of thickness of capsule, zona glomerulosa , zona fasciculata , zona reticularis and medulla were measured. Histological sections were studies by using a research microscope equipped with digital camera and connected to a PC based image analysis system. Sigma Scan Pro (version 4.0, Jendel Scientific, SPSS Inc., Chicago, USA) was used for image analysis.

TEM -Electronmicroscopy

A very small pieces (1mm) of the adrenal cortex and medulla were fixed in 2.5% glutaraldehyde in IM phosphate buffer (pH. 7.3) for 24 hours then post fixed in cold IM phosphate buffered 1 % osmium tetra oxide (pll. 7.3) for 3 hours, rinsed in phosphate buffer for 30 minutes then dehydrated **(**Hayat,1986). Semi-thin sections were stain by Toluidine blue. Ultra thin sections were obtained and mounted on copper grids then stained with uranyl acetate and lead citrate. For the TEM in college of Agriculture, research unit, Cairo University. By using Sumy Electron Optics SEO at 25 Kv.

Results

The buffalo adrenal gland was consisted of cortex and medulla. The cortex of the gland was surrounded by a well-developed capsule (Figure 1). The thickness of the capsule varied according to the age of the animals. It was thin (242.6 ± 12.1 µm) in young and thick (265.3 ± 13.2 µm) in the adult age. The capsule composed of collagen fibers (Figure 2) which was numerous in the outer part of the capsule and smooth muscle fibers which was more numerous closer to the cortex (Figure 3). Connective tissue trabeculae from the capsule penetrate into the cortex, reticular network widely distributed throughout the gland.

The adrenal cortex consisted of three distinct zones, the zona glomuralis, zona fasiculata and zona reticularis. The zona glomuralis consisted of columnar cells in the form of arcuata or arcades. The thickness of the zona glomuralis was (385.5 ± 13.9 µm) in young animals while (415.7 ± 14.6 µm) in adult animals. It appeared as curved cords that its convex surface directed toward the capsule. The nuclei of the columnar cells were oval in shape with intense basophilic reaction, while the cytoplasm was eosinophilic (Figure 4). The columnar cells of the arcuata contained numerous round mitochondria widely distributed all over the contour of the cytoplasm and smooth endoplasmic reticulum , while few rough endoplasmic reticulum was located (Figure 5). The zona fasiculata was the widest zone. The thickness of

the zona fasciculata was (865.6 ± 17.5 µm) in young animals while (912.8 ± 18.3 µm) in adult animals. It consisted of cords of one or two cell thickness separated from the adjacent one by extensive blood sinusoids (Figure 6). The cords consisted of vacuolated cuboidal cells with pale eosinophilc cytoplasm and large basophilic nuclei. (Figure 7).The cuboidal cells of the zona fasiculata showed numerous round and oval mitochondria , small round electron-lucent lipid droplets , well developed Golgi complex (Figure 8) and smooth endoplasmic reticulum.

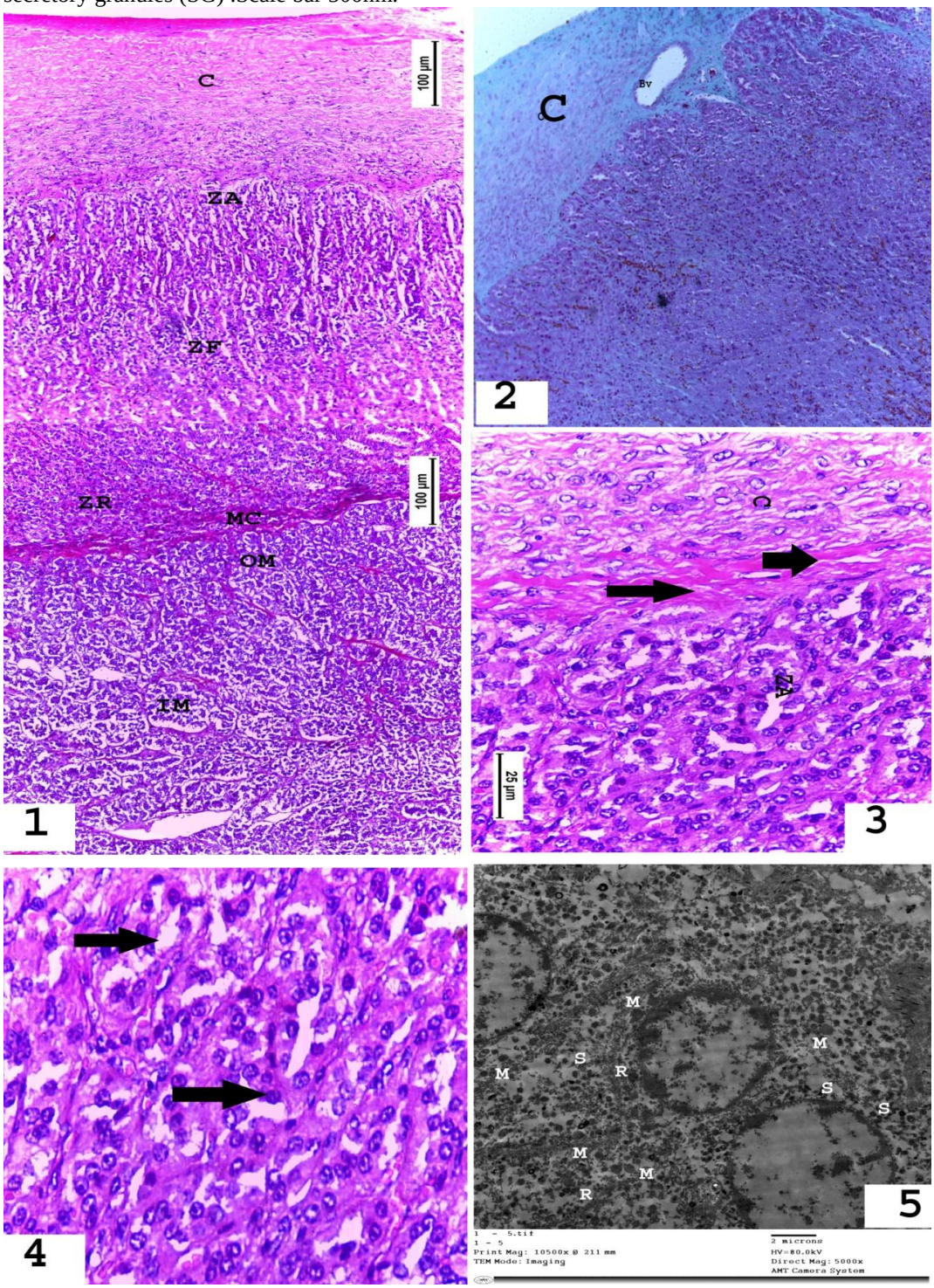
The zona reticularis was consisted of anastomosing cords of cuboidal cells (Figure 9), around extensive network of blood sinusoids .The cell cytoplasm was dense eosinophilc and has no vacuolation like fasicuolata (figure 10). The cells contained round mitochondria , Golgi complex and less vacuoles (Figure 11). The thickness of the zona reticularis was (432.2 ± 14.3 µm) in young animals while (471.3 ± 15.8 µm) in adult animals.

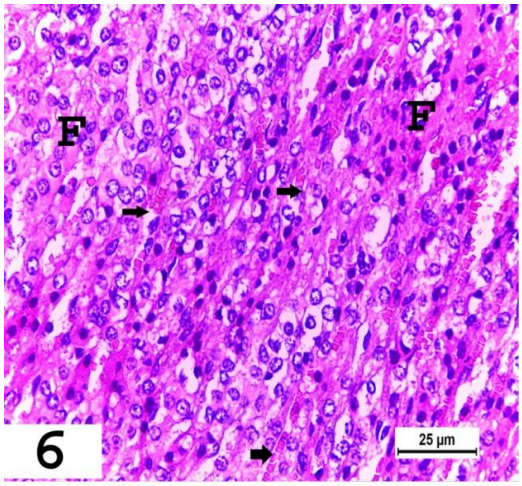
The medulla has irregular distinct separating capsule from the cortex (Figure 12). The thickness of the medulla was (457.8 ± 15.2 µm) in young animals while (482.5 ± 16.6 µm) in adult animals. The medulla arranged in the form of acini separated from each other by extensive network of reticular fibers. The adrenal medulla arranged in the form of outer and inner zone between them extensive network of blood sinusoids were located (Figure 13).

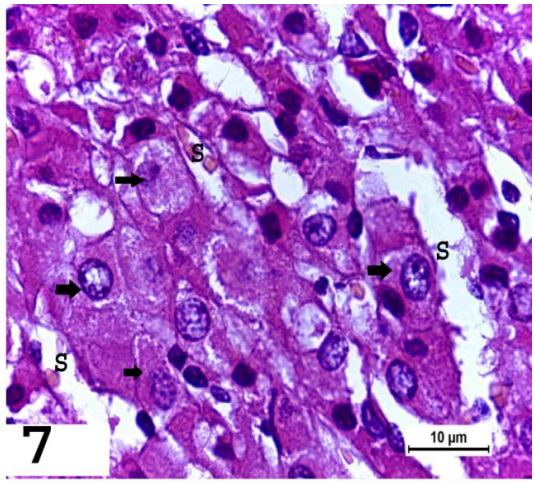
List of Figures

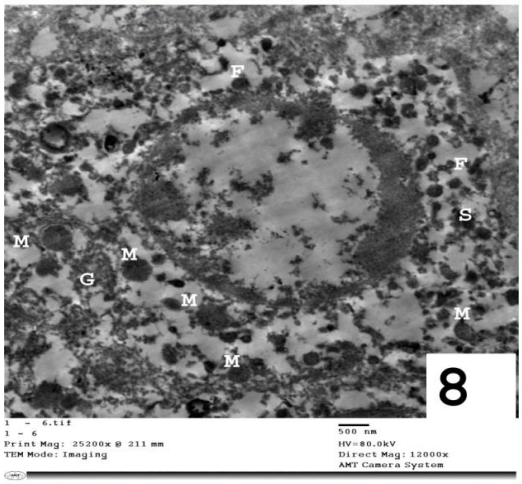
Fig.1: photomicrograph of the buffaloes adrenal gland showing the whole gland, capsule (C) , zona arcuata (ZA), zona fasiculata (ZF), zona reticularis (ZR), medullary capsule (MC), outer part of the medulla (OM) and inner part of the medulla (IM). H&E.Scale bar 25um; ***Fig.2:*** photomicrograph of the buffaloes adrenal gland showing the collagen fibers capsule (C). Crossmons.Scale bar 25um; ***Fig.3:*** photomicrograph of the buffaloes adrenal gland showing the smooth muscle fibers of the capsule (arrow) H&E.Scale bar 25um; ***Fig.4:*** photomicrograph of the buffaloes adrenal gland showing the zona glomuralis(arcuata) (arrow) H&E.Scale bar 10um; ***Fig.5:*** Electromicrograph (TEM) of the buffaloes adrenal gland showing the columnar cells of the zona arcuata, mitochondria (M), rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S).Scale bar 2um; ***Fig.6:*** photomicrograph of the buffaloes adrenal gland showing the zona fasiculata (F), blood sinusoids (arrow) H&E. Scale bar 25um; ***Fig.7****:* photomicrograph of the buffaloes adrenal gland showing the zona fasiculata cuboidal cells (arrow) H&E.Scale bar 10um; ***Fig.8:*** Electromicrograph (TEM) of the buffaloes adrenal gland showing the cuboidal cells of the zona fasiculata, mitochondria (M), rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S), golgi apparatus (G) and fat vacuoles (F) .Scale bar 500nm; ***Fig.9:*** photomicrograph of the buffaloes adrenal gland showing the zona reticularis , (R) . H&E. Scale bar 25um; ***Fig.10:*** photomicrograph of the buffaloes adrenal gland showing the zona reticularis cuboidal cells (arrow) and blood sinusoids (S). H&E. Scale bar 10um; ***Fig.11:*** Electromicrograph (TEM) of the buffaloes adrenal gland showing the cuboidal cells of the zona reticularis, mitochondria (M), rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S) .Scale bar 500nm; ***Fig.12:*** photomicrograph of the buffaloes adrenal gland showing the medullary capsule that separate between the cortex and medulla (arows) . H&E. Scale bar 25um; ***Fig.13:*** photomicrograph of the buffaloes adrenal gland showing extensive blood sinusoids between the medullary cells (Arrows), adrenaline cells (A) and nor-adrenaline cells (NA) . Toludine blue. Scale bar 10um; ***Fig.14:*** photomicrograph of the buffaloes adrenal gland showing the adrenaline secreting cells, these cells were large columnar cells with dense eosinophilic cytoplasm and large oval basophilic nuclei (A) . H&E. Scale bar 25um; ***Fig.15:*** Electromicrograph (TEM) of the buffaloes adrenal gland showing the adrenaline secreting cells, mitochondria (M), rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S) and secretory granules(SG) .Scale bar 2um; ***Fig.16:*** photomicrograph of the buffaloes adrenal gland showing nor adrenaline secreting cells, these cells were large columnar cells with less eosinophilic cytoplasm (NA). H&E. Scale bar 10um; ***Fig.17:***

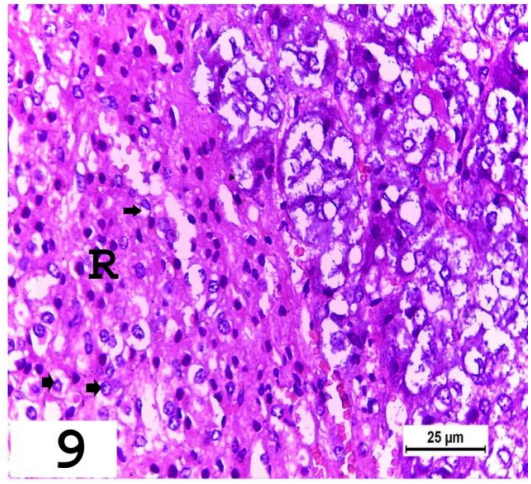
Electromicrograph (TEM) of the buffaloes adrenal gland showing the nor adrenaline secreting cells, mitochondria (M), rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S) and secretory granules (SG) .Scale bar 500nm.

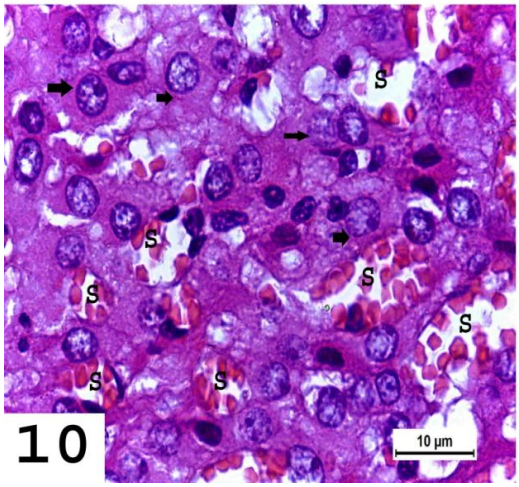


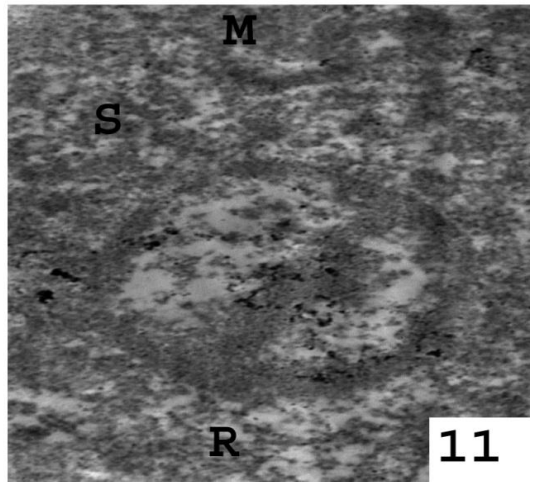

F
F
6
25 µm

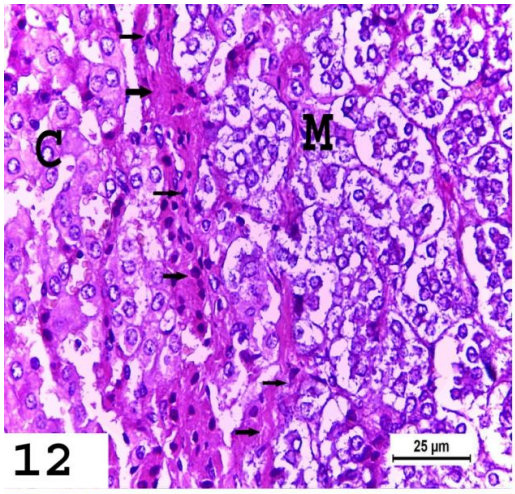

S
S
S
7
10 µm

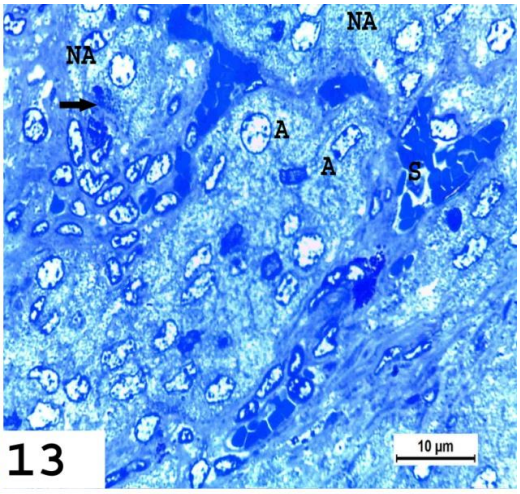

F
F
S
M
M
G
M
M
M
8
1 - 6.tif
1 - 6
Print Mag: 25200x @ 211 mm
TEM Mode: Imaging
500 nm
HV=80.0kV
Direct Mag: 12000x
AMT Camera System

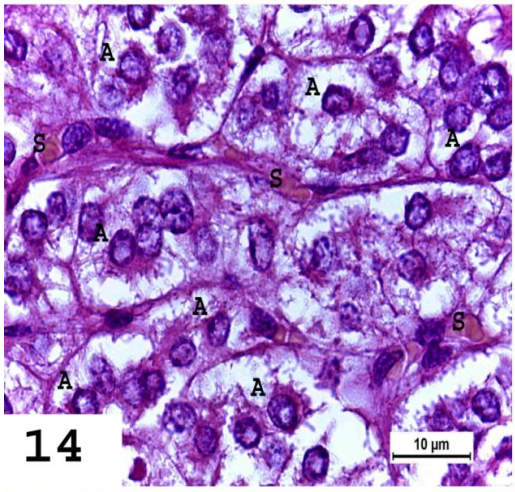

R
9
25 µm

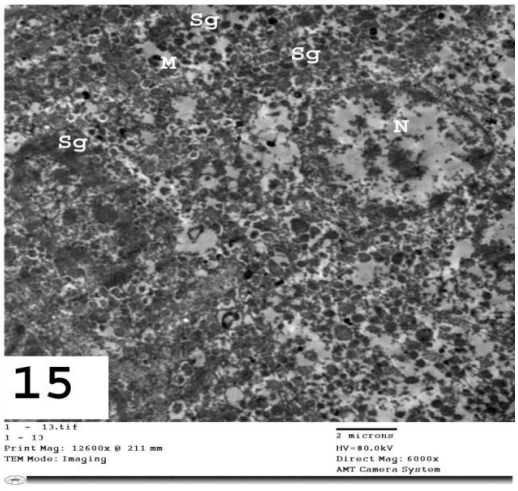

S
S
S
S
S
S
S
S
10
10 µm

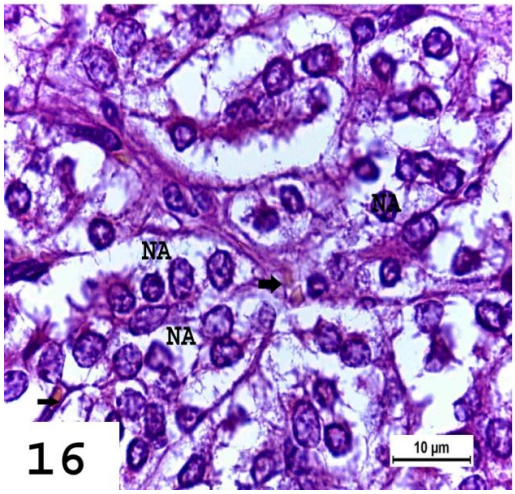

M
S
R
11

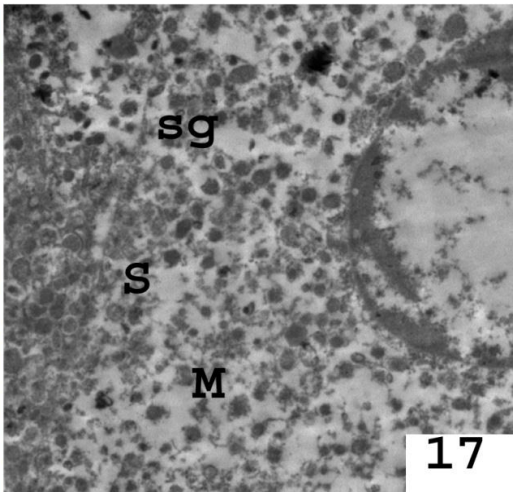

C
M
25 µm
12


NA
NA
A
A
S
10 µm
13


A
A
A
S
S
A
A
A
S
A
A
10 µm
14


Sg
M
Sg
N
Sg
15
1 - 13.tif
1 - 13
Print Mag: 12600x @ 211 mm
TEM Mode: Imaging
2 microns
HV=80.0kV
Direct Mag: 6000x
AMT Camera System


NA
NA
NA
16
10 µm


sg
S
M
17

The outer zone consisted of large columnar cells with dense eosinophilic cytoplasm and large oval basophilic nuclei (Figure 14). The cells arranged in the form of acini. The cells appear pale with toulidine blue (fig.13). These cells considered to be adrenaline secreting cells. These cells showed small, round electron-dense oval granules with variable densities and size. Mitochondria are present but less numerous than that of the fasicuolata cells (Figure 15)

The inner cells are polyhedral in shape small in size and stained less eosinophilic than the outer cells (figure 16). The granules of the inner cells appeared dark blue with toulidine blue stain (figure 13). These cells considered nor- adrenaline secreting cells. The nor-adrenaline cells contain bigger irregularly shaped electron-dense, oval granules with an intensely electron-dense core (Figure 17). The granule may sometimes be characterized by a clear space between the membrane and the granular contents, with the electron-dense core tending to be eccentrically placed within the membrane.

Table 1. Histomorphometrical measurements of the adrenal gland in young and adult buffaloes, Data presented as means ± standard deviation

	Thickness (μm)	
	Young	Adult
Capsule	242.6 ± 12.1	265.3 ± 13.2
Zona glomerulosa	385.5 ± 13.9	415.7 ± 14.6
Zona fasiculata	865.6 ± 17.5	912.8 ± 18.3
Zona reticularis	432.2 ± 14.3	471.3 ± 15.8
Medulla	457.8 ± 15.2	482.5 ± 16.6

Discussion

The adrenal gland in mammals is typically made of two concentric layers of glandular tissue: the outer cortex and the inner medulla. These two parts differ from each other by origin, microscopic structure and function. Because of the differences in cell arrangements, the adrenal cortex is divided into three zones: either the zona glomerulosa or the zona arcuata; the zona fasciculata and the zona reticularis. The zona glomerulosa is found in humans and bovine whereas the zona arcuata is described in horses, donkeys, carnivores and pigs (Banks, 1993; Dellmann, 1993; Raviola, 1994; Junqueira and Carneiro, 2005), also the adrenal cortex of all marine mammals described so far was built of three zones: the zona glomerulosa, the zona fasciculata and the zona reticularis (Lucić, 2002; Bragulla et al., 2004, Clark et al., 2005, 2008).While the cortex and medulla was intermingled with each other in camel (Abdalla and Ali 1989). So the buffaloes adrenal gland has zona arcuata as that of the horse and carnivores.

The capsule of the buffalo is thinner than that in the elephant (Kramer et al. 1991) and similar to that of the impala .The presence of smooth muscle in the capsule is not surprising as these fibers have also been reported in adrenal glands of other mammals

(Dellman and Brown 1987and Kramer et al. 1991). Extensive layer of smooth muscle in its capsule and a continuous layer of undifferentiated capsular cells above the zona glomerulosa, which are not found to the same extent in any of the domestic animals (Kramer et al.,1991).

A layer of capsular cells, as reported by (Kramer et al.,1991) in the elephant and by (Dellman and Brown 1987) in the adrenal gland of domestic animals, was not found in the buffalo. It was postulated (Kramer et al., 1991) that the capsular cells in the elephant were responsible for supplying the pool of cells in the cortex. It is possible that in the buffalo, as suggested by (Banks 1986), in domestic animals, the cells of the zona arcuata may take over this responsibility. We noted that the medulla has irregular distinct separating capsule from the cortex. Abdalla and Ali (1989), augmented our findings in the old camel adrenal gland, while (Maria and Kramer,1993) in buffalo reported no line of demarcation between the cortex and the medulla.

The capillaries of the adrenal cortex, as well as those of other endocrine glands, are of the "sinusoid" type (Ronald and Fawcett, 2002). They consist of two cell types: very numerous endothelial cells of the classic type and histiocytes. The latter are encountered mainly in the deeper layers of the zona fasciculata and zone reticularis.

The arrangement of cells in the form of arcades in the zona arcuata is like that of horses, carnivores and pigs and unlike that of other ruminants and man (Banks 1986). There was no transitional area from the zona arcuata to the zona fasciculata, the former cells being found abutting directly on the large foamy cells of the fasciculata. The progression of cells of the zona fasciculata from pale foamy cells to more eosinophilic cells containing less lipid, is of interest. Adrenocortical cells are said to obtain cholesterol both by endogenous synthesis from acetate and by uptake of lipoproteins released by the liver (Gwynne & Strauss 1982). Only exogenous cholesterol is stored in the lipid droplets (Fruhling, Sand, Penasse, Pecheux & Claude 1973) while endogenously synthesized cholesterol is promptly utilized in steroidogenesis.

The prominent mitochondria with tubular to tubulovesicular cristae, the SER and presence of some lipid droplets are typical features of steroid-producing cells (Nussdorfer, 1986). These structural entities can be associated with cholesterol and cholesterol esters stored in the lipid droplets (Moses, Davis, Rosenthal & Garren 1969; Sharawy, Dirksen & Chaffin 1979). These substances are transformed into definitive steroid hormones via a pathway involving enzymes located both in mitochondrial cristae and SER tubules (Tamaoki, 1973).

The adrenal medulla arranged in the form of acini of cells surrounded by network of reticular fibers. Numerous blood sinusoids distributed between the medullary cells. It arranged as outer zone and inner zone containing adrenaline and nor adrenaline cells. The adrenal medulla consists of chromaffin cells which secrete epinephrine and nor-epinephrine under the influence of hypothalamus via the preganglionic sympathetic neurons and the release of these hormones in cases of fear, fight , stress pain ,and the decrease of level of glucose in the blood (Junqueira and Carneiro, 2005).

The adrenaline secreting cells were columnar in shape and located in the outer medulla while the nor-adrenaline secreting cells were polyhedral cells and located in the inner medulla. The same results were supported our findings (Narasimham and Kamat, 1970 and Ibraheem et al., 2009). The adrenal medulla is the core of the adrenal gland, and is surrounded by the adrenal cortex (Dunn , Kudrath , Passo and Wilson 2011). It secretes approximately 20% nor-epinephrine nor-adrenaline) and 80% epinephrine (adrenaline).

The chromaffin cells are the body's main source of the circulating catecholamines epinephrine and nor-epinephrine. Catecholamines are derived from the amino acid tyrosine and these water-soluble hormones are the major hormones underlying the fight-or-flight response. Chromaffin cells possess typical ultrastructural features, most notably large chromaffin granules (Coupland and Tomlinson, 1989).

In the outer medulla the tall, pale chromaffin-positive cells are believed to be adrenaline cells. These gradually give way to the smaller, polyhedral, intensely chromaffin-positive nor adrenaline cells of the inner medulla. This arrangement of the catecholamine-producing cells into an inner and outer zone is found in many domestic animals (Dellman & Brown 1987) and in the elephant (Kramer et al. 1991).

The adrenal gland undergoes, constant dynamic structural changes and is generally well acknowledged that cellular proliferation is preferentially observed at the periphery of the gland (zona glomerulosa) while cell death is increased in zona reticularis. The regulated balance between proliferation and apoptosis is a prerequisite for the integrative functionality of the gland (Wolkersdorfer and Bornstein 1998 and Vinson 2003, 2004).

The adrenal gland, as an organ responding to stress, is subject to dynamic structural changes, including both proliferation as well as cell death. A balance between these two processes is essential for integrity and functionality of this organ. Regulatory systems of the organism and the autocrine and paracrine local networks determine the cell cycle balance (Wolkersdorfer and Bornstein, 1998).

In rat adrenal cortex, the cells composing each zone are continuously renewed by migration of new cells derivated from progenitor cells that differentiate and die. Thus, apoptosis regulation in adrenal gland may be essential for the definition of the functional zonal architecture (Mitani et al., 1994).

The cell proliferation indices are the highest in zona glomuralis and in the outer part of zona fasiculata but the lowest in zona reticularis. (Pawlikowski , Gruszka , Mucha and Mełeń-Mucha 2001) In contrast, the apoptotic index is the highest in zona reticularis (Carsia, MacDonald , Gibney , Tilly and Tilly 1996) . Although the majority of these studies were performed in rodents, the similar observations concern the human adrenal gland (Sasano , Imatani , Shizawa , Suzuki and Nagura 1995). It is suggested that adrenocortical cells renew from the progenitor cells located in zona glomuralis /zona fasiculata boundary and /or in sub capsular layer.

The adrenal cortex plays a role of "biological clock" all factors which modulate the adrenocortical cell turnover. It may have an important long-term influence on the processes of maturation and aging. It includes, among others, stress and activity of the rennin-angiotensin system, because ACTH and angiotensin peptides strongly affect the adrenocortical cell turnover (Hornsby,2002).

References

1. Abdalla M.A and Ali A.M (1989): Morphometric and histological studies on the adrenal glands of the camel (Camelus dromedaries). Acta Morphol Neerl Scand. 26(4):269-81.
2. Banks, J.W (1986): Applied veterinary histology. 2nd edition. Williams and wilkins, Baltimore.
3. Banks, J. W (1993): Applied Veterinary Histology, 3rd edn. St. Louis, Baltimore, Boston, Chicago, London, Philadelphia, Sydney, Toronto: Mosby Year Book
4. Bancroft, J.D; Cook,H.C; Stirling, R.W and Turner,D.R(1994): Manual of histological techniques and their diagnostic application' .2nd Ed.,Churchill Livingstone, Edinburgh, London, New York Boyd,

5. Bicknell, A.B (2002): Identification of adrenal protease that cleaves programma–MSH: the drawing of a new era in adrenal physiology. J. Endocrinol, 172: 405–10.
6. Bragulla, H., R. M. Hirschberg, U. Schlotfeldt, M. Stede, and K.-D. Budras (2004): On the structure of the adrenal gland of the common seal (Phoca vitulina vitulina). Anat. Histol. Embryol.33, 263–272.
7. Carsia RV, MacDonald GJ, Gibney JA, Tilly K.I and Tilly JL (1996):Apoptotic cell death in the rat adrenal gland: an in vivo and in vitro investigation.Cell Tissue Res; 283:247–254.
8. Clark, L. S., D. C. Pfeiffer, and D. F. Cowan (2005): Morphology and histology of the Atlantic bottlenose dolphin (Tursiops truncatus) adrenal gland with emphasis on the medulla. Anat. Histol. Embryol.34, 132–140.
9. Clark, L. S., D. F. Cowan, and D. C. Pfeiffer (2008): A morphological and histological examination of the pan-tropical spotted dolphin (Stenella attenuata) and the spinner dolphin (Stenella longirostris) adrenal gland. Anat. Histol. Embryol.37, 153–159.
10. Coupland, R.E and Tomlinson, A (1989): The development and maturation of adrenal medullary chromaffin cells of the rat in vivo: a descriptive and quantitative study. Int. J. Dev. Neurosci. 7, 419–438.
11. Dellman, H.D and Brown E.M (1987): Textbook of veterinary histology. Lea and febiger, philadelphia 17.
12. Dellmann, H.D (1993)*:* Endocrine system*. In: Textbook of Veterinary* Histology, 4th edn (H.-D.Dellmann ed). Philadelphia: Lea and Fabiger, pp. 270–284.
13. Dunn R. B, Kudrath W., Passo S.S and Wilson L.B. (2011): "10". Kaplan USMLE Step 1 Physiology Lecture Notes. pp. 263–289.
14. Fruhling, J., Sand, G., Penasse, W., Pecheux, F and Claude, A (1973): Correlation entre la morphologie et ie contents lipidique des corticosurrenales de cobaye, du rat et du boeuf. J. Ultrastruc. Res. 44: 113-133.
15. Gallo-Payet, N and Payet, M.D (2003): Mechanism of action of ACTH: beyond cAMP. Microscopy Research and Technique 61 275–287.
16. Guyton, A. C. and J. E. Hall (1996): Textbook of Medical Physiology, 9th Ed., W. B. Saunders Company, Philadelphia, USA, pp: 227-236.
17. Gwynne, J.T and Strauss, J.F (1982): The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocrinol. Rev. 3: 299-329.
18. Hayat, M. A. (1986): Basic techniques for transmission electron microscopy. 1st ed. Academic Press, Inc. Florida.
19. Hornsby, P.J (2002): Aging of the human adrenal cortex Ageing .Res Rev ; 1:229–242.
20. Ibraheem , A.H. , Jameel , O.J and Jumaa ,Q.S (2009): Histological study for the identification and distribution of medullary (chromaffin) cells in the adrenal gland of camel. Department of Anatomy , College of Vet .Medicine, Tikrit University
21. Junqueira, L. C and J. Carneiro (2005): In: Basic Histology: Textbook, and Atlas, 10th edn (InCroatian, Ž.Bradamante and Lj.Kostović-Knežević eds). Zagreb: Školska knjiga
22. Kramer, B., Teixeira, M and Hatitngh, J (1991): The histology of the adrenal gland of the african elephant, loxodonta africana. S. Afr. J. 2001. 26: 193-198.
23. Lucić, H (2002): Histological and histochemical characteristic of some organs of the striped dolphin (Stenella coeruleoalba) from the Adriatic Sea, M.Sc. Thesis (in Croatian). Faculty of Veterinary Medicine, Zagreb: University of Zagreb
24. Maria Teixeira and Beverley Kramer (1993): The adrenal gland of the African buffalo, Syncerus caffer:a light and electron microscopic study. S. AfT. I. Zoo!. 1993,28(1)
25. Mitani F, Suzuki H, Hata J, Ogishima T, Shimada H and Ishimura Y (1994): A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. Endocrinol 135: 431-438.
26. Moses, H.L., Davis, W.W., Rosenthal, A.S and Garren, L.D (1969): Adrenal cholesterol: localization by electronmicroscope Autoradiography. Science 163: 1203-1205.
27. Narasimham, C. and Kamat, D.N. (1970).A comparative study on the distribution of medullary (chromaffin) cells in the vertebrate adrenal glands . Cytologia . 35: 409-414.
28. Nussdorfer, G.G (1986): Cytophysiology of the adrenal cortex. Int. Rev. Cytol. 98: 1-405.
29. Pawlikowski M, Gruszka A, Mucha S and Mełeń-Mucha G (`2001): Angiotensins II and IV stimulate the rat adrenocortical cell proliferation acting via different receptors Endocrine Regulations 2001; 35:139–142.

30. Rainey, W.E (1999): Adrenal zonation: clues from 11beta-hydroxylase and aldosterone synthase. Molecular and Cellular. Endocrinology 151 151–160.
31. Raviola, E (1994): Adrenal gland. In: Bloom and Fawcett, a Textbook of Histology (D. W.Fawcett ed). New York, London: Chapman & Hall, pp. 503–515.
32. *Ronald P. Jensh and Don W. Fawcett (2002): Bloom and Fawcett's Concise Histology (2nd Edition). Mixed media product.*
33. Saavedra, J. M. and P. B. Trimmermans, (1994): Angiotensin receptors. Plenum Publishing Crop. New York, USA.
34. Sasano H, Imatani A, Shizawa S, Suzuki T and Nagura H (1995):Cell proliferation and apoptosis in normal and pathological human adrenal Mod Pathol 1995; 8: 11–17.
35. Sewer, M and Waterman, M (2003): ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microscopy Research and Technique 61 300–307.
36. Sharaway, M., Dirksen, T and Chaffin, J (1979): Increase in free cholesterol content of the adrenal cortex after stress: radioautographic and biochemical study. Am. J. Anal. 156: 567-576.
37. Tamaoki, B.T (1973): Steroidgenesis and cell structure. Biochemical pursuit of sites of steroid biochemistry. J. Steroid biochem.4: 89-118.
38. Vinson, G.P (2003): Adrenocortical zonation and ACTH. Microscopy Research and Technique 61 227–239.
39. Vinson, G.P (2004): Glomerulosa function and aldosterone synthesis in the rat. Molecular and Cellular Endocrinology 217 59–65.
40. Willenberg, H.S., Bornstein, S.R., Hiroi, N., Poth, G., Goretzki, P.E., Scherbaum and W.A.,Chrousos, G.P (2000): Effects of a novel corticotropin-releasing-hormone receptor type I antagonist on human adrenal function. Mol. Psychiatr. 5, 137–141.
41. Wolkersdorfer, G and Bornstein, S (1998): Tissue remodelling in the adrenal gland.Biochemical Pharmacology 56 163–171.

THE INFLUENCE OF HYDROCHLORIC DROTAVERINE ON THE HAEMATOLOGICAL AND METABOLIC PROFILE IN HORSES

Laurenţ Ognean, Viorica Chiurciu, Cristina Ştefanuţ, Nicodin Iosif Fiţ, Alexandra Arion, SebastianTranca, Radu Harşan
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca
Faculty of Veterinary Medicine, 3-5 Mănăştur Street, 400372, Romania
lognean@yahoo.com

Abstract

The purpose of this experiment was to evaluate the changes in the hematological and biochemical profile of horses, treated with drotaverine hydrochloride (Drotavet). The investigations were performed on 15 clinically healthy horses, with the age between 2-10 years, divided in three equal groups. The product was administered according to the following protocol: group 1 – 200 mg (10 ml) of hydrochloric drotaverine administered three times a day, six hours apart; group 2 – 320 mg (16 ml) hydrochloric drotaverine, intramuscular administration, three times a day, 8 hours apart and group 3 – 160 mg (8 ml) of hydrochloric drotaverine, one single dose. Before and after the treatment, clinical and laboratory tests were performed. The laboratory tests included the following hematological parameters: hematocrit, hemoglobin, total number of erythrocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total and differentiated leukocyte count. The biochemical parameters determined were: aspartate aminotransferase (AST), bile acids (BA), creatine phosphokinase (CK), uric acid (UA), glucose (GLU), Calcium (CA), phosphorus (PHOS), total protein (TP), albumins (ALB), globulin (GLOB), potassium (K+), and sodium (NA+). Following the statistical evaluation of the individual and mean values recorded in the clinical, hematological and biochemical tests carried out before and after the treatment, the values recorded were in the physiological ranges for the 3 groups of horses. Analyzing the values recorded, it can be observed that al of the animals used had good health statuses, that maintained throughout he experimental period. Group 1 presented a mild state of anemia, revealed by the low values of hematocrit and hemoglobin, compared to the normal physiological values (32-48%, and 10-18 g/dl); the anemia was not confirmed by the total erythrocyte count, whose value was in the normal physiological range (6-12x10^{12}/l). Regarding the local tolerance, no adverse reactions were observed after administering the therapeutically dose or the drotaverine hydrochloride overdose via IM or IV administration. The subcutaneously administration led instead to edema and mild local allergic reactions. The use of drotaverine hydrochloride in therapeutically doses and overdoses did not led to adverse systemic (general) reactions, immediate (due to administration, absorption and metabolization of the product) or delayed, in horses used for pre-clinical testing of Drotavet.

Keywords*: horse, Drotavet, testing, hematology, metabolic profile*

Introduction

Drotaverine is a hydrated derivative of papaverine, having a spasmolytic function and acting directly on the smooth muscles. Its' pharmacological activity is based on the increasing levels of cyclic AMP from the smooth muscle by inhibiting the phosphodiesterase (Pap et al., 1997; Bolaji et al., 1996). Also, Drotaverine also exhibits inhibitory effect on thrombocytes, this effect also being correlated with the phosphodiesterase inhibiting effect (Kapui, et al., 1991a), the effect of drotaverine being 3-10 times stronger compared to pentoxifylline (Kapui et al., 1991b; Kapui et al., 1992).

Due to the particularities of drotaverines' metabolization and excretion, the doses must be adjusted when administered to patients with kidney and liver diseases, although detailed studies regarding the dosage regimen were not carried out.

The main goal of this paper was to evaluate the changes of the hematological and biochemical parameters in horses, after drotaverine hydrochloride (Drotavet) administration.

Materials and methods

The testing was carried out on 3 groups of horses, clinically healthy and with an age between 2-10 years. The most important exclusion criteria were: hypersensitivity to drotaverine or other drugs belonging to the same chemical and pharmacological group, significant acute sickness in the last 14 days, treatments using other drugs (barbiturates, phenothiazines) that could have seriously affected the vital signs in the last 30 days, major surgical procedures on the GI tract, any pathological state that could influence absorption, distribution, metabolism and excretion of the substances used, gestation/lactation.

The groups were formed as follows:

- Group 1 (n=5) – a therapeutically dose of Drotavet, for injection (200 mg, 10 ml), administered subcutaneously, 3 times, at an interval of 6 hours;
- Group 2 (n=5) – double dose of Drotavet, for injection (320 mg, 16 ml), administered IM, 3 times, at an interval of 8 hours;
- Group 3 (n=5) – a therapeutically dose of Drotavet, for injection (160 mg, 8 ml), administered IV, in a single dose.

The animals were clinically examined (including the standard clinical exam and laboratory screening) before the experiment begun, over a period of 7 days preceding the study. In day 0, before the experiment begun, the animals were observed in term of behavior, were weighted and the laboratory test were repeated. Day 1 corresponds to the administration of the product, including: clinical tests, administering the single and repeated doses of Drotavet. The final tests were carried out 12 hours after administering the last dose of the drug product and consisted in repeating the clinical, hematological and biochemical tests. The animals were monitored in order to record the possible adverse reactions. The observation period was extended for 15 days starting after the last dose was administered, to record the possible late adverse reaction that could occur.

The following hematological parameters were determined: hematocrit, hemoglobin, erythrocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total and differentiated leukocyte count; the biochemical parameters determined were: aspartate aminotransferase (AST), bile acids (BA), creatine phosphokinase (CK), uric acid (UA), glucose (GLU), Calcium (CA), phosphorus (PHOS), total protein (TP), albumins (ALB), globulin (GLOB), potassium (K+), and sodium (NA+). Data analysis was carried out following the methods described by Reece (1996) and Ghergariu et al., (2000). The values recorded before treatment, were used as control values.

The individual and mean data were statistically analyzed following the EMEA guides and ANSVSAs' Order No. 186.

Results and discussions

After analyzing the data recorded in the clinical, hematological and biochemical tests carried out before and after treatment, it could be observed that most of the values recorded were in the normal physiological ranges, for all 3 groups of horses. It can also be noticed that all of the animals used had a good health status, which was maintained throughout the experimental period.

The hematological and biochemical results from the animals that received Drotavet in normal dose and in a double dose, compared to the control group are expressed as mean ± standard deviation and are presented in tables 1, 2 and 3.

Table 1. Mean values of the erythrocyte parameters recorded at product testing

Group No.		*1*	*2*	*3*
		Mean ± SD	*Mean ± SD*	*Mean ± SD*
Before treatment / /initial				
Ht(%)		*28,18±2,617*	*29,60±2,085*	29,89±0,995
Hb(g/dl)		*10,12±1,139*	*10,66±0,950*	10,50±0,245
Erythrocyte count (T/l)		*7,26±0,890*	*6,82±0,566*	6,55±0,400
Mean erythrocyte constants	MCV/(fl)	*38,98±2,062*	*43,44±0,559*	45,81±3,430
	MCH(pg)	*13,98±0,781*	*15,63±0,294*	16,07±0,662
	MCHC (g/dl)	*35,87±1,197*	*35,98±0,770*	35,16±1,436
After treatment / final				
Ht(%)		*25,92±2,558*	*28,55±2,370*	27,88±3,487
Hb(g/dl)		*9,30±1,160*	*10,30±0,834*	9,96±0,669
Erythrocyte count (T/l)		*6,86±0,940*	*6,71±0,650*	6,30±0,645
Mean erythrocyte constants	MCV/(fl)	*37,94±1,875*	*42,57±0,715*	44,22±2,233
	MCH(pg)	*13,58±0,506*	*15,36±0,474*	15,86±0,773
	MCHC (g/dl)	*35,81±1,000*	*36,08±0,636*	35,95±2,663

The evolution of the erythrocyte parameters in group 1 (table 1), was characterized by lower hematocrit and hemoglobin values, compared to the normal physiological values (32-48%, and 10-18 g/dl). The mean values were situated between 25.92% and 28.18% and 9.30 and 10.12 g/dl, with variations between 23.45% - 32.18% and 8.3 – 11.9 g/dl, respectively, with little influence from the experimental variables. This apparent tendency towards anemia was not confirmed by the evolution of the erythrocyte count, the mean values for this parameter being in- the normal physiological range (6-12x10^{12}/l), with individual values varying between 6.05-8.63x10^{12}/l. the mean erythrocyte constants showed small variations compared to the reference values of MCV (13-19 pg), MCH (34-58 fl) and MCHC (31-37 g/dl). So, the values recorded before and after the treatment were very close, for all the 3 parameters: MCV 13.98-13.58 pg; MCH 38.98-37.94 fl and MCHC 35.87-35.82 g/dl.

For group 2, the evolution of the erythrocyte parameters was characterized by the mean values of hematocrit and hemoglobin that were in the normal physiological range (32-48% and 10-18 g/dl), the variation recorded (26.99-33.23%, and 9.5-12,3g/dl) not being related to any experimental variable. Regarding the total erythrocyte count, the mean values were in-between the physiological limits (6-12x10^{12}/l), varying between 6.31-7.87x10^{12}/l. the mean erythrocyte constants also presented minor fluctuations, compared to the reference values for MCV (13-19 pg), MCH (34-58 fl) and MCHC (31-37 g/dl). Their values recorded before and after treatment were very close (15.63-15.36 pg; 43.44- 42.57 fl and 35.98-36.08 g/dl).

The evolution of the erythrocyte parameters, in group 1, was characterized by the mean values of hematocrit and hemoglobin that were in the normal physiological range (32-48%

and 10-18 g/dl), the variation recorded (25.41-33-96%, and 9.2-10.8 g/dl) not being related to any experimental variable. Similar aspects were observed in the total erythrocyte count (5.52-7.28 T/l), that showed values in the normal physiological range (6-12 T/l). Also, the mean erythrocyte constants did not present any major differences before an after treatment, varying between 16.07-15.86 pg, 45.81-44.23fl and 35.16-35.95 g/dl, compared to the reference values of MCV (13-19 pg), MCH (34-58 fl) and MCHC (31-37g/dl).

Table 2. Mean values of the leukocyte parameters recorded in the pre-clinical testing of the product

Group No.		*A 1*	*A 2*	*A 3*
		Mean ± SD	*Mean ± SD*	*Mean ± SD*
Before treatment / initial				
Leucocytes (G/l)		*12,91±3,210*	*10,08±2,370*	*8,03±1,755*
Leukocyte count (%)	Neutrophils	*37,40±9,864*	*45,20±7,155*	*48,60±13,957*
	Eozinophils	*3,40±1,517*	*5,20±0,447*	*5,00±2,345*
	Bazophils	*0,20±0,447*	*0,20±0,447*	*0,40±0,548*
	Limfocytes	*50,20±9,121*	*40,00±7,517*	*39,40±14,502*
	Monocytes	*8,80±0,447*	*9,20±1,643*	*6,60±2,881*
After treatment / final				
Leukocytes (G/l)		*11,46±2,502*	*8,43±2,370*	*7,958±4,414*
Leukocyte count (%)	Neutrophils	*38,60±8,325*	*43,40±3,435*	*44,40±10,738*
	Eozinophils	*3,25±1,500*	*6,40±2,191*	*3,80±3,033*
	Bazophils	*0,20±0,447*	*0,00±0,000*	*0,40±0,548*
	Limfocytes	*49,60±5,771*	*44,20±7,120*	*42,80±10,330*
	Monocytes	*6,60±2,302*	*8,00±3,536*	*9,00±2,121*

The evolution of the leukocyte parameters in group 1 (table 2) indicated a decreasing trend of the total leukocyte count (from 12.91 to 11.46 G/l), without exceeding the physiological limits (6-12 G/l). From the individual and mean values recorded for leukocyte sub-populations, it can be noticed that the mean values of neutrophils (37,4 – 38,6%) varied between the physiological limits (20-74%); similar distributions were recorded for the eosinophil subpopulations, where the means id not exceed the superior limit (15%), the highest individual value being recorded before treatment. The dynamic of the lymphocyte population was characterized by small variations (from 36.00 to 60.00%), situated in-between the physiological limits (35-75%). The monocytes percentage was close to the superior limit of the physiological range, being higher before treatment (8.8%) compared to the post treatment period (6.6%), but without any signs of monocytosys. Regarding the basophils, their numbers were scarce (1% before and after treatment).

The evolution of the leukocyte parameters in group 2 was characterized by a decrease of the total leukocyte count (from 10.08 to 8.43 G/l), the values recorded being situated in the normal physiological range (6-12 G/l). The individual and mean values recorded for the leukocyte sub-populations revealed variations within the normal physiological range. The mean values for the neutrophils (45.2– 43.4 $x10^9$/l) varied within the physiological limits (20-74 %); similar distributions were recorded for the eosinophils (5,2-6,4 %) and monocytes

(9,2-8,0 %) subpopulations, with values situated between the physiological limits. The dynamic of the lymphocyte population was also characterized by small variations (from 40.00 to 44.20%), with values situated within the physiological limits (35-75 %).

The evolution of the leukocyte parameters in group 3, indicates the same decreasing trent in the total leukocyte count (from 8.03 to 7.95 G/l), without exceeding the normal physiological range (6-12 G/l). A special relevance can be attributed to the individual and mean values of the neutrophils (48.6– 44.4%) that included variation within the normal physiological range (20-74%). Similar distributions were recorded for the eosinophils (5-3.8%) and monocytes (6.6-9%) populations. The mean values of the monocytes did not exceed the upper limit (10%) and did not show any tendency toward monocytosis. The dynamic of the lymphocyte population was also characterized by small variations (from 39.4 to 42.80%), with values situated within the physiological limits (35-75 %).

Table 3. Mean values of the biochemical parameters, recorded in the pre-clinical testing of the product on adult horses

Group No.	*1*	*2*	*3*
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
Before treatment / initial			
TP (g/l)	*59.60 ± 1.673*	*67.60 ± 6.986*	*69.80 ± 5.975*
Alb (g/dl)	*3.10 ± 0.265*	*2.64 ± 0.265*	*2.86 ± 0.134*
Glob (g/l)	*36.40 ± 5.857*	*41.60 ± 3.66*	*41.20 ± 2.775*
GLU (mmol/l)	*4.06 ± 0.428*	*3.66 ± 1.716*	*5.02 ± 0.130*
AST (U/l)	*339.60 ± 45.698*	*318.60 ± 21.721*	*320.20 ± 21.052*
GGT (U/l)	*19.40 ± 3.130*	*22.00 ± 10.654*	*20.00 ± 1.871*
CK (U/l)	*349.00 ± 40.348*	*388.40 ± 54.784*	*392.00 ± 8.803*
CRE (µmol/l)	*66.40 ± 8.562*	*86.80 ± 8.983*	*120.00 ± 44.345*
TBIL (µmol/l)	*18.40 ± 1.817*	*14.60 ± 6.066*	*22.40 ± 5.413*
BUN (mmol/l)	*5.84 ± 1.092*	*6.02 ± 1.270*	*6.48 ± 0.581*
CA (mmol/l)	*3.35 ± 0.459*	*2.922 ± 0.579*	*3.06 ± 0.195*
NA+ (mmol/l)	*135.60 ± 8.735*	*135.40 ± 5.177*	*134.60 ± 5.683*
K+ (mmol/l)	*4.10 ± 0.797*	*5.10 ± 1.351*	*4.48 ± 0.856*
TCO2 (mmol/l)	*20.80 ± 1.643*	*22.20 ± 1.789*	*23.00 ± 3.536*
After treatment / final			
TP (g/l)	*66.60 ± 7.197*	*75.00 ± 3.240*	*71.60 ± 3.647*
Alb (g/dl)	*7.88 ± 11.254*	*2.82 ± 0.179*	*3.06 ± 0.241*
Glob (g/l)	*40.00 ± 4.950*	*43.00 ± 4.301*	*41.80 ± 8.701*
GLU (mmol/l)	*5.40 ± 1.177*	*5.06 ± 0.336*	*5.14 ± 0.397*
AST (U/l)	*316.20 ± 32.299*	*318.60 ± 21.373*	*306.40 ± 15.372*
GGT (U/l)	*19.80 ± 0.837*	*23.80 ± 8.198*	*27.80 ± 4.147*
CK (U/l)	*401.60 ± 14.792*	*346.00 ± 115.358*	*392.40 ± 16.906*
CRE (µmol/l)	*98.68 ± 40.678*	*117.00 ± 25.942*	*102.20 ± 8.899*
TBIL (µmol/l)	*19.30 ± 8.423*	*21.00 ± 4.950*	*22.20 ± 3.347*
BUN (mmol/l)	*7.52 ± 1.429*	*6.14 ± 0.195*	*6.08 ± 0.965*
CA (mmol/l)	*2.96 ± 0.195*	*2.88 ± 0.308*	*2.82 ± 0.179*
NA+ (mmol/l)	*136.40 ± 6.348*	*134.20 ± 4.868*	*130.80 ± 3.194*
K+ (mmol/l)	*4.18 ± 0.923*	*4.22 ± 0.804*	*3.82 ± 0.665*
TCO2 (mmol/l)	*20.80 ± 1.924*	*25.40 ± 1.817*	*20.40 ± 2.074*

The evolution of the biochemical parameters for group 1, presented in table 3, revealed normal characteristics of the metabolic profile in all recreational horses, with more or less important changes in the determined indices. The evolution of protein concentration was characterized by a constant value, the individual fluctuations ranging from 54 to 72 g/l, all values being below the upper physiological limit (80 g/l). The mean albumin concentration (3.10-7.88 g/dl) and albumin concentration (36.40-40.00 g/l) included individual values that are in normal physiological ranges, both before and after treatment. Also, the blood sugar concentration (4,06-5,4 mmol/l), AST (339,6-316,2 U/l), GGT (19,40-19,80 U/l), total bilirubin (18,40-19,30 µmol/l) and creatinine levels (66,4-98,68 µmol/l) were situated in the normal physiological ranges. The blood urine nitrogen showed mean (5,84-7,52 mmol/l) and individual values close to the upper physiological limit (8,9 mmol/l) while the sodium (135,6-136,4 mmol/l), calcium (3,35-2,96 mg/dl), potassium (4,10-4,18 mmol/l) and creatin phosphokinase (349,0-401,6 U/l) values did not show any major changes. Also, the total carbon dioxide (20,8 mmol/l) showed values within the normal physiological range.

In group 2, the biochemical parameters were characterized by variations of the mean and individual values. The serum protein levels had a constant value, with individual variations ranging from 58.0 to 80.0 g/l, situated under the upper limit of the normal physiological range. The mean serum albumin (2.64-2.82g/dl) and globulin (41.60-43.00 g/l) concentrations were in-between the normal physiological ranges, both before and after the treatment. Also, the blood sugar concentration (3.66-5.06 mmol/l), AST (318.6 U/l), GGT (22-23.8 U/l), total bilirubin (14.6-21.0 µmol/l) and creatinine levels (86.8-117.0 µmol/l) were situated in the normal physiological ranges. The blood urine nitrogen showed mean (6,2-6,14 mmol/l) and individual values close to the upper physiological limit (8.9 mmol/l). On the other hand, the sodium (135.4-134.2 mmol/l), calcium (2.922-2.88 mg/dl), potassium (5.1-4.22 mmol/l) and creatine phosphokinase (388,4-346,0 U/l) values did not show any major changes. Also, the total carbon dioxide (22.2- 25.4 mmol/l) showed values within the normal physiological range, close to the index cited in Merck Veterinary Manual

In group 3, the evolution of the biochemical parameters revealed small but non-significant changes in the individual and mean values of the determined indices. The evolution of protein concentration was characterized by a constant value, the individual fluctuations ranging from 60.0 to 76.0 g/l, all values being below the upper physiological limit (80 g/l). The mean albumin (2.86-3.06g/dl) and globulin (41.2-41.8 g/l) concentrations were in-between the normal physiological ranges. Also, within the normal physiological ranges, cited in Merck Veterinary Manual, fell other biochemical indices, like blood sugar concentration (5,02-5,14mmol/l), AST (320,2-306,4 U/l), GGT (20,0-27,8 U/l U/l), total bilirubin (22,4-22,2µmol/l µmol/l) and creatinine levels (120,0-102,2µmol/l). The blood urine nitrogen showed mean (6,48-6,08 mmol/l) and individual values close to the upper physiological limit (8.9 mmol/l) while the sodium (134,6-130,8 mmol/l), calcium (3,06-2,82 mg/dl mg/dl), potassium (4,48-3,82 mmol/l) and creatin phosphokinase (392,0-392,4 U/l U/l) concentrations expressed only minor changes, without statistical significance. Regarding the mean values of the blood total carbon dioxide concentration, it can be noticed that the values recorded before and after treatment are close to each other (20,4 - 23,0 mmol/l) and close to the cited reference index (23,0 mmol/l).

Conclusions

By correlating the results recorded in the tests carried out, it can be noticed that most of the physiological indices were situated within the normal ranges, indicating a good general

tolerance to drotaverine hydrochloride.

Regarding the local tolerance, it can be noticed that no adverse effects were recorded after administering the therapeutically doses and the overdoses, by IM and IV administration. Administering the product subcutaneously, led to adverse reaction in 4 out of 5 cases, characterized by a mild swelling of the inoculation area, accompanied by discomfort and discreet pain.

Based on the results recorded in the clinical, hematological and biochemical tests, Drotavet administering in therapeutic and double doses did not led to systemic adverse reactions, immediate (due to administration, absorption and metabolizing of the product) or delayed (during a period of clinical observation, extended over 15 days from the administration of the last dose). The products' dosing in therapeutically and double doses, by IM and IV administering, was also well tolerated, no local allergic reaction being recorded.

Based on the results recorded in the clinical, hematological and biochemical tests, we appreciate that Drotavet product is well tolerated, no matter the physiological state of the target animals; the product can be administered to horses from the most sensitive categories. The animals exhibited a high tolerance to the double-dosage of Drotavet, increasing the products' efficacy potential.

References

1. Reece W.O. *Functional anatomy and physiology of domestic animals*, Lippincot, 2005
2. GHERGARIU S. POP A., LASZLO K., MARINA SPÂNU (2000). Manual de laborator clinic veterinar. Editura All Educaţional.
3. Pap A, Topa L, Balgha V, et al: Drotaverine antagonizes spasm of Oddi's sphincter provoked by morphine in man (abstract). Gastroenterology 1997; 112(4):A519.
4. Bolaji OO, Onyeji CO, Ogundaini AO, et al: Pharmacokinetics and bioavailability of drotaverine in humans. Eur J Drug Metab Pharmacokinet 1996; 21(3):217-221.
5. Kapui Z, Tardos L, Blasko G, et al: The mechanism of in vitro antiaggregatory effect of drotaverine-acephyllinate (Depogen) (abstract). Thromb Haemost 1991a; 65(6):1079.
6. Kapui Z, Tardos L, Blasko G, et al: Stimulation of prostacyclin release by drotaverine-acephyllinate (Depogen), drotaverine and pentoxifylline (abstract). Thromb Haemost 1991b; 65(6):1334
7. Kapui Z, Hermecz I, Sarkadi B, et al: Comparative studies of drotaverine-acephyllinate (Depogen) and pentoxifylline (Trental). Thromb Res 1992; 66(6):693-706.

HEMATOLOGICAL AND BIOCHEMICAL AND STUDIES IN DOGS TREATED WITH A PRODUCT BASED ON OXYTETRACYCLINE, NITROFURANE, IODOFORME AND BISMUTH SUB-NITRATE

Laurenţ Ognean, Viorica Chiurciu, Nicodim Iosif Fiţ, Ildikó Barabási, Alexandra Arion, Adrian Vlasiu
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăştur Street, 400372, Romania
lognean@yahoo.com

Abstract

The study aimed to reveal the hematological, biochemical parameters in healthy dogs treated with an ointment of oxytetracycline, nitrofurane, iodoform and bismuth sub-nitrate. For the experiment we used two groups of dogs that were treated with a different amount of ointment. The first group (1A; n=10) was treated for 5 consecutive days applying the ointment once every day on intact healthy skin from the interdigital, axilary and inguinal areas of the right side. The second group (2A; n=10) was treated the same way, but the ointment was applied twice daily. A control group (n=10) was kept in the same conditions during the tests to serve as comparison. After applying the ointment daily as stated in the test protocol, clinical examinations were conducted to monitor the local as well as the general reactions produced by the product. These examinations took place after 1, 24, 48 and 120 hours from the first administration of the product, grading the results from 0 to 4 according to the Draize (1971) scale for erythema and/or edema. The final tests were conducted after 10 to 20 hours from the last administration of the product, consisting of a general examination of the subjects and blood sample collections for hematology and biochemistry investigations. The results revealed that the product didn't have an adverse effect on the packed cell volume and hemoglobin levels of the test subjects, these parameters remained in the reference range of the species. The mean value of PCV was 46.74% (37.06% - 53.67%). The mean value of hemoglobin was 16.34 g/dl, the (13.4 g/dl - 17.9 g/dl). The product did not induce post-therapeutic anemia red blood cell count remaining between 5.71 and 7.75 10^{12}/l. All other hematological parameters were in reference range. The metabolic profile showed that the mean values of alkaline phosphatase, kreatin phosfokinase and phosphor were higher than the normal physiological values, before and after the trial. AST, GGT, total proteins, globulins, ureic nitrogen, and calcium, levels were all in reference range. In conclusion the tested product did not modify significantly any hematological or biochemical parameters even when applied in a double dose.

***Keywords**: Pododermin, normal/double dose, hematology, biochemistry, dogs*

Introduction

Bacterial resistance to tetracycline develops in time especially in individuals that underwent repeated and/or long term treatment with the substance. This effect is plasmicide mediated and is induced trough prolonged exposure to the antibiotic agent. Resistance appears when active concentrations are not reached within the bacterial cell due to deficiencies in the active transport system or reduced membrane permeability. The microorganisms' resistance to nitrofurane is thought to be influenced by some genes that influence the activity of H nitoreductase (RdxA) and H flavin oxidoreductase (FrxA).

The purpose of this study was to demonstrate the therapeutic safety of an ointment containing oxytetracyclin, nitrofurane, iodoforme and bismuth nitrate in dogs, applied in normal and in double dose. The products effect on the organism was evaluated through hematological and biochemical blood tests as well as daily evaluations of the treated areas using the Draize scale.

Materials and methods

This study was conducted on privately owned dogs from Cluj-Napoca as well as dogs belonging to the Faculty of Veterinary Medicine from Cluj-Napoca and the data obtained was examined by several departments of this institution including Physiology, Pharmacology, Microbiology and Clinical Laboratory.

Biological material

The tests subjects with ages over 5 months, weighing over 1 kilogram were split into three groups. The subjects were treated with an ointment (Pododermin) of the following composition: 3g hidrocloric oxytetracyclin, 1g nitofuran, 10g iodoform, 1g salicylic acid, 6g subnitric bismuth.

The groups were divided as follows:

- Group 1 (n=10) – subjects from the first group had the product applied once a day for 5 days on intact healthy skin on the right front and hind limbs in the interdigital, axilar and inguinal area;
- Group 2 (n=10) – subjects from the first group had the product applied twice a day for 5 days on intact healthy skin on the right front and hind limbs in the interdigital, axilar and inguinal area;
- Group 3 (n=10) – subjects were kept in the same conditions as the first two groups, but were not treated, serving as control group;

The dogs included in this study were chosen after they underwent clinical examinations, hematological and blood chemistry tests 7 days before the beginning of the study.

The study followed a strict timetable divided into days and hours.

Study protocol. Day 0 preceded the products use, when the subjects behavior was monitored, including feeding and drinking habits, the dogs were weighed and clinically reevaluated. Days 1 to 5 the product was administered as described before according to each groups protocol. Subjects were examined for any general and/or local reactions to the product and any local reactions such as erythema or edema was scored from 0 to 4 according to the Draize scale after 1, 24, 48 and 120 hours from the first administration of the product. During the entire duration of this study, subjects have had free access to food and water. A final examination was conducted 10-20 hours post-therapeutically, after the last administration of the product (Pododermin), including clinical evaluation and repeating all blood tests. All subjects were kept for observation 10 days after the last administration of the product; individuals from the control group were housed in the same living conditions as the test subjects throughout this study. The monitored parameters were as follows: from hematology PCV- packed cell volume, Hb- hemoglobin, total erythrocyte number, MCV- mean cell volume, MCH- mean corpuscular hemoglobin, MCHC- mean corpuscular hemoglobin concentration, Leucocytes, Neutrolfiles, Monocytes, Eozinofiles, Bazofiles, Limfocytes, PLT – platelate count; from blood chemistry ALB – albumine; AST – aspartate aminotransferase; ALP – alkaline phosphatase; CK – creatin fosfokinaza; CA - calcium; GGT – gamma glutamil transferase; TP – total proteins; GLOB – globuline; BUN – blood ureea nitrogen; CK – creatine kinase; PHOS - phosfore. Reference ranges were taken from Merck's Veterinary Manual, Ghegariu et al. (2000), Veterinary clinical laboratory Handbook and Reece (1996) Physiology of domestic animals.

Results and discussions

Hematology and blood chemistry test results from the three groups of dogs participating in the study of an ointment containing hydrocloric oxytetracyclin, nitofuran, iodoform, salicylic acid, subnitric bismuth are shown in tables 1, 2, 3 and 4.

The complete blood cell count test results did not change significantly in any of the three groups that took part in this study. Only small fluctuations were observed in the hematological parameters tested between the pre- and post-treatment stages of the study. All parameters were within the reference ranges shown in The Merck Veterinary Manual.

The white blood cell count revealed a mild drop in the total leucocytes number in all three groups, but values stayed within reference range. The leucocyte formula did not reveal any major changes in the percentage of the different white blood cell types in any group. Any changes between the initial and final stages of the study were within reference range.

The blood chemistry test results revealed more less significant changes of some of the parameters in all three groups of dogs. Alcaline phosphatase values were above the reference range at the end of the treatment period. Gamma-glutamile transferase values remained constant at a level under 5 U/l throughout the study. Creatin phosphokinase levels were elevated in all three groups taken into study before and after the treatment period. Aspartat aminotransferase values remained within the cited reference range. Total protein, globulin and blood urea nitrogen levels presented mild fluctuations that remained in the normal value spectrum for canines. Albumin and globulin values present also minor changes, with levels still remaining within the cited reference range. Calcium was found to be at a normal value in both pre- and post-therapeutic stages of the study, whereas phosphor levels were elevated throughout the study in all three canine groups.

Table 1. Mean hematology values from the pre- and post-therapeutic stages of the study in the three groups of dogs

Group		*1*	*2*	*3*
		Mean+ standard dev.	*Mean+ standard dev.*	*Mean+ standard dev.*
Pre-therapeutic/initial				
PCV(%)		*46,05±4,47*	*45,85±3,61*	46,12±4,94
Hb(g/dl)		*16,34±1,27*	*16,71±1,40*	16,02±1,58
Erythrocyte nr.(T/l)		*7,16±0,59*	*7,45±0,56*	6,64±1,02
Mean corpuscular values	MCV/(fl)	*64,53±3,53*	*63,04±4,37*	70,13±4,68
	MCH(pg)	*23,22±0,70*	*22,88±1,63*	24,62±1,91
	MCHC(g/dl)	*35,56±1,35*	*36,43±0,72*	34,78±1,20
Post-therapeutic/final				
PCV(%)		*46,74±5,10*	*45,66±4,12*	46,26±4,70
Hb(g/dl)		*16,34±1,71*	*16,49±1,65*	16,16±1,45
Erythrocyte nr.(T/l)		*6,95±0,75*	*7,37±0,79*	6,69±0,92
Mean corpuscular values	MCV/(fl)	*67,76±3,30*	*68,03±64,49*	69,85±3,53
	MCH(pg)	*23,9±0,82*	*23,52±23,20*	24,50±1,61
	MCHC(g/dl)	*35,00±1,37*	*34,58±35,97*	34,99±1,04

Reference from Merck Veterinary Manual (2010)-**PCV**: 37-55; **Hb**: 12-18; **Erythrocyte nr.**: 5,5-8,5; **MCV**: 60-77; **MCH**: 19,5-24,5; **MCHC**: 32-36.

Table 2. Mean white blood cell count values from the pre- and post-therapeutic stages of the study in the three groups of dogs

Group		*1*	*2*	*3*
		Mean+ standard dev.	*Mean+ standard dev.*	*Mean+ standard dev.*
Pre-therapeutic/initial				
Leucocytes (G/l)		*12,09±2,55*	*13,26±1,81*	*11,11±1,59*
Leucocytes formula (%)	Neutrophile	*65,5±2,98*	*65,3±3,30*	*64,8±2,74*
	Eozinophile	*4,3±1,63*	*6,8±1,13*	*5,9±1,44*
	Basophile	*0±0*	*0±0*	*0±0*
	Lymphocyte	*22,4±2,87*	*21,2±3,70*	*22±3,97*
	Monocyte	*7,9±1,19*	*6,7±2,31*	*7,3±1,49*
Post-therapeutic/final				
Leucocytes (G/l)		*11,81±3,04*	*12,43±1,92*	*11,03±1,14*
Leucocytes formula (%)	Neutrophile	*66,1±2,60*	*66,3±1,82*	*65,7±2,40*
	Eozinophile	*4,4±0,96*	*7,4±1,07*	*5,7±1,25*
	Bazophile	*0±0*	*0±0*	*0±0*
	Limphocyte	*21,7±2,62*	*20,1±2,51*	*21,7±2,26*
	Monocyte	*7,8±1,13*	*6,2±1,31*	*6,9±1,52*

Reference from Merck Veterinary Manual (2010)-**Leucocytes**: 6-17; **Neutrophile**: 60-70; **Eozinophile**: 2-10; **Bazophile**: 0; **Limphocyte**: 12-30; **Monocyte**: 3-10.

Table 3. Mean blood chemistry values from the pre- and post-therapeutic stages of the study in the three groups of dogs

Group		*1*	*2*	*3*
		Mean+ standard dev.	*Mean+ standard dev.*	*Mean+ standard dev.*
Pre-therapeutic/initial				
TP	(g/dl)	5,74±0,34	5,84±0,53	6,3±0,86
ALB	(g/dl)	3,4±0,44	3,34±0,30	3,22±0,24
GLOB	(g/dl)	2,36±0,33	2,38±0,38	2,74±0,41
GGT	(U/l)	>5	>5	>5
ALP	(U/l)	111,6±14,15	103,6±6,11	94,6±10,53
AST	(U/l)	39±3,08	38,4±3,21	39±7,07
CK	(U/l)	311,8±85,00	223,8±66,98	134,4±46,25
BUN	(mg/dl)	16,4±3,97	19,2±2,68	19,6±4,16
CA	(mg/dl)	10,44±1,54	10,22±1,24	10,82±1,09
PHOS	(mg/dl)	9,1±1,14	7,76±1,45	8,36±0,96
Post-therapeutic/final				
TP	(g/dl)	6,1±0,63	6,4±0,72	6,24±0,59
ALB	(g/dl)	3,42±0,33	3,48±0,36	3,44±0,24
GLOB	(g/dl)	2,52±0,43	2,54±0,39	2,5±0,35
GGT	(U/l)	>5	>5	>5
ALP	(U/dl)	118±17,68	107,2±6,80	103±8,86
AST	(U/l)	40,2±2,59	39,6±2,07	41,2±1,48
CK	(U/l)	277,6±99,16	207,6±74,51	159,2±29,08
BUN	(mg/dl)	18,6±4,04	19±0,39	19,6±3,36
CA	(mg/dl)	10,92±1,93	10,54±1,47	10,94±1,48
PHOS	(mg/dl)	9,54±1,52	7,72±0,96	8,84±1,47

Reference from Merck Veterinary Manual (2010)-**TP**: 5,5-7,5; **ALB**: 2,6-4,0; **GLOB**: 2,1-3,7; **ALP**: 10,6-101; **AST**: 8,9-49; **CK**: 14-120; **BUN**: 8,8-26; **CA**: 8,7-11,8; **PHOS**: 2,9-6,2

Mean platelet count results show that in group 1 and 3 platelet levels were lower post-therapeutic when compared to the beginning of the study. In group 2 platelet count values were elevated in the post-therapeutic stages of the study. These changes in mean platelet count remained nonetheless in reference range in all three groups. Results obtained for this parameter demonstrate that the product used in the study does not interfere with the subjects' coagulation factor. Local tolarance or modifications were graded from 0 to 4 according to Draizer's scale demonstrated the absence of any adverse effect on the skin when the product was applied in normal and in a double dose.

Table 4. Mean platelet count values from the pre- and post-therapeutic stages of the study in the three groups of dogs

Groups		1	2	3
		Mean + standard dev.	*Mean + standard dev.*	*Mean + standard dev.*
Pre-therapeutic/initial				
Platelet count	(10^9/l)	354,2±59,33	297,8±61,85	309,1±59,10
Post-therapeutic/final				
Platelet count	(10^9/l)	326,4±92,32	316,2±53,13	307,7±48,44

*Reference from Merck Veterinary Manual (2010)-**Platelet count**: 200-900

Test results gathered from hematological, blood biochemistry and local reaction examinations demonstrated that the product tested in this study does not cause any general or local adverse reactions, when applied in normal or double dose. Generalized adverse reactions were non existened on reexamination performed 10 days after the last administration of the product, indicating that the ointment was well tolareted and metabolized by the subjects. Local reactions such as erytheama, papulae, edema and prurit were also absent in all groups treated, the product was absorbed quickly and efficiently. Taking into account the dogs natural reaction of licking the treated areas, the ointmen must be applied in a thin layer untill it is well absobed localy and the subject needs to monitored for 5 minutes after administration to prevent it from licking the treated area.

Conclusions

Pre- and post-therapeutic test results obtained from the three groups of dogs participating in the study as well as the statistic calculations performed on the gathered data showed that the majority of the tested parameters (hematology and blood chemistry) were in refference range. From a clinical point of view it is relevant to mention that all subjects that took part in the study were stable throuout the course of the entire study periode regarding: temperature, general health, apetite, normal growth spur, hematological and blood biochemistry parameters and the absence of any adverse reactions noticible clinicaly. According to the data aquiered thru the various test performed on the subjects participating in the study, the tested product can be used safelly in dogs in the recomended dosage as well as the double amount of the reccomended dosage, without producing any adverse reactions.

Bibliography

1. GHERGARIU S. POP A., LASZLO K., MARINA SPÂNU (2000). Manual de laborator clinic veterinar. Editura All Educaţional.
2. MOELLERING, R.C. (1983). *In vitro* antibacterial activity of the aminoglycoside antiobiotics. *Rev. Infect. Dis.,* 5(2): S212-S231.
3. NECHIFOR M, BUIUC D, DIACONU E, POIATĂ A, TESLARIU E, FILIP C, NEGRU A, ANTONESCU C. (1992) Pharmacological studies of sulbactam and its association with semisynthetic beta-lactam antibiotics. Rev Med Chir Soc Med Nat Iasi.; 96(1-2):51-5.
4. PINTEA V., CODRUŢ M., MANTA D.A.M SALAGEANU G., (1982) - Fiziologie Medical-Veterinară; Ed. Didactică şi pedagogică, Bucureşti
5. REECE O. W. (1996). Physiology of domestic animals.Blackwell Publishing
6. RICHEZ, P. (1994) Antibacterial activity of tetracycline oxytetracylcine against human gut flora. Study No. SVL 029. Unpublished report from Datavet Veterinary Drug Consultants. Submitted to WHO by Intervet International, Boxmeer, Netherlands
7. SNELL, J.F., GARKUSCHA, R., & ALLEN, E.L. (1957). Radioactive Oxytetracycline II. Distribution in C-57 BL Mice (preliminary experiments). *Antibiot. Ann.,* 502-506.
8. URAM, J.A., FRENCH C.E., BARRON, G.P., & SWIFT, R.W. (1954). The effect of high levels of terramycin or streptomycin on growth, reproduction and lactation of the rat. *J. Nutr.,* 481-492.

ULTRASTRUCTURAL STUDY OF THE NICTITATING GLAND IN DOGS

Cazacu P. Petru
CSE Equester Riding Club, Iaşi, Al. Viilor no. 22, 707515
cazacupetru@gmail.com

Abstract

The purpose of this study is to describe the ultrastructural characteristics of tubuloalveolar epithelial cells of the nictitating gland in dogs, especially secretory vesicles. Nictitating membrane was harvested from both eyes from two adult dogs. Incision and dissection of the bulbar and palpebral conjunctiva and underlying structures to its complete isolation were performed. Small portions from sectioned area were harvested and immediately prefixed in buffered glutaraldehyde, postfixed in buffered osmium tetraoxide, rapidly dehydrated in graded ethanols and embedded in epoxy resin for electron microscopy. Ultra thin sections were contrasted with uranyl acetate followed by lead citrate and examined under a Philips CM 100 electron transmission microscope.The secretion vesicles of the epithelial cells of the nictitating gland are presented as serous type vesicles (with intense and uniform electron density) and mucosal type vesicles (with low electron density). Therefore, the secretion product of the nictitating gland in dog is mixed, sero-mucous.The secretory vesicles size ranged from 0.39 to 1.09 μm.

Keywords: *Nictitating gland, secretory vesicles, ultrastructure, dogs*

Introduction

In dogs the nictitating gland (*third eyelid superficial gland*) is located on the distal portion of the third eyelid and on the bulbar part of nictitating membrane cartilage, nictitating gland progresses to a few millimeters from the horizontal edge of the nictitating membrane cartilage (Morgan *et al*., 1993 and Samuelson, 1999, as cited in Lazard, 2004; Cazacu *et al*., 2005). Nictitating gland is a tubuloacinar structure, exocrine, whose sero-mucous secretion is eliminated through several excretory ducts that open through orifices located in the deep posterior face of the nictitating membrane (Kaswan, 1985, as cited in Delafon, 2002).

The nictitating gland in dog is the second important lachrymal gland after the main lachrymal gland, regarding the tear film secretion, respectively its aqueous fraction (Severin, 1973, as cited in Chadieu, 1994). The aqueous layer contributes to the viscosity of the tear film [14]. It contains lactoferrin, lysozyme, secretory IgA, IgG, IgM, albumin, transferrin, ceruloplasmin, glycoproteins, participating in the defences of the ocular surface. Also, contains nutrients for the avascular cornea, namely inorganic salts, glucose, oxygen and proteins [4, 11, 14, 20]. The aqueous layer contains growth factors involved in corneal regeneration processes, whose levels increase in glandular secretion product when the corneal epithelium is damaged [32].

Secretory nictitating gland insufficiency with different etiologies causes lack of lubrication of the cornea with exposure to the biotic or inert aggressive agents, failure to initiate processes of defences through the inadequacy of antibacterial and growth factors, these causes keratoconjunctivitis *sicca*.

Description of normal histological and histochemical aspects of the nictitating gland was performed in several mammals' species: in wolves (*Canis lupus lupus*) [22], dogs (*Canis familiaris*) [6-10, 19], red foxes (*Vulpes vulpes*) [22], cats (*Felis silvestris catus*) [10], horses (*Equus caballus*) [10], pigs (*Sus scrofa domestica*) [26, 29], One-Humped Camels (*Camelus dromedarius*) [1, 25], roe deers (*Capreolus capreolus L*.) [28]. An ultrastructural study of the

nictitating gland in dog was not identified in romanian or foreign bibliography consulted. There are few studies on the Harderian gland in some species: in *black sea bottlenose dolphin (Tursiops truncatus ponticus)* [3], *albino rats* [5, 17, 18], several species of gerbils (*Gerbillus gerbillus*, *Meriones crassus*, *Meriones lybicus*, *Psammomys obesus*, *Ctenodactylus vali*) [16, 18], gundi (*Ctenodactylus vali*) [18], rabbit (*Oryctolagus cuniculus*) [30], and the nine-banded armadillo (*Dasypus novemcinctus*) [31].

The purpose of this study is to describe the ultrastructural characteristics of tubuloalveolar epithelial cells of the nictitating gland in dogs, especially secretory vesicles. Similar studies were not identified in romanian or foreign bibliography consulted.

Materials and Methods

Nictitating membrane was harvesting from both eyes of two dogs, a male dog, Pekingese breed, 3 years old, 6 kg and a female of mongrel dog, 5 years old and 8 kg. The bodies of dogs were originated from various private veterinary practices (from Iasi county) and prosecture of the Faculty of Veterinary Medicine from Iasi.

Bilateral excision of the nictitating membrane and gland was performed by incision and dissection of the bulbar and palpebral conjunctiva and underlying structures to its complete isolation. Only nictitating membranes with normal clinical appearance were harvesting.

The nictitating membranes were trimmed and mid-sagittally sectioned. Small portions from sectioned area were harvested and immediately prefixed in buffered glutaraldehyde, postfixed in buffered osmium tetraoxide, rapidly dehydrated in graded ethanols and embedded in epoxy resin for electron microscopy. Ultra thin sections were contrasted with uranyl acetate followed by lead citrate and examined under a *Philips CM 100* electron transmission microscopes.

Data were analyzed using descriptive statistical indicators (arithmetic mean, standard error of the mean, 95% confidence interval, standard deviation, minimum and maximum value) using SPSS 10.0 software (SPSS Inc., 2000).

Results and Discussions

Glandular epithelial cells with pyramidal aspect, joined to each other via junctions, forming secretory units with a central lumen. The cells are laid on a basal lamina and are attached by hemidesmosomes type junction (fig. 4; fig. 5 and fig. 6).

The nucleus of the epithelial cells present peripheral and island chromatine (fig. 1 and fig. 2), with average size of 4.61/3.12 μm (tab. 1). Rough endoplasmic reticulum (RER) is located at the base of glandular epithelial cell and perinuclear (fig. 4; fig. 6 and fig. 7). Secretory vesicles are located dorsal of nucleus and less on its sides, to be exocitate in the acini lumen (fig. 1; fig. 2; fig. 4; fig. 5 and fig. 7). These vesicles often are serous with intense and uniform electron density (fig. 2 and fig. 3) and mucous rare with low electron density, bright, with slightly granular material (fig. 4). Secretory vesicle sizes are in range from 0.39 to 1.09 μm (tab. 1).

Ultrastructural study of the nictitating gland of the dog shows and describes, in particular, the appearance of secretory vesicles. According to Pavelka and Roth (2005), which characterizes the electron appearance of different types of secretory vesicles, serous secretory vesicles appear as vesicles with intense and uniform electron density and mucous secretory vesicles appear as vesicles with low electron density, bright and less granular material. Glandular epithelial cells of the nictitating gland of dog are laid on a basal lamina and are

attached by hemidesmosomes type junction. Glandular epithelial cells present the intracellular structures characteristic for exocrine glandular epithelial cells – nucleus, RER, mitochondria and secretory vesicles. Secretory vesicles of epithelial cells of the nictitating gland are serous (with intense and uniform electron density) and mucous (with low electron density).

Table 1. Ultra morphometric aspects of the nucleus and of the secretory vesicles of the epithelial cells of the nictitating gland in dog (µm)

Cytological structures		$x \pm SE_x$	95% CI	s	Min	Max
Nucleus	*Øm*	3,12±0,33	2,26-3,98	0,82	1,95	4,23
	ØM	4,61±0,48	3,37-5,85	1,17	2,86	6,19
Secretory vesicles		0,68±2,538E-02	0,63-0,73	1,18	0,39	1,09

Note: $x \pm SE_x$ – arithmetic mean±standard error of the mean; *95% CI*– confidence interval with 95% probability; *s* – standard deviation; *Min* – minimum value; *Max* – maximum value; *ØM* – large diameter; *Øm* – small diameter.

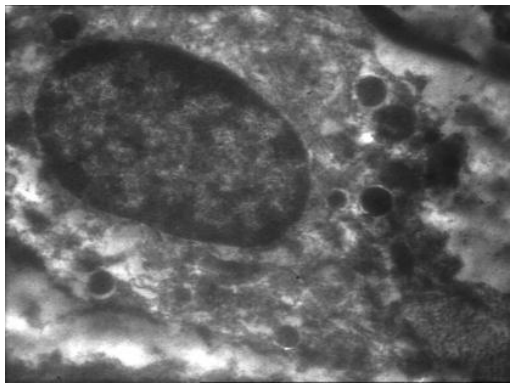


Fig. 1. Epithelial cells of the nictitating gland in dogs. Large ovoid nucleus with peripheral chromatin and serous supranuclear vesicles with intense and uniform electronic density. TEM; x 3400.

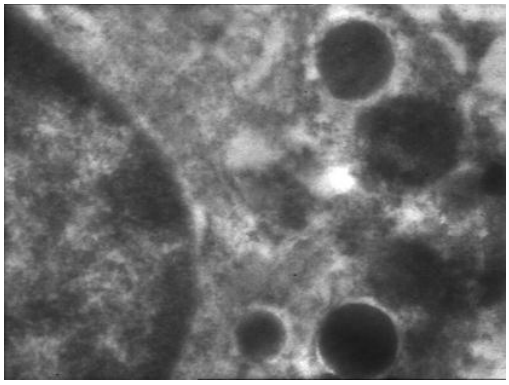


Fig. 2. Epithelial cells of the nictitating gland in dogs. Nucleus with peripheral heterochromatin prepared and serous supranuclear vesicles are present with intense and uniform electronic density. TEM; x 8700.

In the romanian and foreign bibliographic material consulted, I have not found ultrastructural descriptions of the canine nictitating gland, only the paper of Martin *et al*. (1988) on the ultrastructure of the *main lacrimal gland* of the dog. The authors suggest the possibility of three types of secretory epithelial cells in glandular acini as follows: cells with secretory vesicles with low electron density (mucosal type), the second type of cells with secretory vesicles that appear as lipid vesicles, and the third type cells with fine granular vesicles, bright, not sure if they are an artifact or just a stage of maturation of the first type of secretory vesicles. However, due to histological similarities (compound tubuloacionous glands) and histochemical (sero-mucous glands in dogs) of two canine lacrimal glands, main

and nictitating, the study by Martin *et al*. (1988) should be considered. Thus, the research cited is understood that the *main lacrimal gland* would a mixed muco-lipid secretion, which contradicts theories and studies that classify the *main lacrimal gland* of the dog as mixed sero-mucous gland [2, 12, 13, 21]. Other ultrastructural studies should be undertaken simultaneously on both lacrimal glands by the same group of researchers, using the same techniques and interpreted the same microscope to determine the exact type of secretory vesicles, epithelial cell type and type of secretion for each gland in part.

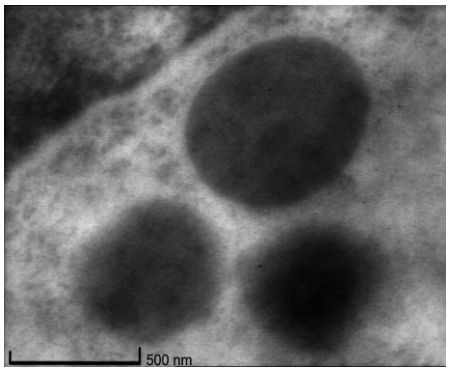


Fig. 3. Epithelial cells of the nictitating gland in dogs. Serous secretory vesicles with intense and uniform electron density. TEM; x 16000.

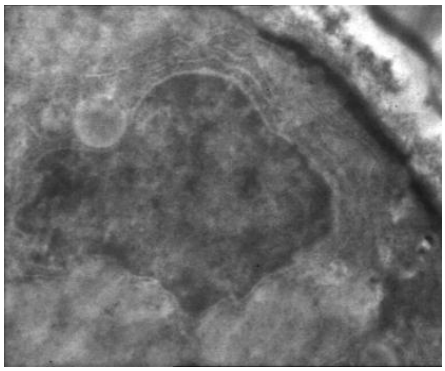


Fig. 4. Epithelial cells of the nictitating gland in dogs. Nucleus with peripheral chromatin prepared, while at the basal pole hemidesmosomes and basal lamina observed, lateral nucleus are two mucous secretions vesicle with low electronic density, light, slightly granular material and under nucleus RER tubules is observed. TEM; x4400.

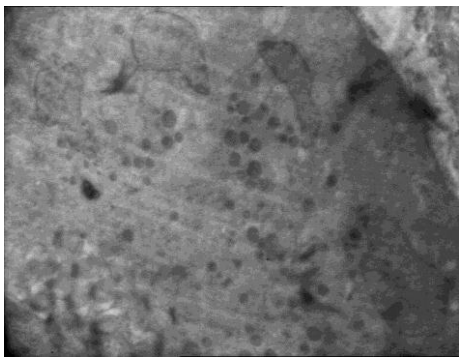


Fig. 5. Epithelial cells of the nictitating gland in dogs. Four glandular epithelial cells of a secretory unit. Peripheral nuclei are prepared in the lower third of the cells and supranuclear secretory vesicles are observed. TEM; x 1100.

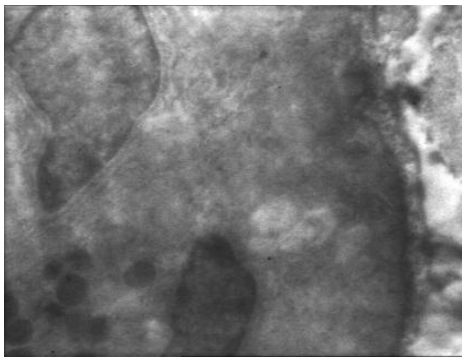


Fig. 6. Epithelial cells of the nictitating gland in dogs. Lower third of two epithelial cells. The nuclei with peripheral chromatin surrounded by the RER cistern and above of the nucleus serous vesicles are observed with high-density electrons. Basal cell pole rests on basal lamina which is attached by hemidesmosome. TEM; x 2400.

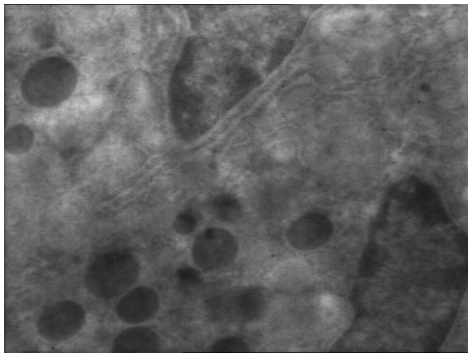


Fig. 7. Epithelial cells of the nictitating gland in dogs. Lower third of two epithelial cells. The nuclei have peripheral heterochromatin prepared, surrounded by the RER cistern and above of nucleus serous vesicles are observed with high-density electrons. TEM; x 3400.

Conclusions

1. The secretory vesicles of the epithelial cells of the nictitating gland are presenting as serous type vesicles (with intense and uniform electron density) and mucosal type vesicles (with low electron density). Therefore, the secretion product of the nictitating gland in dog is mixed, sero-mucous.
2. The secretory vesicles size ranged from 0.39 to 1.09 μm.

References

1. Al-Ramadan Y.S., Ali M.A., 2012 – *Morphological Studies on the Third Eyelid and its Related Structures in the One-Humped Camel (Camelus dromedarius)*. J. Vet. Anat.; Vol. 5; No. 2; p. 71-81.
2. Bacha J.W. Jr., Bacha M. Linda, 2000 – *Color atlas of veterinary histology*. 2nd ed., Lippincott Williams & Wilkins, Baltimore.
3. Bodiak N.D., Stepanova L.V., 1994 – *Harderian gland ultrastructure of the black sea bottlenose dolphin (Tursiops truncatus ponticus)*. J Morphol. May; 220(2), p. 207-221.
4. Borisevich V.B., 1976 – *Lacrimal glands of dogs and cattle in the light of histologic and histochemical findings*. Arkh Anat Gistol Embriol, Sep;71(9), p. 12-17.
5. Brownscheidle C.M., Niewenhuis R.J., 1978 – *Ultrastructure of the harderian gland in male albino rats.* Anat Rec. Mar;190(3), p. 735-753.
6. Cabral P.V., Laus L.J., Dagli Z.L.M., PereiraT.G., Talieri C.I., Monteiro R.E., Mamede V.F., 2005 – *Canine lacrimal and third eyelid superficial glands' macroscopic and morphometric characteristics*. Ciência Rural, Santa Maria, v35, n.2, p. 391-397.
7. Cazacu P., Cotea C., 2007 – *Morphological and cytochemical particularities of nictitating gland in large breed dogs*. Lucrări ştiinţifice, Medicină Veterinară section, USAMV, Iaşi, vol. 50 (9), p. 135-143.
8. Cazacu P., Cotea C., 2008 – *Morphological and micromorphometric characteristics of the third eyelid and nictitating gland in the dog*. Lucrări ştiinţifice, Medicină Veterinară section, USAMV, Iaşi, part I, vol. 51 (10), p. 22-26.
9. Cazacu P., Cotea C., Solcan Carmen, 2005 – *Morfologia glandei superficiale (glanda nictitantă) a pleoapei a treia la câine (Canis familiaris)*. Lucrări ştiinţifice, Medicină Veterinară section, USAMV, Iaşi, p. 150-156.
10. Cazimir Iuliana, 1998 – *Cercetări histologice comparate privind membrana nictitantă şi glanda Harder la diferite specii de animale*. Dissertation, USAMV, Faculty of Veterinary Medicine, Bucureşti.
11. Chaudieu G., 1994 – *L'appareil lacrymal*. Prat. Méd. Chir. Anim. Comp., 29, p. 421-446.
12. Cornilă N., 2001 – *Morfologia microscopică a animalelor domestice*. Vol. II, ALL Medica Publishing, Bucureşti.
13. Cornilă N., Manolescu N., 1995 – *Structura şi ultrastructura organelor la animalele domestice*, Ceres Publishing, Bucureşti.
14. Davidson J. Harriet, Kuonen J. Vanessa, 2004 – *The tear film and ocular mucins*. Veterinary Ophthalmology 7, 2, p. 71–77.

15. Delafon Claire, 2002 – *Contribution à l'étude des modifications histologiques de la glande nictitante de chiens atteints de kératoconjonctivite sèche après un traitement local à la cyclosporine A 0,2 %*. These pour obtenir le grade de Docteur Veterinaire, École Nationale Vétérinaire d'alfort, La Faculte de Médecine de Créteil, France.
16. Djeridane Y., 1992 – *The harderian gland of desert rodents: a histological and ultrastructural study*. J Anat. Jun;180; p. 465-480.
17. Djeridane Y., 1994 – *The harderian gland and its excretory duct in the Wistar rat*. A histological and ultrastructural study. J Anat. Jun;184 (Pt 3), p. 553-566.
18. Djeridane Y., 1996 – *Comparative histological and ultrastructural studies of the Harderian gland of rodents*. Microsc Res Tech. May 1;34(1), p. 28-38.
19. Dugan S.J., Severin G.A., Hungerford L.L., Whitely H.E., Roberts S.M., 1992 – *Clinical and histologic evaluation of the prolapsed third eyelid gland in dogs*. J Am Med Assoc; Dec 15; 201 (12), p. 1861 – 1867.
20. German A.J., Hall E.J., Day M.J., 1998 – *Measurement of IgG, IgM and IgA concentrations in canine serum, saliva, tears and bile*. Vet. Immunol Immunopathol., Jul 8; 64(2), p. 107-121.
21. Hamor R.E., Ehrhart E.J., 2006 – *Eye*. In: *Dellmann's Textbook of Veterinary Histology*, 6th ed., Eurell J.A. and Frappier B.L. (Eds.); Blackwell Publishing, Ames, Iowa, USA.
22. Hraste A., Janicki Z., Zobundzija M., Mihelic D., Jakovac M., Rinck J., 1997 – *Histological and histochemical characteristics of the Glandulae profundae plicae semilunares conjunctivae among some carnivores*. Z. Jagdwiss. 43, p. 120-124.
23. Lazard P., 2004 – *Chirurgie de la luxation de la glande lacrymale superficielle de la membrane nictitante chez le chien: nouvelle technique microchirurgicale*. Revue Méd. Vét., 2004, 155, 2, p. 82-86.
24. Martin C.L., Munnell J., Kaswan R., 1988 – *Normal ultrastructure and histochemical characteristics of canine lacrimal glands*. Am. J. Vet. Res., Sep; 49(9); p. 1566-1572.
25. Mohammadpour A.A., 2009 – *Morphological and histological study of superior lacrimal gland of third eyelid in camel (Camelus dromedarius)*. Iranian Journal of Veterinary Research, Shiraz University,Vol. 10, No. 4, Ser. No. 29, p. 334-338.
26. Munkeby B.H., Smith H.J., Winther-Larssen E.H., Bjørnerud A., Inge Bjerkås I., 2006 – *Magnetic resonance imaging of the Harderian gland in piglets*. J. Anat., 209, p. 699–705.
27. Pavelka M., Roth J., 2005 – *Functional Ultrastructure; An Atlas of Tissue Biology and Pathology*. Springer, Wien, NewYork.
28. Nawrot J.K., Marycz K., Czogała J., Kujawa K., Janeczek M., Chrószcz A., Brudnicki W., 2013 – *Morphology of the lacrimal gland and superficial gland of the third eyelid of Roe deer (Capreolus capreolus L.)*. Pak. Vet. J, 33(2); p. 139-144.
29. Rotariu Nadia, Cotea C., Solcan Carmen, 2005 – *Mofologia glandei Harder (glanda profundă a membranei nictitante) la porc (Sus scrofa domesticus).* Lucrări Ştiinţifice, Medicină Veterinară section, USAMV, Iaşi, vol. 48, p. 179-183.
30. Shirama K, Satoh T, Yokoyama Y, Kano K, Kitamura T, Yamada J., 1996 – *Ultrastructural study on the harderian gland of the rabbit* (*Oryctolagus cuniculus*). Folia Morphol (Warsz); 55(3), p. 133-41.
31. Weaker F.J., 1981 – *Light microscopic and ultrastructural features of the Harderian gland of the nine-banded armadillo*. J Anat. Aug; 133(Pt 1), p. 49-65.
32. Woo H.M., Ellison Bentley E., Campbell S.F., Marfurt C.F., Murphy C.J., 2005 – *Nerve growth factor and corneal wound healing in dogs*. Experimental Eye Research; 80; p. 633–642.

CYTOLOGICAL STUDY OF THE NORMAL NICTITATING GLAND IN DOGS

CAZACU P. Petru
CSE Equester Riding Club, Iaşi, Al. Viilor no. 22, 707515
cazacupetru@gmail.com

Abstract
The cytological study of normal nictitating gland of dogs and establishing of normal morphological characteristics of the units and secretory epithelial cells, it is important for cyto- and oncocytopathological diagnosis of the morbid processes of this gland. Nictitating membrane was harvesting the from both eyes of 5 dogs, then it were trimmed and mid-sagittally sectioned and cytological slides were performed by touch imprint smear and tissue scrapings. For staining the cytological slides were used following stains: Haematoxylin-Eosin (HE) for cytology, May-Grunwald-Giemsa (MGG) and acid periodic Schiff-hematoxylin (PAS-H). Glandular epithelial cells in cytological slides appear as clusters of epithelial cells and/ or secretory units with acidophile cytoplasm and granular aspect, which surrounds a large and dense nucleus, often eccentrically located. Secretory units consist of 7-10 glandular epithelial cells.

Keywords: *Nictitating gland, normal cytology, cytological slides, dogs.*

Introduction

In dogs the nictitating gland (*third eyelid superficial gland*) is located on the distal portion of the third eyelid, fixed by a support conjunctiva tissue to the periorbital fascias (Constantinescu, 2005, cited in Cabral, 2008) and on the bulbar part of nictitating membrane cartilage, nictitating gland progresses to a few millimetres from the horizontal edge of the nictitating membrane cartilage (Morgan *et al*., 1993 and Samuelson, 1999, cited in Lazard, 2004; Cazacu *et al*., 2005). Nictitating gland is a tubuloacinar structure, exocrine, whose sero-mucous secretion is eliminated through several excretory ducts that open through orifices (polistomatic gland), located in the deep posterior face of the nictitating membrane (Kaswan, 1985, as cited in Delafon, 2002).

The nictitating gland in dog is the second important lachrymal gland after the main lachrymal gland, regarding the tear film secretion, respectively its aqueous fraction. The aqueous fraction of the tear film has a thickness of 7 μm in dogs (external fat fraction – 0.1 μm and internal mucosal fraction – 2 μm), being secreted by the lachrymal gland at a rate of 61%, 35% by the nictitating gland and 3% by the accessory conjunctival glands of Krause and Wolfring (Severin, 1973, cited in Chadieu, 1994). According to Gelatt (2000), nictitating gland produces 30-50% of the aqueous fraction of the precorneal tear film of the dog.

The aqueous layer contributes to the viscosity of the tear film [6]. It contains lactoferrin, lysozyme, secretory IgA, IgG, IgM, albumin, transferrin, ceruloplasmin, glycoproteins, participating in the defence of the ocular surface. Also, contains nutrients for the avascular cornea, namely inorganic salts, glucose, oxygen and proteins [1, 5, 6, 9]. The aqueous layer contains growth factors involved in corneal regeneration processes, such as EGF (Epidermal Growth Factor), HGF (hepatocyte Growth Factor), TGF-β (transforming growth factor), KGF (Keratocyte Growth Factor) and NGF (Nerve Growth Factor), whose levels increase in glandular secretion product when the corneal epithelium is damaged [14].

Secretory nictitating gland insufficiency with different etiologies, causes lack of lubrication of the cornea with exposure to the biotic or inert aggressive agents, and failing to

initiate the processes of defence through the inadequacy of antibacterial and growth factors, these resulting in appearance of keratoconjunctivitis *sicca*. Some morbid processes as protrusion of the gland of the third eyelid, eversion of the third eyelid, follicular conjunctivitis, follicular hyperplasia and tumoral processes, involves the nictitating gland directly or indirectly.

Description of normal cytological aspects of the nictitating gland in dog is important for cytopathological diagnosis of the morbid processes of this gland.

The purpose of this study is to describe the characteristics of normal cytology of the nictitating gland of the dog in cytological slides, performed for the first time; similar studies were not identified in local or abroad bibliography consulted.

Materials and methods

Nictitating membrane was harvested from both eyes of 5 dogs of different breeds (mongrel dogs, Pekingese and German Shepherd), 3 males and 2 females, weighting between 2-12 kg and aged 3 months to 6 years. The corpses of dogs were from various private veterinary practices (from Iasi county) and prosecture of the Faculty of Veterinary Medicine from Iasi.

Bilateral excision of the nictitating membrane and gland was performed by incision and dissection of the bulbar and palpebral conjunctiva and underlying structures to its complete isolation. Only nictitating membranes with normal clinical appearance were harvested.

The nictitating membranes were trimmed and mid-sagittally sectioned, then cytological slides were performed by touch imprint smear and tissue scrapings. For staining the cytological slides were used the following stains: Haematoxylin-Eosin (HE) for cytology, May-Grunwald-Giemsa (MGG) and acid periodic Schiff-haematoxylin (PAS-H). Subsequently, the cytological slides were examined at the optical microscope *MC300 Micros Austria*.

Results and Discussions

Epithelial cells of the nictitating gland in cytological slides appear as clusters of epithelial cells and/ or secretory units with acidophil cytoplasm surrounding the large nucleus, dense, often located eccentrically (fig. 1; fig. 2 and fig. 3). The staining with periodic acid Schiff-haematoxylin (PAS-H) reveals the positive reaction of cytoplasm contents (fig. 7 and fig. 8). Glandular epithelial cells have pyramidal appearance and slightly rounded apical pole (fig. 3; fig. 4 and fig. 5). The cytological slides, which show secretory units, these consist of 7-10 glandular epithelial cells (fig. 1; fig. 4 and fig. 6). These morphological features consists of normal secretory epithelium/ normal nictitating gland.

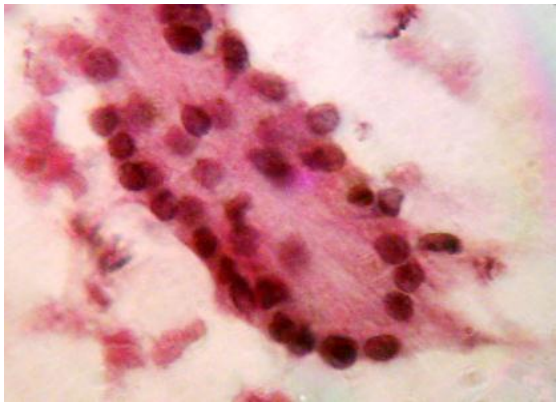


Fig. 1. Epithelial cells of the nictitating gland in dogs. Cluster of epithelial cells with eosinophilic cytoplasm and the nucleus often located in an eccentric position. TI (Touch imprint). HE stain; x 900.

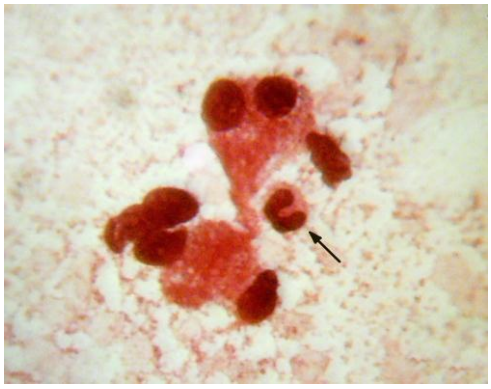


Fig. 2. Epithelial cells of the nictitating gland in dogs. Epithelial cells with eosinophilic cytoplasm and nucleus often located in an eccentric position. Near the epithelial cells can be observed an unsegmented neutrophil (arrow). TI (Touch imprint). HE stain; x 900.

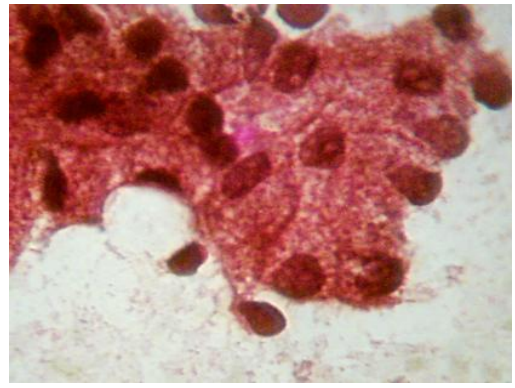


Fig. 3. Epithelial cells of the nictitating gland in dogs. Epithelial cells with eosinophilic and granular-appearing cytoplasm that surrounds a dense, often eccentric nucleus. TI (Touch imprint). HE stain; x 900.

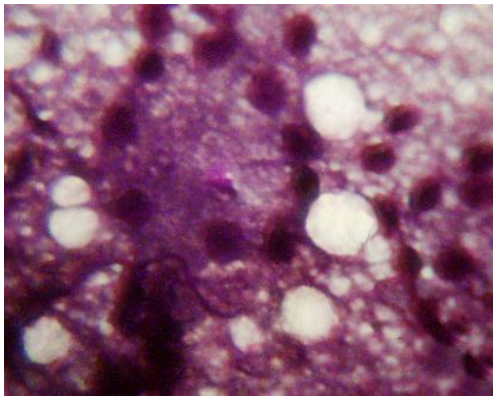


Fig. 4. Epithelial cells of the nictitating gland in dogs. Tubuloacinar unit composed of nine pyramidal glandular epithelial cells with slightly rounded apical pole and the cytoplasm with granular appearance. Spherical nuclei are large and located in base of the cells. Tissue scraping. MGG stain; x 900.

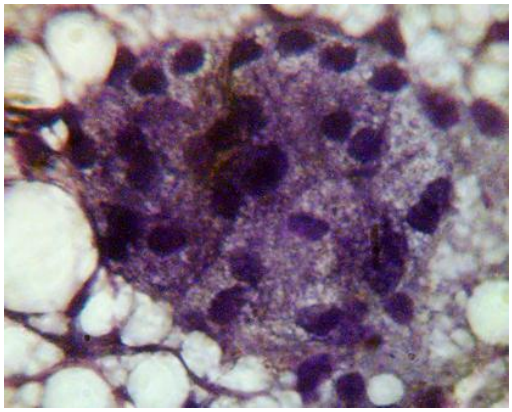


Fig. 5. Epithelial cells of the nictitating gland in dogs. Cluster of glandular epithelial cells with pyramidal appearance, granular cytoplasm and spherical and large nuclei. Tissue scraping. MGG stain; x 900.

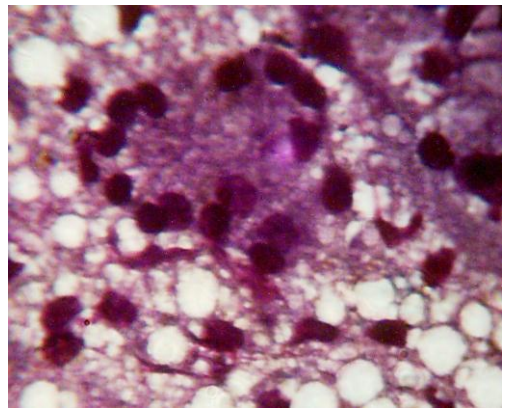


Fig. 6. Epithelial cells of the nictitating gland in dogs. Cluster of glandular epithelial cells with granular cytoplasm and spherical and large nuclei. Tissue scraping. MGG stain; x 900.

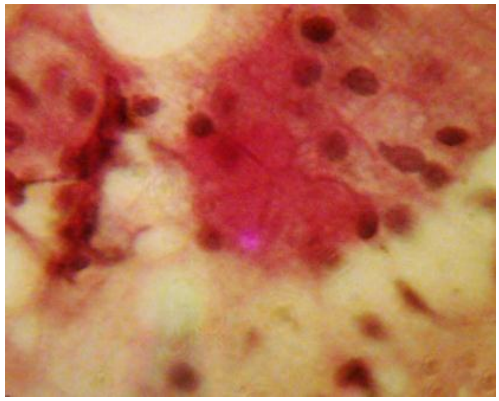


Fig. 7. Epithelial cells of the nictitating gland in dogs. Cluster of glandular epithelial cells with PAS-positive granular cytoplasm and spherical and large nuclei. Tissue scraping. PAS-H stain; x 900.

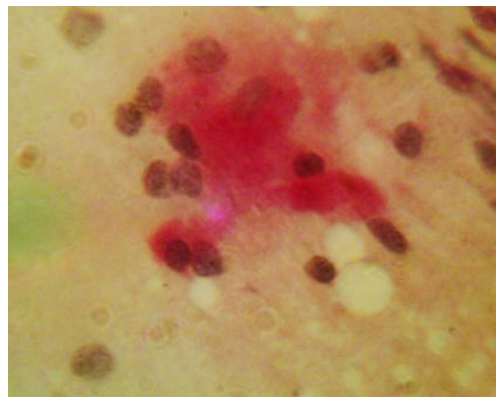


Fig. 8. Epithelial cells of the nictitating gland in dogs. Cluster of glandular epithelial cells with PAS-positive granular cytoplasm, spherical and large and eccentric nuclei. Tissue scraping. PAS-H stain; x 900.

The normal cytological aspects of dog nictitating gland in cytological slides (touch imprint and tissue scrapings) have not been described so far in local and abroad comsulted literature. There is only one case report (Kamaromy *et al*. 1997, cited in Raskin, 2009) of nictitating gland adenocarcinoma diagnosed in cat by demonstration of malignant epithelial cells in cytological preparations obtained from excised biopsy tissue. Nictitating gland tumor processes can be varied: squamous cell carcinoma, lymphoma, hemangioma, hemangiosarcoma, adenocarcinoma of the nictitating gland and apocrine adnexoma with squamous metaplasia [3, 11, 13]. Therefore, the cytological study of normal nictitating gland in dogs and establishing the normal morphological characteristics of the units and secretory epithelial cells, is important for cytopathological diagnosis of the morbid processes of this gland. So, the morphological and cytological normal characteristics of the epithelial cells of

the nictitating gland are: the glandular epithelial cells in cytological slides appear as clusters of epithelial cells and/ or secretory units with acidophil cytoplasm and granular aspect, which surrounds a large and dense nucleus, often eccentrically located. Glandular epithelial cells had pyramidal appearance and slightly rounded apical pole and the secretory units consisted of 7-10 glandular epithelial cells.

Conclusions

1. Glandular epithelial cells appear in cytological slides in form of epithelial cells and/or secretory units clusters with acidophilic cytoplasm and granular aspect, surrounding a large, dense, often eccentrically located nucleus.
2. Glandular epithelial cells have pyramidal appearance and slightly rounded apical pole, and the secretory units consist of 7 to 10 glandular epithelial cells.

References

1. Borisevich V.B., 1976 – *Lacrimal glands of dogs and cattle in the light of histologic and histochemical findings*. Arkh Anat Gistol Embriol. Sep;71(9), p. 12-17.
2. Cabral P.V., Watanabe E., Tostes A.R., Simonelli M.S., Laus L.J., 2008 – *Third eyelid gland protrusion in dogs. An experimental model proposal*. Ciência Rural, Santa Maria, v38, n.7, p. 1920-1924.
3. Carr A.P., Griffey M., Müller R.S., 1992 – *An unusual tumor of the nictitating membrane gland of dogs – a case report*. Dtsch Tierarztl Wochenschr.;99(11), p. 465-466.
4. Cazacu P., Cotea C., Solcan Carmen, 2005 – *Morfologia glandei superficiale (glanda nictitantă) a pleoapei a treia la câine (Canis familiaris)*. Lucrări ştiinţifice, Medicină Veterinară section, USAMV, Iaşi, p. 150-156.
5. Chaudieu G., 1994 – *L'appareil lacrymal*. Prat. Méd. Chir. Anim. Comp., 29, p. 421-446.
6. Davidson J. Harriet, Kuonen J. Vanessa, 2004 – *The tear film and ocular mucins*. Veterinary Ophthalmology 7, 2, p. 71–77.
7. Delafon Claire, 2002 – *Contribution à l'étude des modifications histologiques de la glande nictitante de chiens atteints de kératoconjonctivite sèche après un traitement local à la cyclosporine A 0,2 %*. These pour obtenir le grade de Docteur Veterinaire, École Nationale Vétérinaire d'alfort, La Faculte de Médecine de Créteil, France.
8. Gelatt N.K., 2000 – *Essentials of Veterinary Ophthalmology*. 1st edition, Lippincott Williams & Wilkins, Philadelphia.
9. German A.J., Hall E.J., Day M.J., 1998 – *Measurement of IgG, IgM and IgA concentrations in canine serum, saliva, tears and bile*. Vet. Immunol Immunopathol., Jul 8; 64(2), p. 107-121.
10. Lazard P., 2004 – *Chirurgie de la luxation de la glande lacrymale superficielle de la membrane nictitante chez le chien: nouvelle technique microchirurgicale*. Revue Méd. Vét., 2004, 155, 2, p. 82-86.
11. Maggs J.D., 2008 – *Third eyelid*. In: *Slatter's Fundamentals of veterinary ophtalmology*, Maggs D., Miller P., Ofri R. (Eds.), 4th edition, Saunders, *Philadelphia.*
12. Raskin E. Rose, 2009 – *Eyes and Adnexa*. In: *Canine And Feline Cytology: A Color Atlas And Interpretation Guide*, Raskin E. Rose, Meyer D. (Eds.), 2nd edition, Saunders, Missouri, USA.
13. Sbarbati Andrea, Calderan Laura, Nicolato Elena, Marzola Pasquina, Lunati E., Donatella B., Bernardi P., Osculati F., 2002 – *Magnetic resonance imaging of the rat Harderian gland*. J. Anat. 201, p. 231-238.
14. Woo H.M., Ellison Bentley E., Campbell S.F., Marfurt C.F., Murphy C.J., 2005 – *Nerve growth factor and corneal wound healing in dogs*. Experimental Eye Research; 80; p. 633–642.

BLOOD HARVESTING IN THE TELEOST FISH

Alexandru Lataretu, Elena Mitranescu, Florin Furnaris, Valer Teusdea
University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, District 5, 050097,Bucharest, Romania
alex_lata@yahoo.com

Abstract
Due to the continuous evolution of fish industry, researchers had to develop and implement new medical diagnosis methods for fish health monitoring. One of these methods is represented by blood examinations. Blood examinations are important tools for hematological, biochemical, parasitological and bacterial assessments. In order to obtain relevant blood samples it is imperative to use a feasible blood harvesting technique. Thus, in this study different harvesting procedures reported in the literature, and also a comparison between two different anticoagulants like heparin and ethylenediaminetetraacetic acid (EDTA) are presented.

Key words*: fish, hematology, blood harvesting, anticoagulants*

Introduction

According to Noga E. (2010), harvesting blood from a fish with less than 8 cm in length usually implies a great risk of killing him. Blood samples, appropriate for a complete blood count, can be safely harvested from fish weighing minimum 100 g (Stoskopf M., 1993). The procedure should ideally be completed in less than 30 seconds, because holding the fish out of the water for prolonged periods can lead to respiratory troubles and electrolyte abnormalities (Thrall et al., 2012). Also according to Branson E. J. (2008), in order to reduce the fish disturbance generated by stress (especially when assessing the cortisol level), the time passed between the primary perturbation of the fish (ex: catching the fish) and the blood harvesting procedure, has to be less than five minutes. If this is not possible, the harvesting procedure can initiate a stress response or increase the magnitude of an already existing stress response (Pickering et al. 1982).

For the hematologic assessment, it is recommended to harvest blood on heparin or EDTA as anticoagulant. Heparin has the following disadvantages: it appears to induce a clumping tendency in leukocytes and thrombocytes, and when using a Romanowsky staining methodology, heparin can cause a blue shade to the blood smears (Thrall et al., 2012). Furthermore, if the clotting process has already started in the sample, heparin may not prevent coagulation due to the alternative pathways of this process (Noga E., 2010). Disadvantages of EDTA are also noted: hemolysis can occur in some fish species (Thrall et al. 2012) or when used in association with tricaine sedation or anesthesia (Thrall et al. 2012; Noga E. 2010). This last problem can be minimized by cooling the sample to 4˚C and/or rapidly drawing the blood smear (Noga E. 2010). The hemolytic effect of EDTA on common carp red blood cells is also described by Walencik J. & Witeska M. (2007).

The use of anesthesia is controversial: some authors don't recommend chemical anesthesia in fish because of the possible complications (Noga E., 2010), while others (Branson E. J. 2008) recommend a light sedation of the fish before the blood harvesting procedure.

Regarding the harvesting methodology, the literature recommends several techniques:

For larger fish (more than 8 cm), blood can be collected using a hypodermic needle and syringe, from the caudal vein or artery and by heart puncture (Branson E., 2008; Stoskopf M., 1993) or dorsal aorta puncture (Department of Fisheries and Oceans, Canada, 2004). Some specialists recommend using a syringe coated with Li-heparin to reduce the chance of micro-clotting in the blood sample (Stoskopf M., 1993), in comparison with others who don't mention in their studies such a procedure. Regarding the possibility of sample dilution (especially in small samples), Stoskopf M., (1993) states that in practical work, no major problems have been noted.

Harvesting blood via a capillary tube

This method is recommended when the fish is less than 8 cm in length and it consists of the following steps: the anesthetized fish is placed on a level surface, the base of the tail is amputated and blood is collected from the caudal vessels using a capillary tube (Noga E., 2010). The fish should be humanely killed immediately. According to the same author, caution should be taken when using this method for biochemistry analyses, due to the possible tissue fluid contamination of the sample.

Another technique related in several studies (Bry C. & Zohar Y., 1980; Tashjian D. & Hung S.) for harvesting blood and monitoring fish physiological parameters is represented by dorsal aorta catheterization.

This study focused on two issues: testing the harvesting methodology reported in the literature and a comparison of two anticoagulants (EDTA and heparin) widely used in fish medicine, discussing the hemolytic effect of EDTA in contrast with heparin, based on blood smear observations via light microscopy.

Materials and methods

Two common carps (*Cyprinus carpio*) and two rainbow trouts (*Oncorhynchus mykiss*) were used in this experiment. Blood was harvested from the following puncture zones: caudal vessels via the lateral and ventral approach techniques, cardiac and dorsal aorta, using a syringe with a 23 G hypodermic needle.

The fish were minimally sedated with MS - 222 prior to blood sampling as there was no danger of handling stress due to the short air exposure and blood harvesting time (under 30 seconds).

The blood was collected in Li-heparin and EDTA 2 ml containers for each fish.

The ventral approach (figure 1) consisted in placing the tip of the needle at the ventral median line and in the middle portion of the fish tail peduncle, and slightly inserting the needle cranial and caudal until the tip reached the vertebras (Stoskopf M., 1993).

The lateral approach of the caudal vessels (figure 2) consisted of positioning the needle slightly under the lateral line, in the middle area of the tail. The needle was oriented cranially, at a 45^{o} angle and inserted just under the vertebras (Stoskopf M., 1993).

The cardiac approach (figure 3) was performed in the ventral part of the fish by inserting the needle to the "bony notch formed by the bones creating the posterior margin of the opercular cavity" and directing it dorsally, until reaching the heart (Stoskopf M., 1993).

The dorsal aorta (picture 4) approach was performed by inserting the needle with the tip oriented caudal on the dorsal median line of the mouth, immediately after the juncture of the second gill arch (Department of Fisheries and Oceans, Canada, 2004).

Fig. 1. Ventral approach of the caudal vessels (original)

Fig. 2. Lateral approach of the caudal vessels (original)

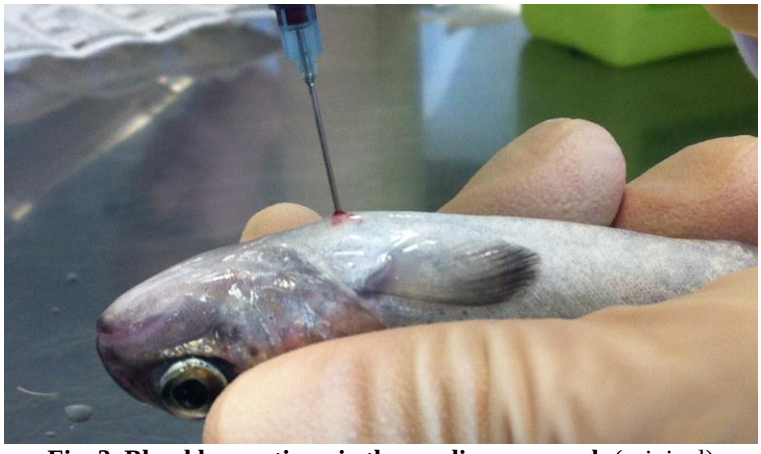


Fig. 3. Blood harvesting via the cardiac approach (original)

Fig. 4. Dorsal aorta blood harvesting (original)

Blood smears were drawn immediately using both EDTA and heparin mixed blood, air dried and stained with a Romanowsky – staining procedure (Diff – Quick stain) for morphologic examination. The smears were examined via light microscopy using the 40 or 100 X magnification.

Results and discussions

The blood harvesting techniques tested in this study permitted the collection of adequate samples of blood, in both terms of quality and quantity, for blood smear examination.

Regarding the anticoagulant trial, following the analyses of the blood films, it was noted that in the heparinized carp blood samples most of the red blood cells presented normal morphological features, with only occasional observed hemolysis (figure 5).

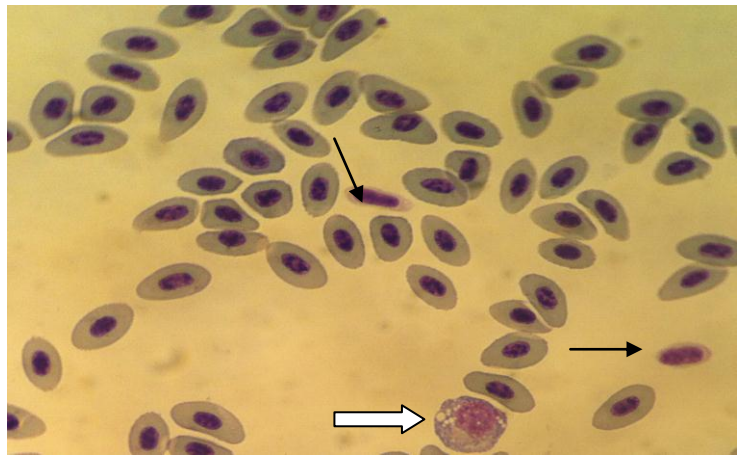


Fig. 5. Smear from heparinized common carp (*Cyprinus carpio*) blood, presenting intact erythrocytes with normal morphological features. A monocyte with multiples vacuoles (large arrow) and two thrombocytes (small arrow) can also be seen. Diff – Quick stain, 100 X (original)

The carp blood samples containing EDTA (figure 6) revealed a pinkish background with few intact but swollen erythrocytes and numerous "naked" erythrocyte nuclei. The nuclei presented a foamy structure, and were increased in size.

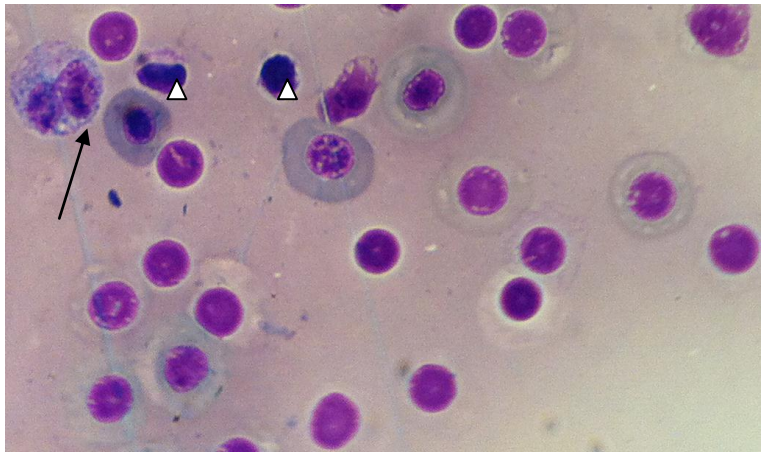


Fig. 6. Smear from common carp (*Cyprinus carpio*) blood harvested on EDTA, revealing round and swollen erythrocytes, and numerous round bare nuclei with a vacuolated structure. A neutrophil (arrow) and two thrombocytes (arrow head) can also be seen (arrow). Diff – Quick stain, 100 X (original)

The rainbow trout blood smears did not reveal any significant *in vitro* changes, the erythrocytes presenting relative similar morphological features between both salmonid EDTA and heparin smears (figures 7 and 8).

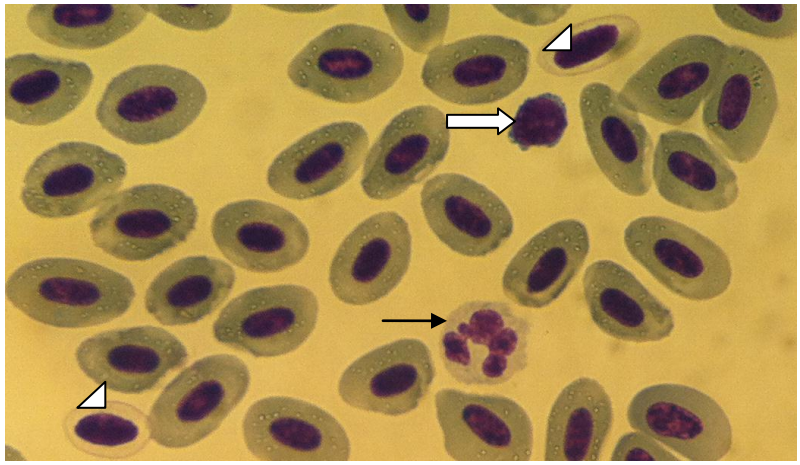


Fig. 7. Smear from Rainbow trout (*Oncorhynchus mykiss*) blood harvested on EDTA, presenting intact red blood cells with normal morphological features. A neutrophil (arrow), two thrombocytes (arrow head) and a small lymphocyte (large arrow) can also be seen. Diff – Quick stain, 100 X (original)

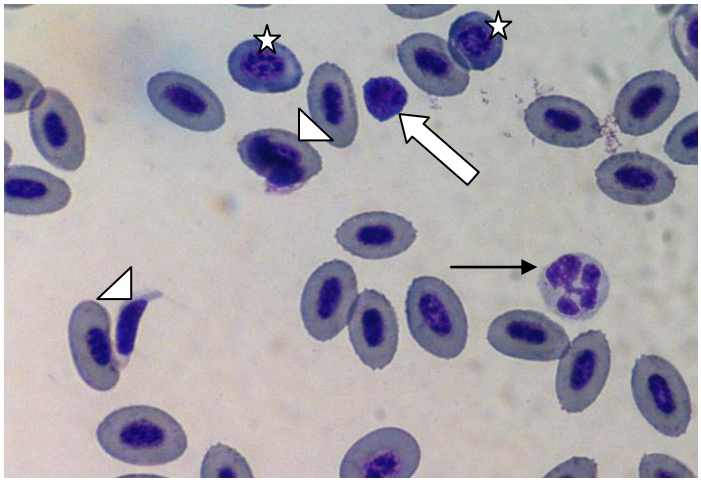


Fig. 8. Smear from Rainbow trout (*Oncorhynchus mykiss*) blood harvested on heparin revealing intact erythrocytes with normal morphological features. A neutrophil (arrow), three thrombocytes (two overlapping; arrow head), two erythrocyte precursors (star) and a small lymphocyte (large arrow) can also be seen. Diff – Quick stain, 100 X (original)

Conclusions

It can be concluded that erythrocytes of different fish species respond differently to certain anticoagulants, in this case EDTA and heparin. Carp red blood cells are, apparently, sensible to EDTA, manifesting morphological changes (cell membrane destruction followed by nuclear structural changes) that lead to hemolysis. In contrast, rainbow trout erythrocytes do not seem to show any significant morphological changes, in blood samples harvested on EDTA in comparison with heparin.

Acknowledgements

This paper has been cofinanced from European Social Fond through Sectorial Operational Program for Human Resources Development 2007 – 2013, Project POSDRU/107/1.5/S/76888, "Doctoral bourses for helping and exploring in agronomical and veterinary medicine field".

References

1. Branson, E. J., Fish Welfare. Blackwell Publishing, pp. 38-39, 2008.
2. Bry C., Zohar Y., Dorsal aorta catheterization in rainbow trout (*Salmo gairdneri*) II. Glucocorticoid levels, hematological data and resumption of feeding for five days after surgery. Reprod. Nutr. Dévelop., 20, (6), pp 1825 – 1834, 1980.
3. Canada Department of Fisheries and Oceans Animal – User Training Template. Blood Sampling of Finfish, 2004.
4. Noga, E.J., Fish Disease, Diagnostic and Treatment, Second Edition. Wiley – Blackwell Publishing, pp 32 – 35, 2010.
5. Pickering, A.D., Pottinger, T.G. & Christie, P., Recovery of the brown trout, Salmo trutta L., from acute handling stress: a time-course study. Journal of Fish Biology, 20, pp 229–44, 1982.

6. Stoskopf, M.K., Fish Medicine. Saunders, pp 64-65, 1993.
7. Tashjian D., Hung S., Non – Invasive surgery techniques in fish research: a review on esophageal intubation, dorsal aorta cannulation, and urinary catheterization in sturgeon. Department of Animal Science, University of California.
8. Thrall, M. A., Weiser, G., Allison, R. W., Campbell, T. W., Veterinary Hematology and Clinical Chemistry, Second Edition, pp 298 – 300. Wiley – Blackwell Publishing, 2012.
9. Walencik J., Witeska M., The effects of anticoagulants on hematological indices and blood cell morphology of common carp (*Cyprinus carpio* L.). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, Vol. 146, Issue 3, pp 331 – 335, 2007.

NEW INSIGHTS INTO THE MORPHOLOGY AND FINE STRUCTURE OF THE CHICKEN VAGINA

C. Todireanu[1], C. Cotea[1], Carmen Solcan[1], P. Cornillie[2]
[1]Faculty of Veterinary Medicine, Iasi, Romania
[2]Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium
Todireanu.Catalin@gmail.com

Abstract

In the present study we bring new insights into the morphology, histology, cytochemistry and fine structure of the chicken vagina. The research was undertaken on 20 Lohmann Brown chickens, aged between 19 and 50 weeks. Samples were taken from different areas of the vagina and were processed for a gross anatomy study, for histology, cytochemistry and scanning electron microscopy. The results show differences in the proportion of different histological structures between the vagina and the other oviduct segments. The primary folds appear very long and thin, with few tubular glands. There are also a lot of secondary and tertiary folds. The tunica muscularis is very well developed in this segment of the oviduct and consists of two layers of smooth muscle: inner circular and outer longitudinal. In the epithelium, the ciliated cells dominate and there are fewer goblet cells than in other segments.

Key words*: anatomy, chicken, histology, SEM*

Introduction

The focus of the present study is on the morphology and histology of the vagina in Lohmann brown chickens in correlation with the observations made using the scanning electron microscropy. The research was conducted due to the lack of data in the literature concerning the morphology,histology and fine structure of the vagina in this hybrid, specialized in egg production. The Lohmann brown hybrid has been chosen because it is one of the best hybrids specialized in egg production.

Material and method

We have taken into study 20 Lohmann Brown chickens, aged between 19 and 50 weeks, from the beginning of the laying period, to the peak of egg production. The birds were slaughtered and the vagina was sampled and fixed in 4% buffered formalin. The vagina was divided into two areas: the anterior zone, towards the uterus and the posterior one, towards the cloaca. The samples taken from the two areas were chiseled and embedded in paraffin, cut at 5 μm and stained HE, HEMB, PAS, Van Gieson, Masson, Novelli and Gomori.

Results and discussions

The vagina is a very muscular duct that continues the shell gland and opens into the cloaca through the proctodeum (1, 3, 5) (fig. 1,3). The exact length of the vagina is between a well-developed muscular sphincter that is located in the posterior end of the shell gland, and the opening in the proctodeum (2, 6).

This segment has a length that varies with age with an average of 13-14 cm.

The vagina is not a strait duct and has flexures and convolutions that are bound together and to the shell gland by very well-developed connective tissue layers that are located on the outside (fig. 2).

The thickness of the vagina is mostly represented by the tunica muscularis (fig. 4, 6). The inner-circular layer is very well developed, being thicker that in any segment of the

oviduct. The outer-longitudinal layer isn't that developed and consists of bundles of smooth muscle fibers scattered throughout the connective tissue stroma (fig. 11).

The tunica mucosa has a lot of narrow primary folding that present also secondary and tertiary branches (fig 5, 7, 9). The lamina propria does not contain tubular glands, therefor the folds are very thin (fig. 12, 13). The vagina opens posteriorly into the cloaca, through the left wall, into the proctodeum.

The surface epithelium of the vagina consists of ciliated cells that are non-secreting and mucous-secreting non-ciliated cells (fig. 8, 15, 16). The ciliated cells are narrow, with an oval-round shaped nucleus situated in the middle of the cell and with cilia at the apical pole (fig. 10, 18). The mucous-secreting cells have an oval nucleus at the base of the cell and numerous secretion granules at the apical pole. The height of the epithelium at the peak of the folds is greater than that of the other segments of the oviduct, and also of the epithelium from the depressions between the folds, where we can find more non-ciliated cells (fig. 14, 17, 19).

The vagina has the same histological structure as the other segments of the oviduct: mucosa, tunica submucosa, tunica muscularis and serosa (4, 7, 8). The tunica submucosa is represent only a fine connective tissue, while the serosa consists of a squamous epithelium on a thin layer of connective tissue.

The scanning electron microscopy images show the multitude of narrow primary folds of the mucosa (fig. 20, 21). Also, using the same technique it is noticeable that there are more ciliated cells at the top of the folds, whereas between the primary folds, the mucous-secreting cells tend to be more in number than the ciliated (fig. 24). There are also secretory granules at the top of the cilia, in the lumen of the vagina (fig. 22, 23).

Fig.1. The ovary and oviduct of a 25 weeks old chicken with the egg mass in the isthmus

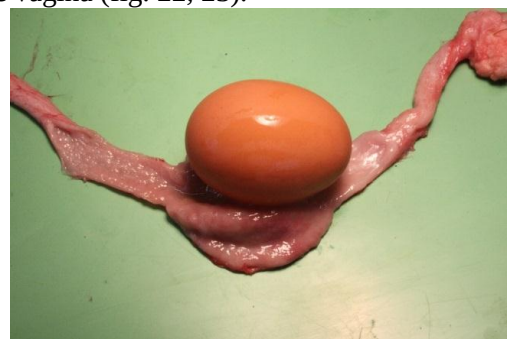


Fig.2. A well developed egg in the uterus, ready to be expelled through the vagina. The folds of the vagina mucosa can also be distinguished

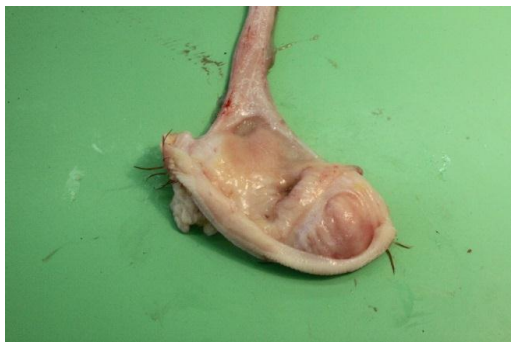


Fig.3. The entrance of the vagina into the cloaca, opening into the proctodeum

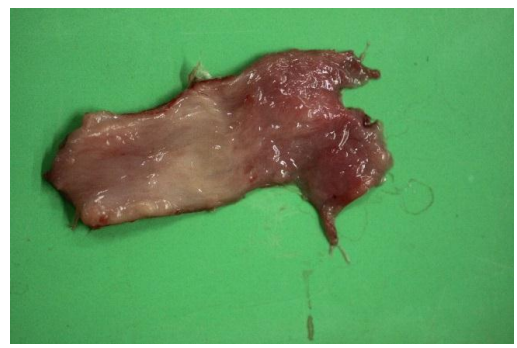


Fig.4.The morphology of the mucosa folds in the vagina and uterus of a 22 weeks old chicken

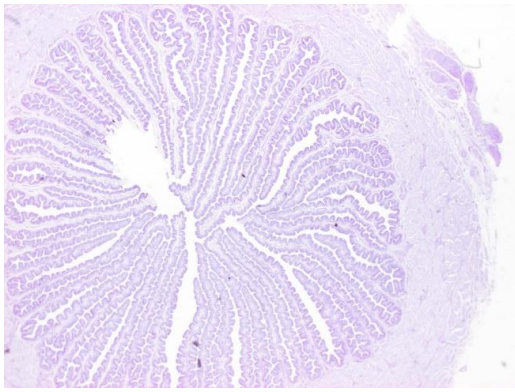


Fig. 5. Numerous very high and thin primary folds in the Lohmann brown chick vagina; PASstain; x 20

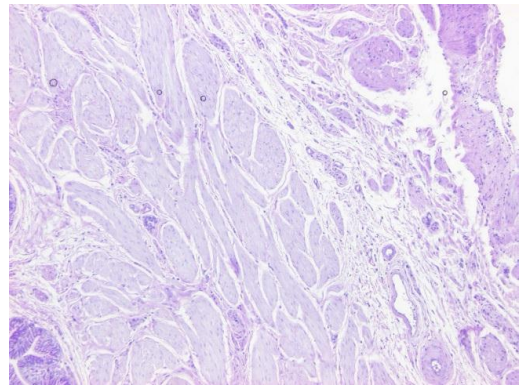


Fig.6. The tunica muscularis of the hen vagina with the two layers of smooth muscle. HEstain; x 100

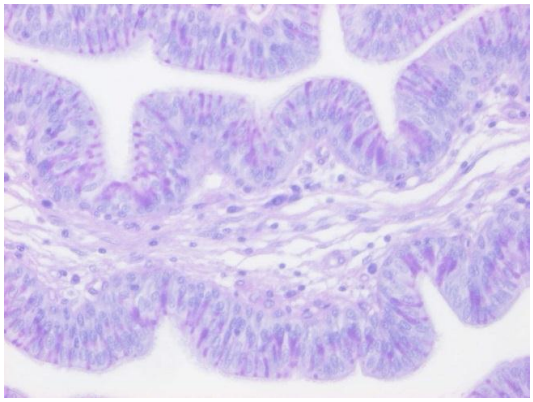


Fig.7. PAS positive secretion in the ciliated epithelium; PAS stain; x 400

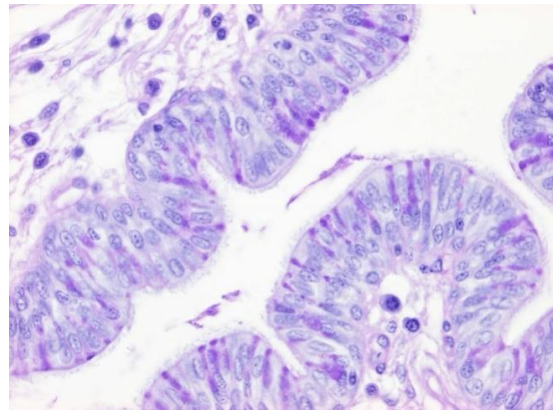


Fig.8. PAS positive secretion in the lumen of the vagina; PAS stain; x 600

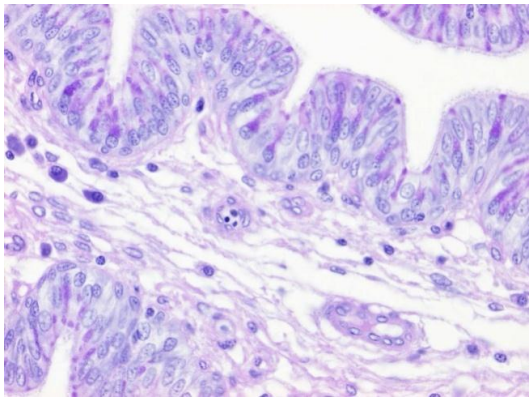


Fig.9. The primary fold of the mucosa with few glands in the lamina propria and the high columnar surface epithelium. PAS stain; x 600

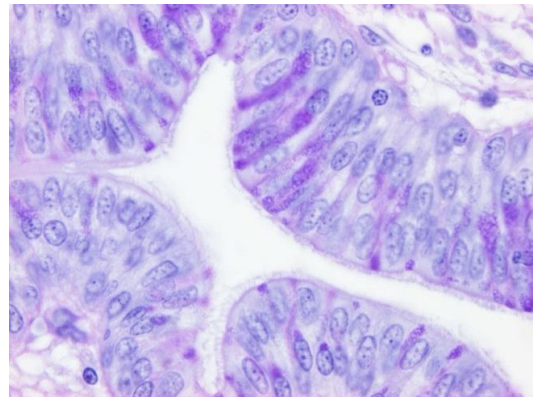


Fig.10. The surface epithelium with numerous ciliated cells and goblet cells with PAS positive secretion at the apical pole; PAS stain; x 1000

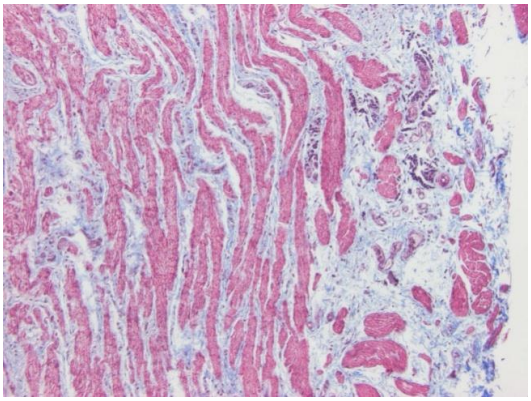


Fig.11.The two layers of smooth muscle: the inner circular layer which is more developed and the outer-longitudinal. Masson's trichromestain; x 100

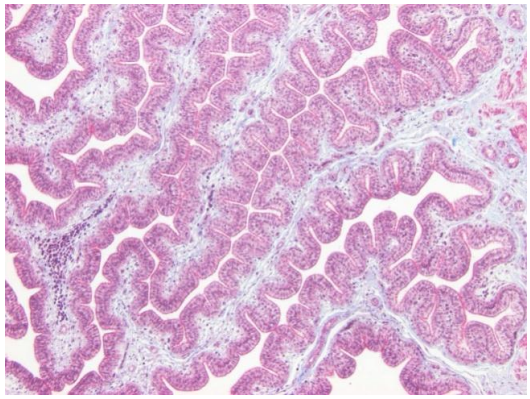


Fig.12. Numerous high primary folds with developed secondary folds. Masson's trichromestain; x 100

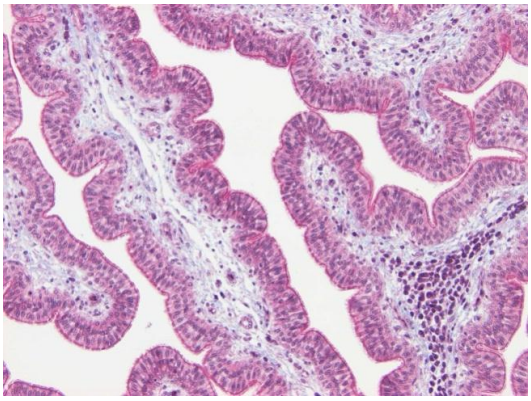


Fig.13. The primary fold with few glands in the lamina propria. Masson's trichromestain; x 200

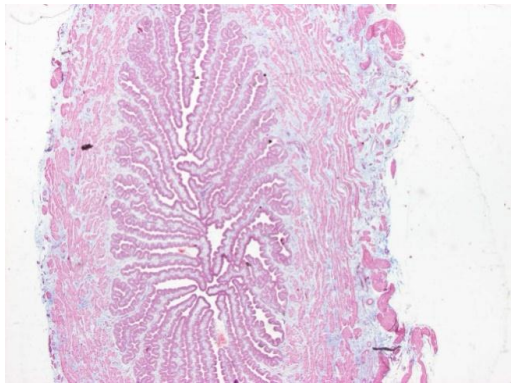


Fig.14.Overview of the hen vagina with a very well developed tunica musculosa and numerous folds. Masson's trichromestain;x20

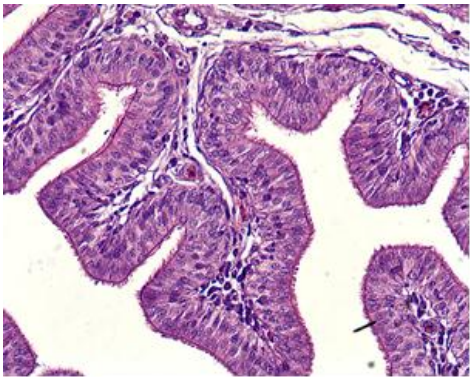


Fig.15.The primary fold of the mucosa with numerous ciliated cells in the epithelium and small blood vessels in the lamina propria; HEMB stain; x200

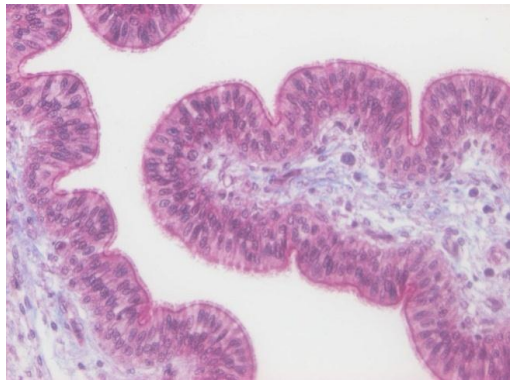


Fig.16. Collagen fibres and blood capillaries in the lamina propria of the mucosa; Masson's trichromestain; x 400

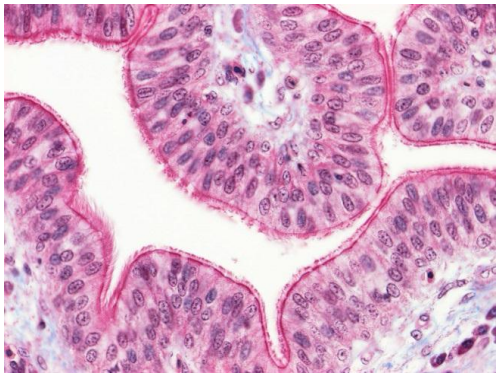


Fig.17.Numerous ciliated cells in the surface epithelium of the secondary folds; Masson's trichromestain; x 600

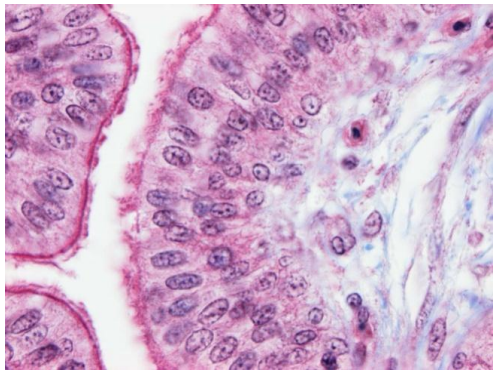


Fig.18. The surface epithelium is structured of ciliated cells and goblet cells. Also, small capillaries can be distinguished in the lamina propria; Masson's trichromestain x 1000

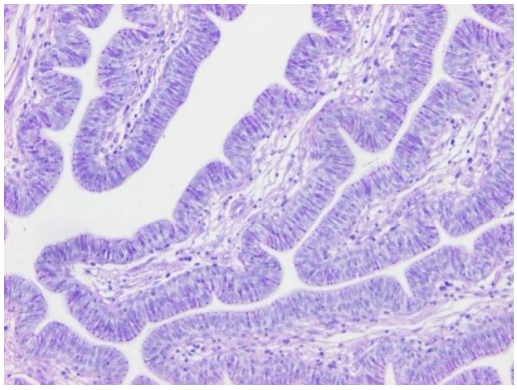


Fig.19. The primary folds have many secondary folds that enlarge the secretory territory of the vagina. PASstain; x 200

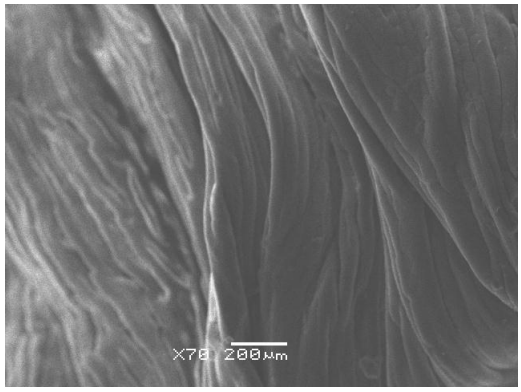


Fig. 20. Numerous folds of the mucosa in the hen vagina; SEM; x70

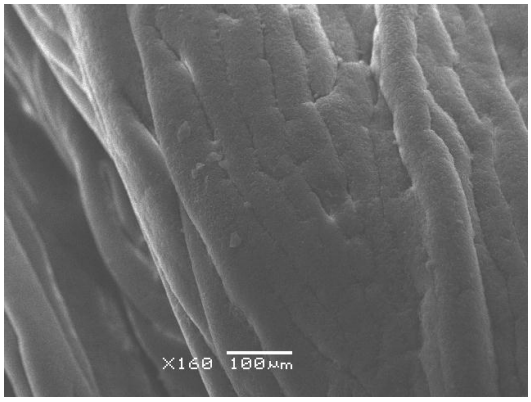


Fig. 21. The appearance of the primary folds of the mucosa with secretion in the lumen; SEM; x160

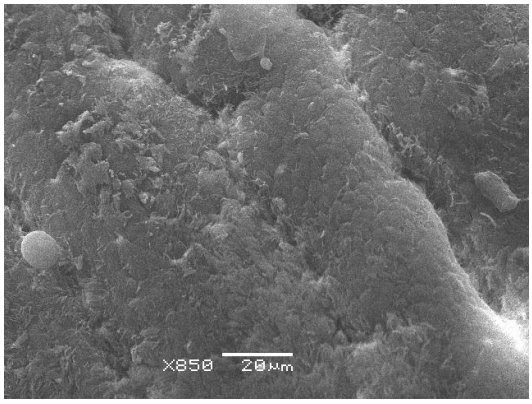


Fig. 22. Ciliated and goblet cells covered by secretion; SEM; x850

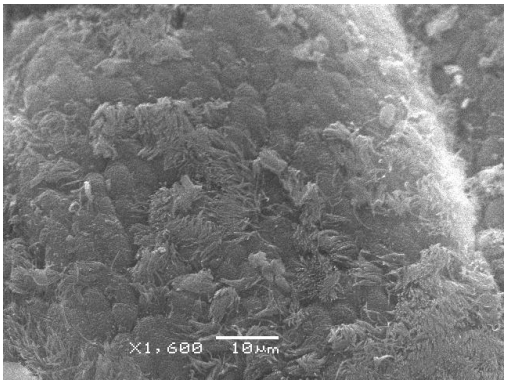


Fig. 23. The goblet cells and ciliated cells alternate in the surface epithelium; SEM; x1600

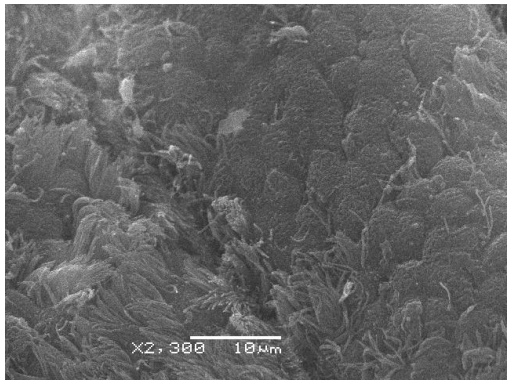


Fig. 24. The ciliated cells have a lot of cilia at the apical pole that help transport the secretion; SEM; x2300

Conclusions

1. The vagina is a very muscular duct that continues the shell gland and opens into the cloaca through the proctodeum
2. This segment is structured of a tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa
3. The thickness of the vagina is mostly represented by the tunica musculosa
4. The inner-circular layer is very well developed, being thicker that in any segment of the oviduct
5. The tunica mucosa has a lot of narrow primary folding that present also secondary and tertiary branches
6. The lamina propria does not contain tubular glands
7. The alternation between the two types of cells in the epithelium can be better observed using the scanning electron microscopy

References

1. Bellairs R., Osmond M., 2005- *The atlas of chick development*, Elsevier Academic Press.
2. 2.Chousalkar K., 2008 – *Ultrastructural changes in the oviduct of the layingh end uring the laying cycle*, CellTissueRes, 332: 349-358.
3. Cotea C.,2013– *Histologie specială*, Ed. Tehnopress, Iaşi.
4. 4.Cotea ., 2013 – *Biologie celulară, embriologie generală, histologie generală*, Ed. Tehnopress, Iași
5. Todireanu C., Cotea C., Solcan Carmen, 2010 – *The morphology and histochemistry of the magnum during the egg passage through the oviduct in hens during the laying cycle*, Lucrări științifice – Medicină Veterinară, vol 53.
6. Todireanu C., Cotea C., Solcan Carmen, 2010 – *The dynamics of the shell membranes formation in the is thmus of theLohmann Brown layinghens*, Lucrări Stiintifice – Medicină Veterinară, vol 53;.
7. Wang X., Ford B. C., Praul C. A., Leach Jr. R. M., 2002 - *Collagen X expression in oviduct tissue during the different stages of the egg laying cycle*, Poultry Science 81:805–808.
8. Whittow C., 2000 – *Sturkie's Avian Physiology (Fifth edition)* Elsevier.

THE MORPHOLOGY OF THE INFUNDIBULUM IN LOHMANN BROWN LAYING HENS IN CORRELATION WITH THE EGG PASSAGE THROUGH THE OVIDUCT

C. Todireanu[1], C. Cotea[1], Carmen Solcan[1], P. Cornillie[2]
[1]Faculty of Veterinary Medicine, Iasi, Romania
[2]Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium
Todireanu.Catalin@gmail.com

Abstract

The aim of the present study is to unravel the gross anatomy, histology, cytochemistry and ultrastructure of the infundibulum in Lohmann brown laying hens and to highlight the differences in the morphology of this segment as the egg mass passes through. The research was undertaken on 20 chickens aged between 19 and 50 weeks. The samples were collected from the two distinct areas of the infundibulum: the funnel and the neck. The samples were fixed in 4% buffered formalin, embedded in parrafin, sectioned at the size of 5 µm and stained HE, HEMB, PAS, Van Gieson, Masson, Novelli and Gomori. The infundibulum consists of a pseudostratified ciliated epithelium and a lamina propria which form the tunica mucosa; a thin layer of connective tissue that structures the submucosa; two layers of smooth muscle that form the tunica muscularis; and the tunica serosa which is formed of mesenchymal cells and connective tissue.

Key words*: anatomy, hen, histology, SEM*

Introduction

The present research is focused on the morphology of the infundibulum in Lohmann brown chickens in correlation with the egg passage through the oviduct. The research was conducted due to the lack of data in the literature concerning the morphology and histochemistry of the infundibulum in this hybrid, specialized in egg production. The Lohmann brown hybrid has been chosen because it is one of the best hybrids specialized in egg production.

Material and method

We have taken into research 20 Lohmann Brown chickens, aged between 19 and 50 weeks, from the beginning of the laying period, to the peak of egg production. The birds were slaughtered and the infundibulum was prelevated and fixed in 4% buffered formalin. The infundibulum was divided into two distinct areas: the funnel and the neck. The samples taken from the two areas were chiseled and embedded in paraffin, cut at 5 µm and stained HE, HEMB, PAS, Van Gieson, Masson, Novelli and Gomori.

Results and discussions

The infundibulum consists of two distinct areas: the funnel and the neck. The funnel is lying very close to the ovary and has the role of capturing the ova (2,4,8,9). This area has very thin walls that converge and form the infundibulum neck which appears as a narrow thin-walled tube, that gradually increses in size and thickness towards the magnum (fig. 2,3).

The length of this segment varies with age, and has an average value of 9 cm (fig. 1). The thickness also increases from the cranial area towards the magnum, and also with age, mainly because of the development of the mucosa folds (fig. 4). The infundibulum consists of a tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa (1,3,5,6,7).

The mucosa is structured from a surface epithelium and a lamina propria. In the surface epithelium we can observe ciliated cells that are non-secreting; goblet cells that are mucous-secreting and non-ciliated; and secretory cells that are located in the depths of the adjacent mucosal folds (fig. 5, 6, 7).

The mucosa of the funnel area is lined with a ciliated epithelium with very few mucous-secreting goblet cells (fig. 8, 10). These cells appear very rarely cranially and increase in number toward the neck of the infundibulum. The ciliated cells are tall and thin, columnar, with a round-oval shaped nucleus that is positioned in the middle of the cell. At the apical pole, these cells poses a tuft of cilia that can easily be distinguished.

The goblet cells appear thicker, with an oval shaped nucleus that is positioned at the base of the cell and with an enlarged apical pole which consists of secretory granules. The secretory material is PAS positive (fig. 11).

In the neck of the infundibulum as it continues caudally, the folds increase in size and become more complex in structure (fig. 15, 16). There are a lot of goblet cells that are mucous-secreting and although there are also numerous ciliated cells, these are obscured by the mucous cells that are filled with granular secretory material at the apical pole.

The lamina propria consists of tubular glands that have low pyramidal shaped cells with granular secretion at the top of the cell. The nucleus appears round and is pushed by the secretory material towards the basal cell membrane (fig. 17).

In this area, the mucosa has primary, secondary and tertiary folds, and as we get closer to the magnum, the folds become simpler and turn into the typical broad longitudinal folds of the magnum.

The second tunica of the infundibulum is the tunica submucosa which consists of connective tissue.

The tunica muscularis is structured from smooth muscle cells, and appears as bundles of muscle cells in the funnel area and as two distinct layers in the neck area (fig. 19). The two layers are inner-circular and outer-longitudinal (fig. 18). The tunica serosa is very thin, made of connective tissue and a squamous epithelium.

Using the scanning electron microscopy we could notice numerous thin epithelial folds (fig.12), and also the fact that the ciliated cells dominate in the epithelium of the funnel area of the infundibulum (fig. 13).

There are also goblet cells that are non-ciliated, but they tend to be obscured by the ciliated cells (fig. 14).

In the neck region of the infundibulum, using SEM we notice a lot of mucous-secreting cells, and fewer ciliated cells (fig. 20). This pattern is more obvious as we go towards the magnum. Also, in this region we can see the openings of the tubular glands from the lamina propria (fig. 21, 22).

At the surface of the epithelium we notice a lot of secretory material in the shape of granules and filaments (fig. 23, 24).

Fig.1Measurements of different parts of the genital system

Fig.2. The funnel of the infundibulum with a very thin structure

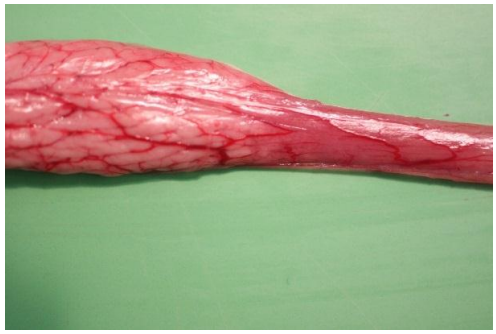


Fig.3. Morphological comparison between the neck of the infundibulum and the anterior part of the magnum

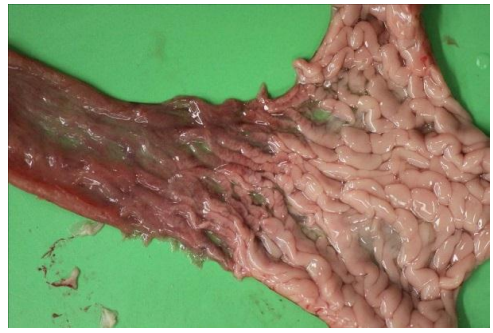


Fig.4. The morphology of the mucosa of the neck of the infundibulum compared with the magnum mucosa

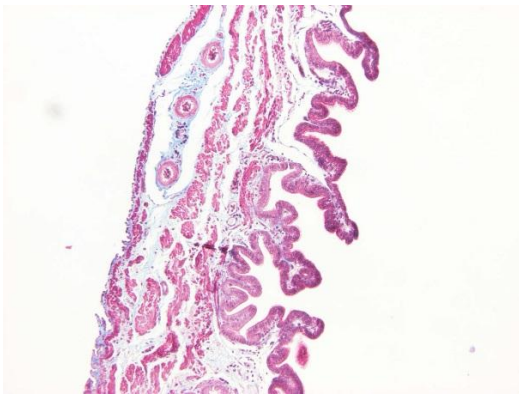


Fig. 5.The funnel area of the infundibulum; Masson's trichrome stain; x20

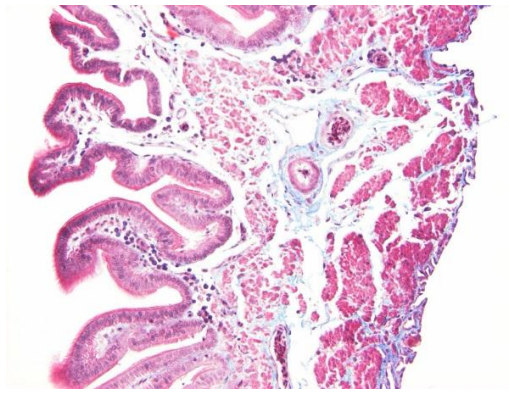


Fig.6. The funnel area has fimbriae that are covered with cilia, Masson's trichrome stain; x 100

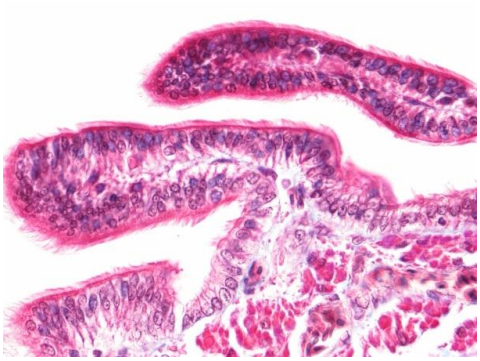


Fig.7. Numerous ciliated cells in the epithelium of the funnel; Masson's trichrome stain; x 200

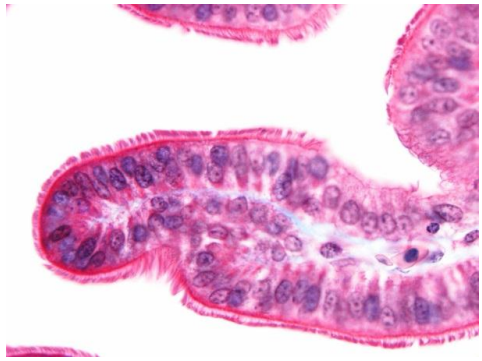


Fig.8. The highly ciliated surface epithelium is underlined by a lamina propria rich in collagen and capillaries; Masson's trichrome stain; x 600

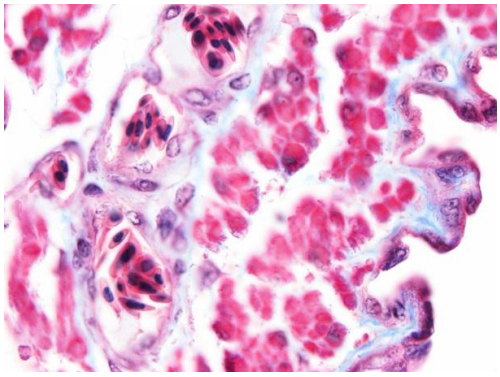


Fig. 9. Between the two layers of smooth muscle there are bigger blood vessels; Masson's trichrome stain; x1000

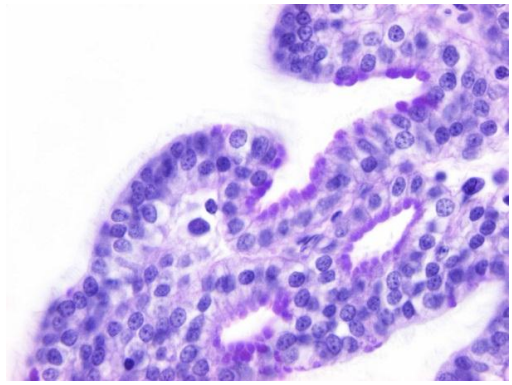


Fig.10.PAS positive secretion at the apical pole of the secretory cells from the epithelium and lamina propria. PAS stain; x600

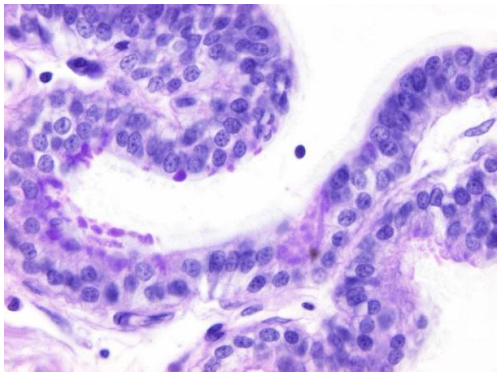


Fig.11. PAS positive secretion being excreted from the apical pole of the goblet cells; PAS stain; x600

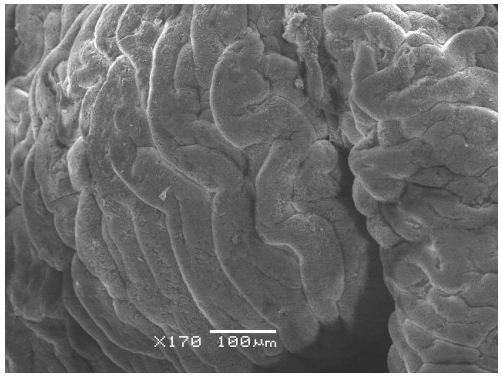


Fig.12. Numerous thin folds in the funnel area of the infundibulum; SEM; x170

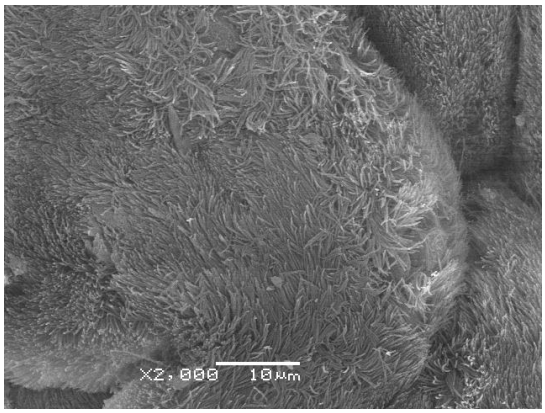


Fig.13.The epithelium presents a lot of ciliated cells with numerous cilia at the apical pole; SEM; x2000

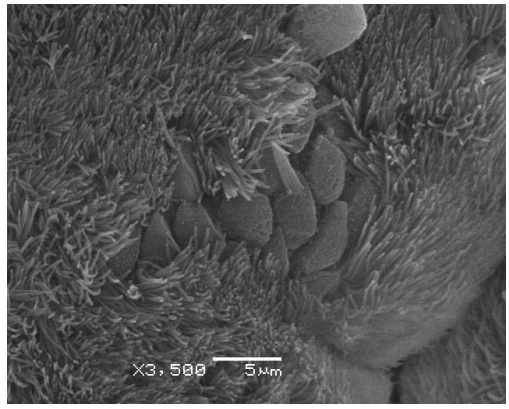


Fig.14. Between the multitude of ciliated cells there are a few goblet cells; SEM; x3500

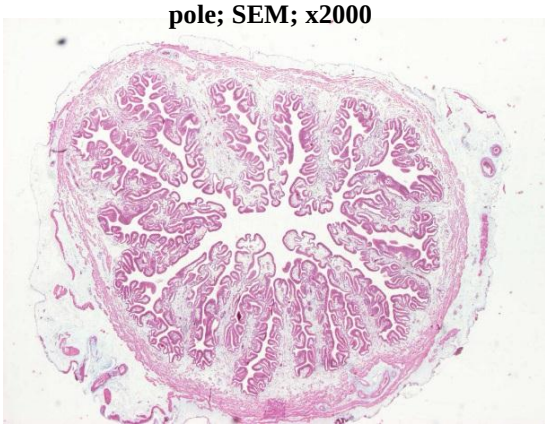


Fig.15. The neck of the infundibulum; Masson's trichrome stain; x20

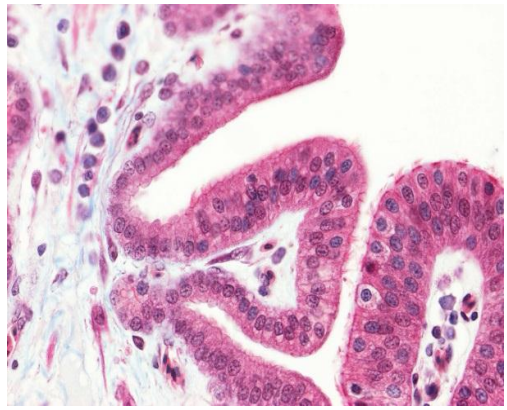


Fig.16.The prime folds of the mucosa with capillaries and collagen in the lamina propria; Masson's trichrome stain; x 200

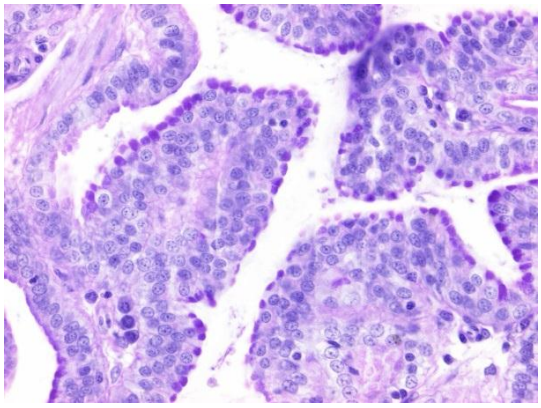


Fig.17. The epithelium presents more goblet cells than the funnel region that excrete their secretory granules in the lumen;PAS stain; x200

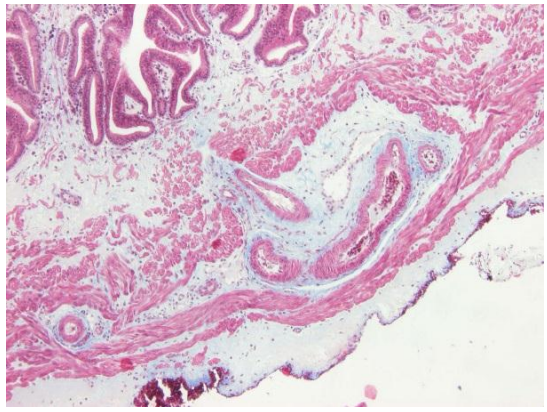


Fig.18. The tunica muscularis is very well developed and between the two layers of smooth muscle presents large blood vessels. Masson's trichrome stain; x 100

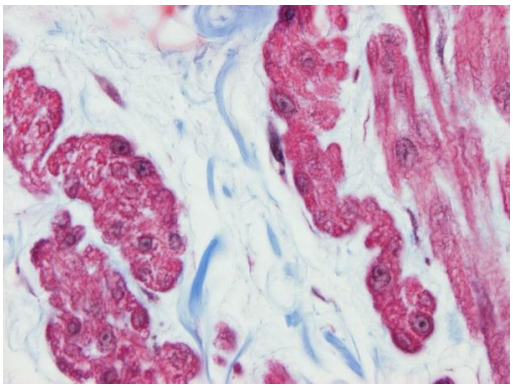


Fig. 19. The tunica muscularis is structured from two layers of smooth muscle: inner-circular and outer-longitudinal; Masson's trichrome stain; x 1000

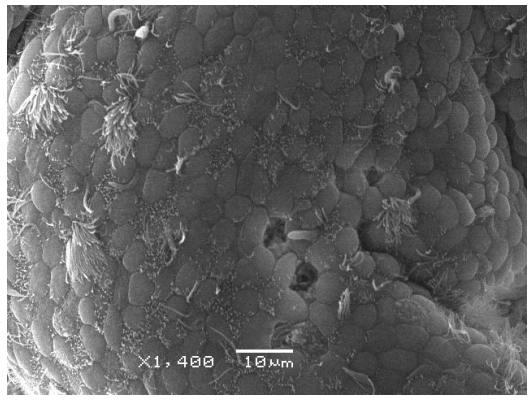


Fig. 20. The surface of the infundibulum neck with few ciliated cells and a lot of secretory cells; SEM; x1400

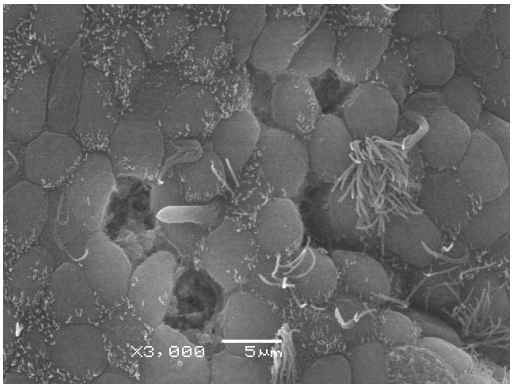


Fig. 21. The surface epithelium with ciliated cells and goblet cells and also tubular gland openings; SEM; x3000

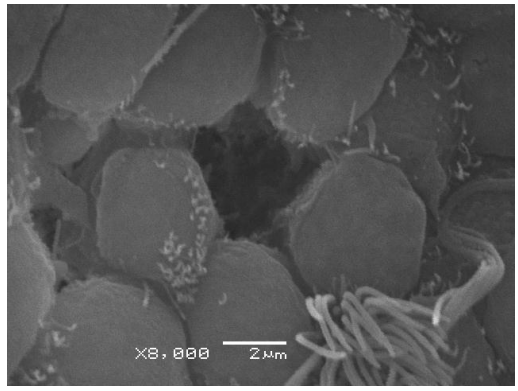


Fig. 22. The cells lining the tubular gland opening in the lumen through the epithelium; SEM; x8000

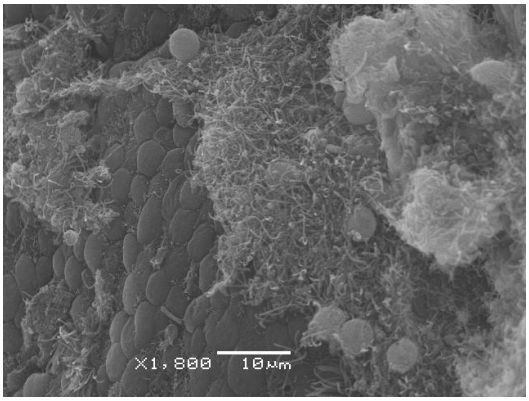


Fig. 23. Secretion granules and filaments at the surface of the epithelium; SEM; x1800

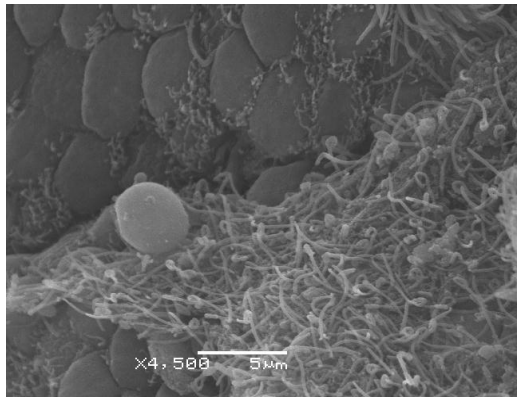


Fig. 24. Secretion granules and filaments over the apical pole of the goblet cells from the epithelium; SEM; x4500

Conclusions

1. The infundibulum consists of two distinct areas: the funnel and the neck;
2. This segment is structured of a tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa;
3. In the surface epithelium of the funnel we can observe ciliated cells that are non-secreting; goblet cells that are mucous-secreting and non-ciliated; and secretory cells that are located in the depths of the adjacent mucosal folds;
4. The mucosa of the funnel area is lined with a ciliated epithelium with very few mucous-secreting goblet cells;
5. In the neck of the infundibulum as it continues caudally, the folds increase in size and become more complex in structure;
6. There are a lot of goblet cells that are mucous-secreting and although there are also numerous ciliated cells;
7. The lamina propria consists of tubular glands that have low pyramidal shaped cells;
8. The second tunica of the infundibulum is the tunica submucosa which consists of connective tissue;
9. The tunica muscularis is structured from smooth muscle cells, and appears as bundles of muscle cells in the funnel area and as two distinct layers in the neck area;
10. The tunica serosa is very thin, made of connective tissue and a squamous epithelium;
11. Using the SEM we could notice numerous thin epithelial folds (fig.1.), and also the fact that the ciliated cells dominate in the epithelium of the funnel area of the infundibulum;
12. In the neck region of the infundibulum, using SEM we notice a lot of mucous-secreting cells, and fewer ciliated cells. This pattern is more obvious as we go towards the magnum.

References

1. Bellairs R., Osmond M., 2005- *The atlas of chick development*, Elsevier Academic Press.
2. Chousalkar K., 2008 – *Ultrastructural changes in the oviduct of the layingh end uring the laying cycle*, CellTissueRes, 332: 349-358.
3. Cotea C.,2013– *Histologie specială*, Ed. Tehnopress, Iaşi.
4. CoteaC., 2013 – *Biologie celulară, embriologie generală, histologie generală*, Ed. Tehnopress, Iași
5. Khokhlov R. Y. (2008) - Morphology of the infundibulum of the oviduct of the sexually mature hens, Int. J. Morphol., *26(4)*:883-886.
6. Todireanu C., Cotea C., Solcan Carmen, 2010 – *The morphology and histochemistry of the magnum during the egg pas sage through the oviduct in hens during the laying cycle*, Lucrări Stiintifice – Medicină Veterinară, vol 53.
7. Todireanu C., Cotea C., Solcan Carmen, 2010 – *The dynamics of theshellmembranesformation in theisthmus of theLohmann Brown layinghens*, Lucrări Stiintifice – Medicină Veterinară, vol 53;.
8. Wang X., Ford B. C., Praul C. A., Leach Jr. R. M., 2002 - *Collagen X expression in oviduct tissue during the different stages of the egg laying cycle*, PoultryScience 81:805–808.
9. Whittow C., 2000 – *Sturkie's Avian Physiology (Fifth edition)* Elsevier.

MORPHOLOGIC PARTICULARITIES OF THE BROWN BEAR (*Ursus arctos*) MAJOR SALIVARY GLANDS

Alexandru Munteanu, Corneliu Cotea,Cătălin Todireanu
Faculty of Veterinary Medicine, Iasi, Romania

Abstract

The brown bear (Ursus arctos) belongs to the Carnivora order, being adapted to an omnivorous diet. The particularities of the brown bear major salivary glands are the same in those of the domestic carnivore : parotid gland is reduced in size, with triangular shape, the mandibular salivary gland is located close to the angle of the jaw and is partially covered by the parotid salivary gland with oval shape, the monostomatic salivary gland is well developed with a elongated form, the polystomatic salivary gland consists of glandular reduced lobules, forming a discontinue string.

Keywords*: morphology, brown bear, salivary glands.*

Materials and methods

The material used has a 4 month cub, that die from unknown causes on which anatomical examination was conducted.

The time between the death of the bears and collection and fixation of tissues was of 2–12 h. Histological sections was 5 μm thick, stained with PAS.

Results and discussions

The salivary glands are paired organs that secrete saliva, via their ducts into the oral cavity. Saliva keep the mucoasa of the mouth moist and is mixed with the food during mastication to lubricate the passage of the food bolus during swallowing and initate chemical digestion of food.

Parotid gland is reduced in size, with triangular shape and yellow-brown color, which lies at the junction of the head and neck, ventral to the auricular cartilage in the retromandibular fossa. The dorsal end portion is double incised, with forked appearance, which includes among its branches the ear concha. By the medial side, the gland comes in contact with the facial nerve, maxillary vascular cord and parotid lymph node. Front side is covered by skin and *Parotidoauricularis muscle***.**

Parotid channel (*Ductus parotideus* / *Stenon*) emerges from the rostral edge of the gland, passes over the lateral surface of the masseter muscle, in his third middle, perforates buccinator muscle and opens in upper lateral oral vestibule, in the plane of the third premolar, at the salivary papilla, which is not very evident (*fig. 1, 2*).

Structurally, the parotid gland is mixed, has both serous and mucous component that are strictly separate. The serous cell is a wedge-shaped or pear- shaped cell, with acidophilic cytoplasm. The rounded nucleus is central or paracentral in position. The lateral margins of the cell is indistinct. The mucous adenomere consist of mucous cells that are pyramidal. The cytoplasm of these cells is foamy and the flattened nucleus are positioned basally (*fig. 6, 7, 8, 9, 10, 11*).

The mandibular salivary gland is located close to the angle of the jaw and is partially covered by the parotid salivary gland. It is oval in shape, with the lobules poorly separated, situated subcutaneously, caudal to the monostomatic salivary gland, between the linguofacial

and maxillary viens. The color is lighter than the parotid salivary gland. It drains by a single large duct, (*Ductus mandibularis* / *Wharton*), wich passes ventral to the mucosa of the floor of the oral cavity, close to the lingual frenulum, to open with the major sublingual duct ,that accompanies, on the sublingual caruncle, small and triangular in shape (*fig. 3, 4, 5*).

Histological structure of the mandibular gland, confirm that it is a mixed gland with both mucous and serous structures (*fig. 12, 13, 14, 15, 16*). The monostomatic salivary gland is well developed with a elongated form, a compact mass that stretches from the posterior third of the tongue to the mandibular salivary gland. The gland have a single draining duct (*Bartholin*) that shows a trajectory parallel and dorsal to the *Wharton'* duct and shares a common opening with the mandibular salivary duct on top of the sublingual caruncle protruding from the prefrenular part of the floor of the oral cavity (*fig. 3, 4, 5*). Both sublingual salivary glands produce a serous-mucoid secretion in which the mucous part dominates (*fig. 17, 18, 19, 20, 21, 22, 23, 24*).

The polystomatic salivary gland consists of glandular reduced lobules, forming a discontinue string. The gland is located more rostrally that the the monostomatic salivary gland and opens through several samller ducts (*Rivinius*). These openings are located on a longitudinal fold in the lateral sublingual recesse (*fig.3,4*).

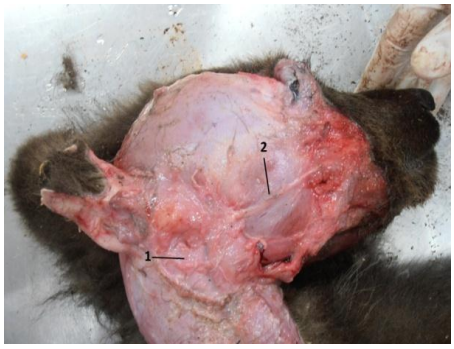


Fig. 1 The parotid salivary gland : 1- gl. parotis; 2- ductus parotideus.

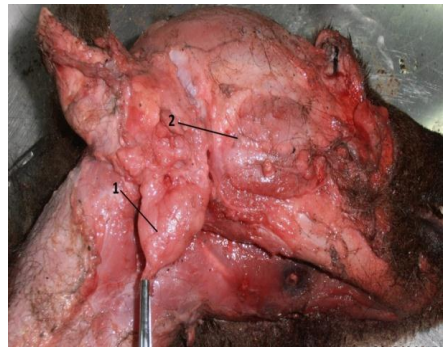


Fig. 2 The parotid salivary gland : 1- gl. parotis; 2- ductus parotideus.

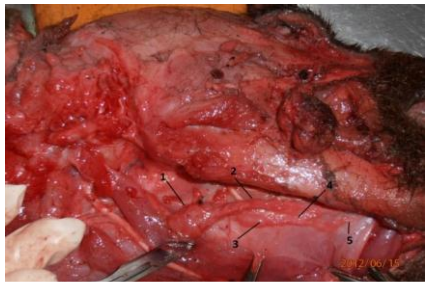


Fig. 3 Major salivary glands: 1- gl. mandibularis; 2- ductus mandibularis; 3- gl. sublingualis monostomatica; 4- ductus sublingualis major; 5- gl. sublingualis polistomatica.

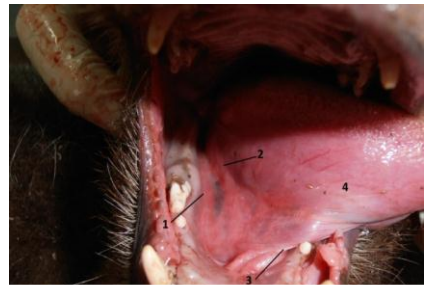


Fig. 4 Lateral sublingual recess: 1- plica sublingualis; 2- gl. sublingualis monostomatica; 3- frenulum linguae; 4- lingua.

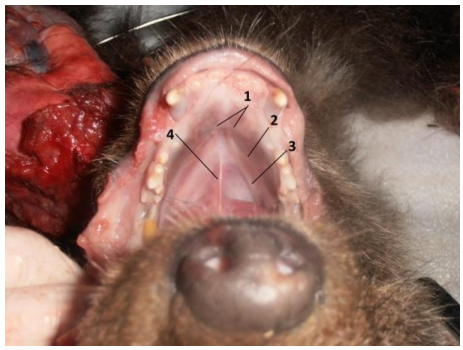


Fig. 5 The sublingual floor: 1- caruncula sublingualis; 2- ductus sublingualis major; 3- ductus mandibularis; 4- frenulum linguae

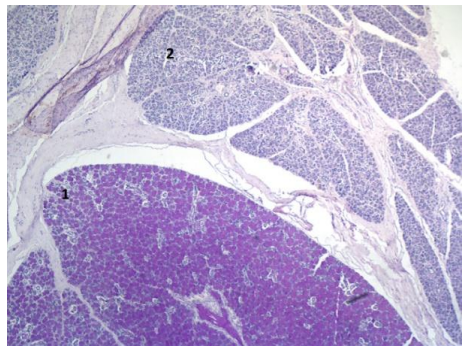


Fig. 6 The separation of the serous acinus region and mucos acinus region in the parotid salivary gland : 1- mucosa region; 2-serous region; PAS stain; x 40.

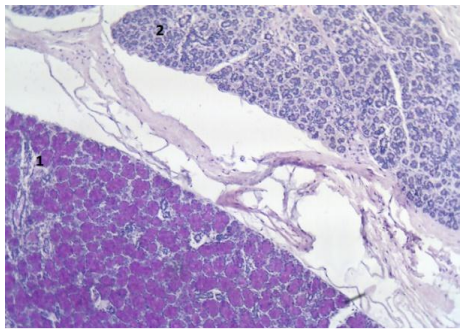


Fig. 7 The separation of the serous acinus region and mucos acinus region in the parotid salivary gland : 1- mucosa region; 2-serous region; PAS stain; x 100.

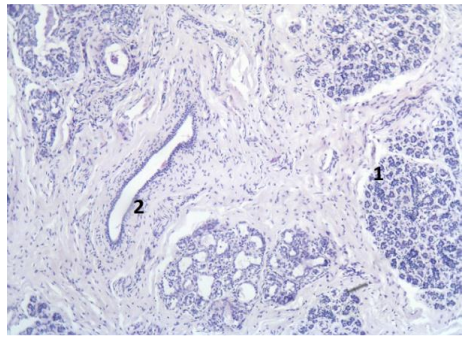


Fig. 8 The parotid salivary gland with serous region : 1 - serous acinus; 2- collector duct; PAS stain; x 100

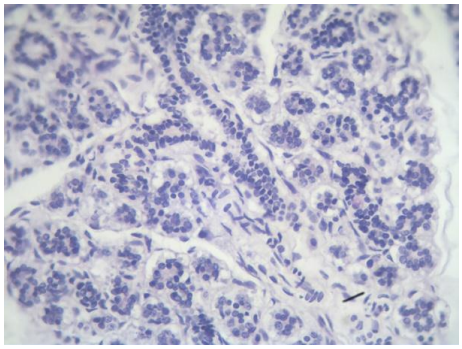


Fig. 9 The parotid salivary gland with serous region and interlobular connective duct; PAS stain; x 400

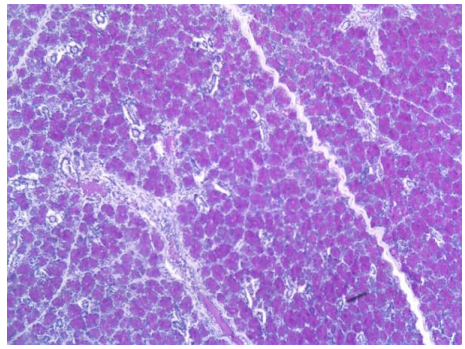


Fig. 10 The parotid salivary gland with mucos acinus ; PAS stain; x 100

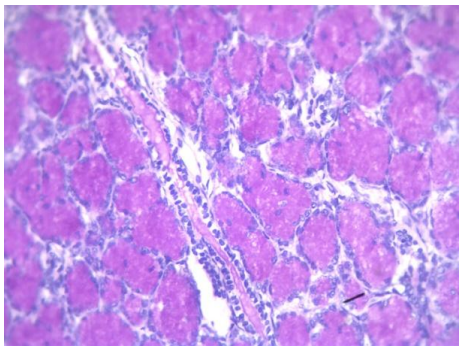


Fig. 11 The parotid salivary gland with mucos acinus ; PAS stain; x 400

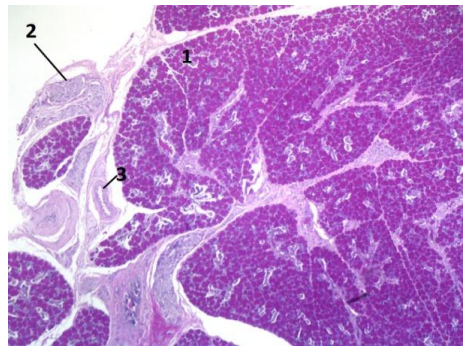


Fig. 12 The mandibular salivary gland : 1- mucos acinus; 2- a nerve ganglion; 3- blood-vessel; PAS stain; x40

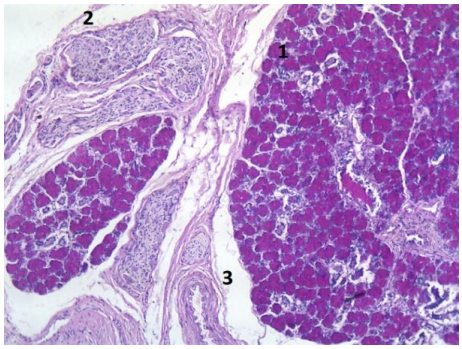


Fig. 13 The mandibular salivary gland : 1- mucos acinus; 2- a nerve ganglion; 3 - blood-vessel; PAS stain; x100

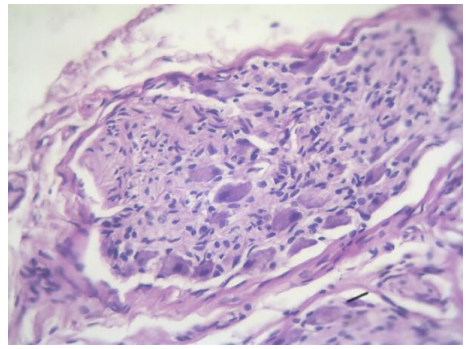


Fig. 14 Nerv ganglion from mandibular salivary gland ; PAS stain; x 400

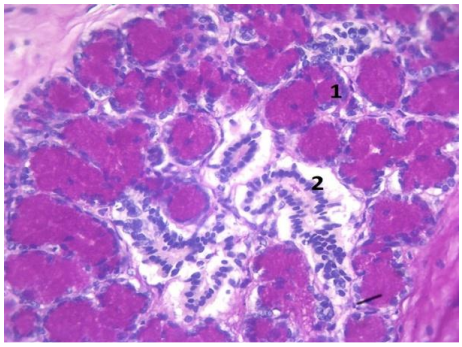


Fig. 15 The mandibular salivary gland with mucos region: 1- mucos acinus; 2- interlobular connective duct; PAS stain; x 400

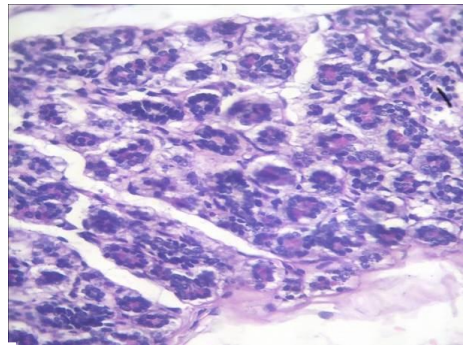


Fig. 16 The mandibular salivary gland with serous region; PAS stain; x 400

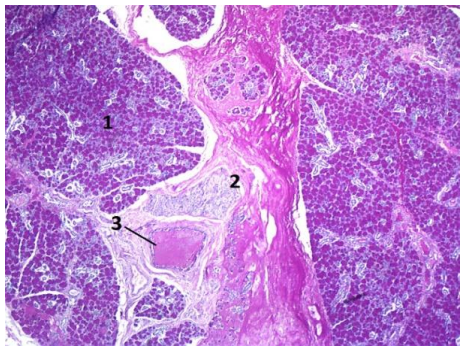


Fig. 17 Sublingual monostomatic salivary gland: 1- mucosa region ; 2- nerv ganglion; 3- collector duct; PAS stain; x 40

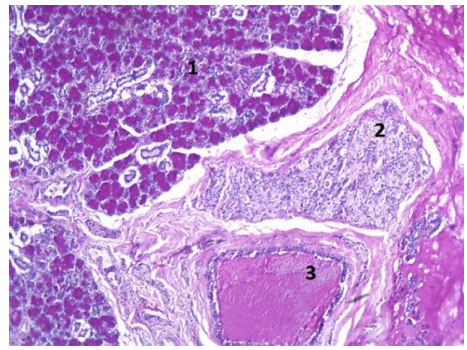


Fig. 18 Sublingual monostomatic salivary gland: 1- mucosa region; 2- nerv ganglion; 3- collector duct; PAS stain; x 100

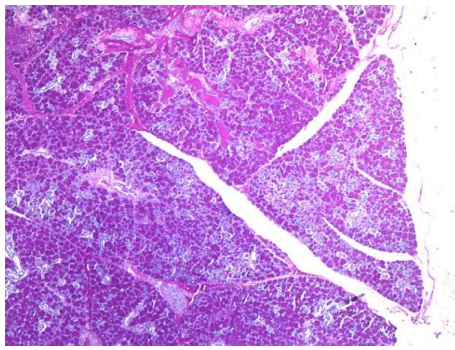


Fig. 19 The lobes of the sublingual monostomatic salivary gland with fringe aspect; PAS stain; x 40

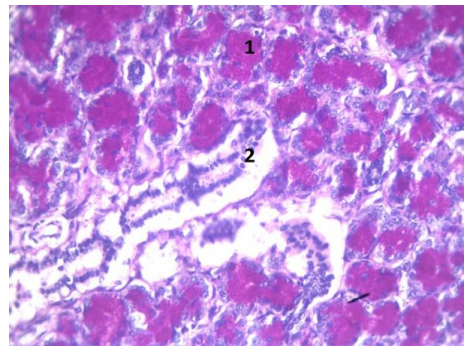


Fig. 20 The sublingual monostomatic salivary gland:1- mucos acinus; 2- interlobular connective duct; PAS stain; x 400

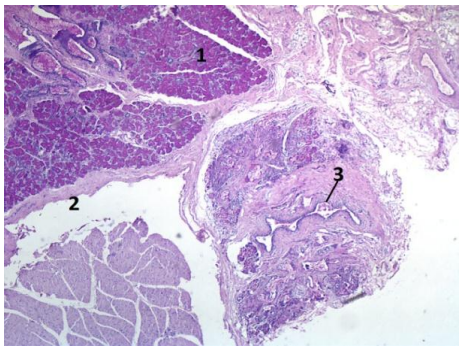


Fig. 21 The sublingual polystomatic salivary gland: 1- mucos acinus; 2- squamous epithelium; 3- collector duct; PAS stain; x 40

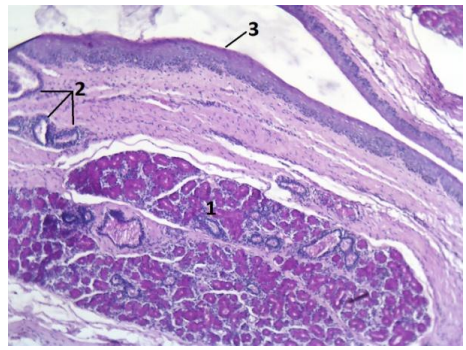


Fig. 22 The sublingual polystomatic salivary gland: 1- mucos acinus; 2- *Rivinius* ducts; 3- squamous epithelium; PAS stain; x 100

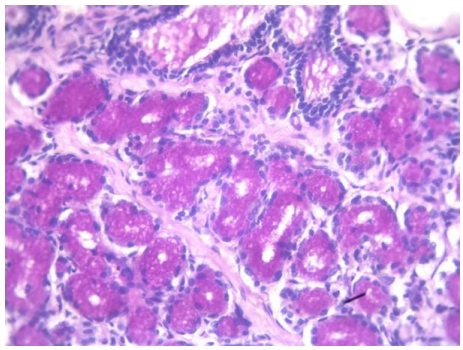


Fig. 23 The sublingual polystomatic salivary gland with mucos acinus and interlobular connective duct; PAS stain; x 400

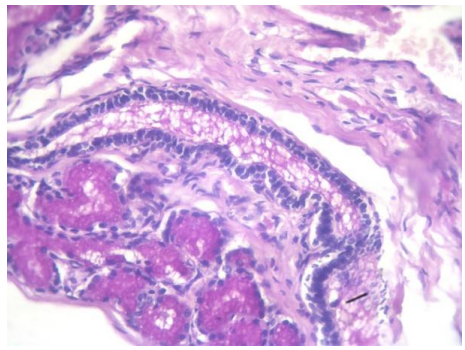


Fig. 24 Interlobular connective duct from sublingual polystomatic salivary gland looking curved; PAS stain; x 400

The salivary glands are developed in correlation with omnivorous diet, similar to those of the domestic carnivores, meaning dog.

Parotid salivary gland is limited topographically similar to that of the dog, with excretory duct with similar paths, directly over the surface of the masseter muscle.

The mandibular salivary gland is more reduced that parotid gland, with a mixt secretion.

Morphologically, are individualized both sublingual salivary glands.

Bartholin and *Wharton* ducts shares a common opening and *Rivinius* openings are located on a longitudinal fold in the lateral sublingual recesse.

Both sublingual salivary glands produce a serous-mucoid secretion in which the mucous part dominates.

References

1. Cotea C. – Histologie Specială, Editura Tehnopress, Iaşi, 2011.
2. Coţofan V., Hriţcu Valentina- Anatomia animalelor domestice volumul II Organologie,Editura Orizonturi Universitare, Timişoara, 2007.
3. Paştea E., V. Coţofan, Şt. Chiţescu, M. Miclea, N. Cornilă, V. Nicolescu, C. Radu, Popovici, R. Palicica- Anatomia comparată a animalelor domestice vol. I, Editura Didactică şi Pedagogică, Bucureşti,1985.
4. Postolache Ferat Aida- Anatomie Veterinară – Splanchnologie- , Editura ” Ion Ionescu De La Brad ”, Iaşi, 2004.
5. Spătaru Mihaela-Claudia - Anatomia Comparată a Animalelor, Editura Alfa, Iaşi, 2009.

CYTOLOGICAL ASPECTS OF FLUID EFFUSIONS IDENTIFIED AND ANALYZED IN GOATS

Andreea-Roxana Ancuta, Andra-Cristina Bostănaru,Sorin-Aurelian Pasca, Corneliu V. Cotea, Gheorghe Savuta
Faculty of Veterinary Medicine, Iaşi
anda181184@yahoo.com

Abstract

The examination of cell populations from effusions represents a useful diagnostic technique, essential in current veterinary practice.Samples are taken from 17 goats following a precise technique depending on the location of the effusion. After cytocentrifugation of the sample, the cytological exam is performed by recognizing the well-known morphological characteristics and in some cases by identifying noticeable criteria of malignity. Effusions are classified depending on the cell type and total protein concentration is divided into three categories: transudate, modified transudate and exudate. The cytosmear background, the morphology of the cells contained within the effusion and/or the identification of formed elements (germs, parasites) allow orientation towards a physiopathological process compatible with one or more categories.Hemorrhagic effusions appear consequently to traumatisms or are secondarily formed after hemostasis disorders. The effusions prominently lymphocytic can be reactional (cardiopathies, compressions of lymphatic vessels, bacterial infections) or tumoral (lymphocytic desquamation).Infectious effusions of bacterial origin are always rich in cells and composed exclusively of neutrophils polymorphonuclear. In the case of gel-forming effusions specific to small ruminants, transudates are cell-poor, with normal blood elements and presenting a fine granular material on the cytosmear background.Normal and abnormal morphologic criteria are indispensable in order to identify cellular atypias and eventually determine the origin tissue of the t encountered cells.

Keywords: *small ruminants, pleural effusion, peritoneal effusion, pericardia effusion.*

Introduction

Cytological examination can be applied to almost all types of injuries (*R. Cowell*, 2008) found in goats, being a safe economical and very useful technique to a diagnostic approach. This allows for a quick response since the time of processing the samples is short and has the orientation towards appropriate complementary examinations in diagnosis certainty. Cytology of cavitary effusions can recognize an inflammatory, traumatic or neoplastic cavity. The main difficulty for effusions remains the similarity between malignant cells and transformed mesothelial cells: then inflammatory and chronic effusions, mesothelial hyperplasia becomes important and atypical, mimicking a tumoral population resulting in degenerated, vacuolated, mitotic cells.

Materials and method

The casuistry came from private farms, from the intensive exploitation system of growth in Iasi County, in Faculty of Veterinary Medicine of Iaşi clinics, within Necropsy and Pathology Laboratory of the Faculty of Veterinary Medicine and the Laboratory of Pathology Anatomy and Cytology of the National Veterinary School of *Alfort*, Franţa - *Ecole Nationale Veterinaire d'Alfort (ENVA).*

For the cytological exams, the effusion samples were collected from three internal cavities: pleural cavity, peritoneal cavity and pericardial cavity from 17 goats, representing 100% of cases with different breeds and ages.

We mention that the animals were presenting various diseases accompanied by accumulation of fluids in the serous cavities. All samples were accompanied by information on the presumptive diagnosis in order to guide and interpret the cytological examination results.

The bacteriological examination was performed for the effusion samples secondary to the microscopically identification of intra and / or extracellular pathogens in 6 goats, representing 26% of the cases.

In order to achieve the cytological preparations, the puncture was performed on serous cavities and the displaying on blades if the liquid obtained by blood cytosmear method, which was subsequently colored with May Grünwald-Giemsa technique.

The puncture was performed without anesthesia, the place of choice being trimmed and disinfect. There were used 22 to 23 G needles attached to 5 ml syringes. The sampling was carried out in sterile tubes with anticoagulant (EDTA) to measure the physical, chemical and microscopically characteristics. The microscopically analysis was initially performed to small objective (x10, x40) and subsequently with the immersion objective (x100) (*Crespeau François, 2010*).

The aim was the identification, morphology and proportion of the main normal cell types, but also the highlighting of cytological criteria of abnormal nucleated cells with different origins, belonging to different cell lines (*Cote C., 2008 Leib MS, Monroe W.E., 1997*).

The bacteriological examination (direct bacterioscopic) was carried out for 35% of cases, respective 6 samples, both from suffering live animals and postmortem, from fresh cadavers, from the collected represented by the puncture fluid, but also from the intestinal contents (in this category are include the samples of the fresh cadavers).

In carrying out the cytosmear, there are followed the next steps: displaying the material as thin layer on a clean glass blade, defatted and sterilized by flaming; the drying of the cytosmear was made at room temperature; fixing the cytosmear through heat, passing the blade three times through the flame of the gas burner; staining the cytosmear was performed by the Gram method and, in some cases, Giemsa method.

For the cytosmears from the organ, the sectioning area of the organ on the blade was wiped, and for the puncture liquid, the displaying was made by placing a drop with Pasteur pipette.

Results and discussions

Cytological study (microscopically) of effusions in goats

Our observations have been made in this phase of research on 17 cases, of which 6 were carriers of parasitic infestations and 10 samples represented by inflammatory processes, of which nonsuppurative inflammations in 4 cases with septic nonsuppurative inflammations (3 cases), and aseptic nonsuppurative inflammations (1 case); septic suppurative and purulent inflammations in 6 cases and 1 case of active effusion associated with tumoral process (Table. 1).

Table 1. The classification of effusions
after *Crespeau F.,* École Nationale Vétérinaire d'Alfort, 2007 (* reference values)

Material	Macroscopical aspect	NTC/µl	PTg/dl
Transudate	colorless, clear	*< 1500	*< 2,5
Modified transudate	Variable color (white - yellow - red)	* 1000 - 7000 1400 - 3600	* 2,5-7,5 2,7-4
Exudate	Variable color, high turbidity, floaters	*> 7000 7100 - 4500	* > 3 4,5 – 8

Aseptic nonsuppurative inflammations were diagnosed in 3 goats, collected from the pleural cavities, the analyzed effusions having the following characteristics:

The clinical examination revealed respiratory distress, mixed dyspnea; biochemical parameters values (CP and NTCN / ml) corresponding to the effusions of inflammatory type (Table 1).

Upon cytological examination, there is identified, for all samplings, an sanguinolent background due to contamination during sampling while accompanied by a soft protein texture; there are revealed numerous normal neutrophils granulocytes, but they are also observed GN more hypersegmented, nondegenerate (no suppurative appearance). Macrophages are also in an increased number, with vacuolated cytoplasm, foam-like, with signs of eritrophagocytosis; there are also identified mature and small lymphocytes. No pathogens were visibly microscopically highlighted (Fig. 1, 2).

Cytological diagnosis is relevant to the aseptic nonsuppurative inflammatory effusions. Although, cytologically, no intra or extracellular pathogens were identified, we can not exclude the possibility of a bacterial infections.

Analyzing the number 10 case, from anamnesis we see that the kid presents joint pains while walking, nocturnal choking conditions. Echocardiographic examination revealed a large amount of effusion. From classical biochemical determinations made, respectively the CP g / l and NTCN / ml values, there are available effusions of exudate type (Table 1).

The cytological examination indicates on a protein background slightly bleeding a mixed cell population, consisting of active macrophages, macrovacoulated, containing hemosideric pigments, eritrophagocitose, large mesothelial, basophile, with spherical nucleus, sometimes with visible nucleolus, both segmented and mature (Fig. 3).

The cytological diagnosis is compatible with an aseptic inflammatory pericardial effusion, secondary to the sero-fibrinous pericardial inflammation.

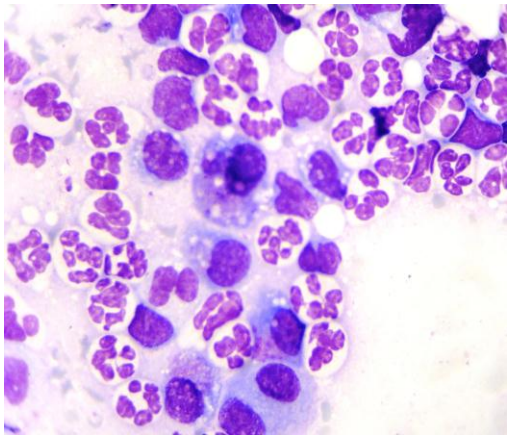


Fig. 1. Kid. Aseptic pleural Exudate. Hypersegmented neutrophiles granulocytes. Interstitial bronchopneumonia Col. MGG, x 1000

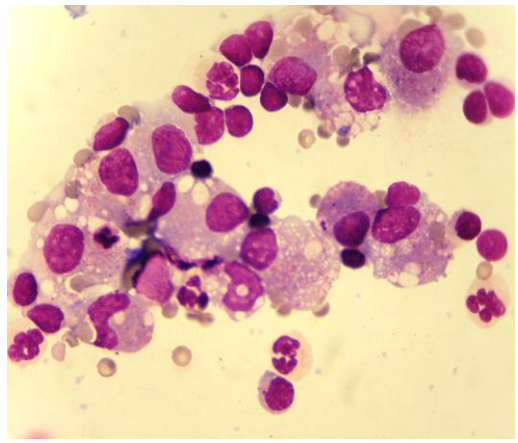


Fig. 2. Kid. Aseptic pleural exudate. Active macrophages (eritrophagocytose). Interstitial bronchopneumonia Col. MGG, x 1000

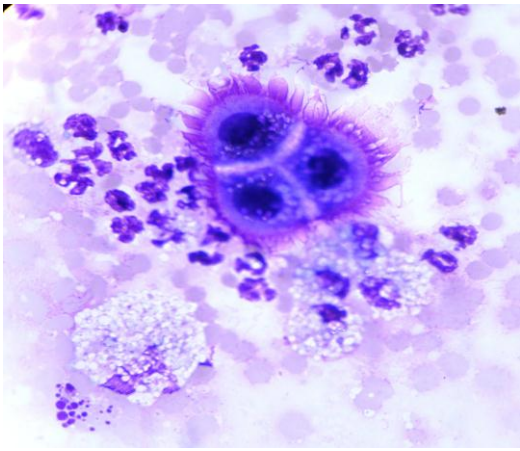


Fig. 3. Kid. Pericardial exudate. Sero-fibrinous pericarditis. Extracellular hemosiderin. Anaerobic enterotoxaemia. Col. MGG, x 1000

Septic suppurative inflammations

The septic suppurative inflammations were found in 2 animals, 1 kid and a goat by samples taken from the chest cavity.

From the anamnesis data, the goats presented advanced state of dehydration, a severe laryngitis and tracheitis. The biochemical determinations made, regarding the quantitative parameters have referenced values available to the effusions of exudate type (Table 1). The bacteriological complementary examination was performed from pleural transfuses.

Upon the cytological examination on an intense proteic background, the preparations contain a polymorphous cell population, composed on degenerate neutrophils granulocytes (piocytes), macrophages and mesothelial cells in approximately equal numbers. There can be

observed, both intracytoplasmic and extracellular, the presence of certain pathogens represented by numerous rod-shaped microorganisms (rods) in the form of clusters, micro-organisms with morphological structure typical for actinobacillus. The cytological diagnosis is compatible for some inflammatory effusions of septic exudate type - strong neutrophilic inflammation associated to purulent bronchopneumonia (Fig. 4).

After the bacteriological diagnosis, there were isolated bacteria belonging to *Arcanobacterium pyogenes* species.

According to the literature, *Arcanobacterium pyogenes* is the most important species of the genus, being a common germ of respiratory tract, genital mucosa and other mucosa of ruminants. By the presence of two neuroaminidase, the germs adhere relatively easy to the epithelial cells of the host and generates infections, mostly purulent.

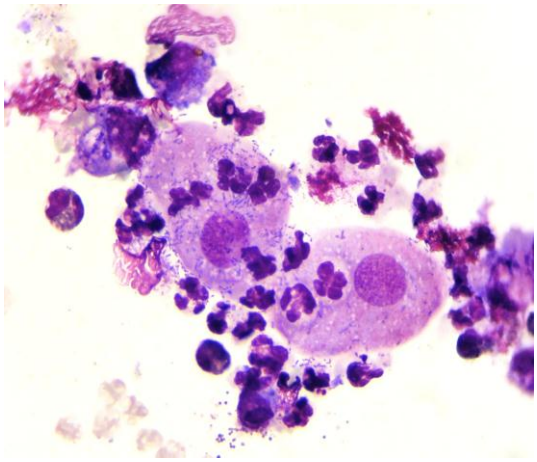


Fig. 4. Kid. Septic pleural exudate. Intra/Extracellular bacilli. Bacterial purulent bronchopneumonia. Col. MGG, x 1000

Other cases of *septic suppurative inflammations* (2 cases) revealed us cloudy bilateral pleural transudates. Clinically, for both animals at the left lung level, there were found vesicular murmur, cough, and dehydration. There were collected exudate samples to obtain the bacteriological examination.

From the classical biochemistry determinations point of view, the effusions fall in the inflammatory exudate type (Table 1).

Upon the cytological examination, microscopically, on an sanguinolent background (due to the contamination while collection) and proteic moderate, there is found a cellular population predominantly neutrophilic, in different stages of degradation – karyolitic neutrophils, degenerated (piocytes) hypersegmented, accompanied by macrophages containing intracytoplasmic nuclear material, lymphoblasts and mature lymphocytes. There are highlighted extracted and / or intracytoplasmic microorganisms with morphologically polymorphic appearance (Fig. 5).

Upon cytological diagnosis, the karyolitic neutrophils and intracellular microorganisms naturally present in infections and in certain bacterial inflammatory

conditions. Under these circumstances, we can speak of a subacute / chronic pleurisy, expressed through a septic suppurative exudate associated with a diffuse purulent pneumonia.

The bacteriological diagnosis is relevant for an pasteurelic infection (Fig. 6).

In the etiology of pasteurellosis in young ruminants it is often involved *M. haemolytica* şi *Pasteurella multocida* (*Paul I., 2005).*

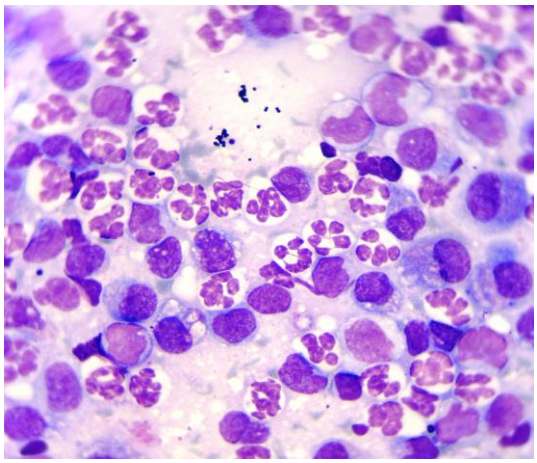


Fig. 5. Kid. Septic suppurative pleural exudate. Fibrinous pleurisy. Col. MGG, x 1000

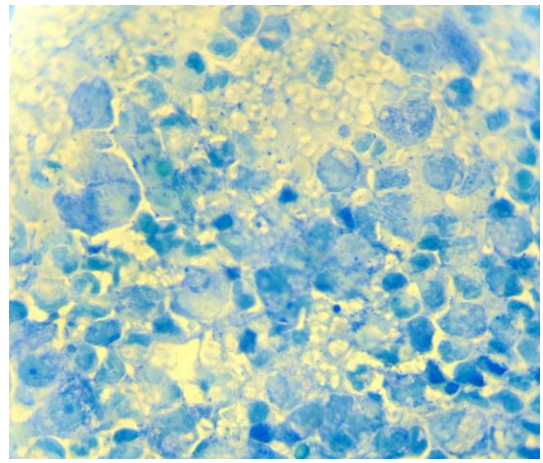


Fig. 6. Kid. Cytosmear from exudate. Pasteurella multocida. Col. Methylene blue, x 1000

Fibrin purulent inflammation (septic suppurative) was observed in two cases, peritoneal effusions, being biochemically employed in the simple effusions type (Table 1).

The clinical examination reveals for both animals signals of ascitic abdomen for about 3 weeks, arthritis and polyarthritis; due to poor state of maintenance.

The cytological examination express on a hemorrhagic background of the preparations, on which there are highlighted various types of cellular populations: numerous neutrophils granulocytes – piocytes, accompanied by active macrophages with cytoplasms in patches vacuolated, rare images of eritrophagocytose, some lymphocytes, rare plasmocytes and some active mesothelial cells (Fig. 7).

The observed germs in these effusions are microaerophilic bacteria, but there are not the main pathogens responsible for transudates development. According to the morphological appearance, there could not be identified as cytological, so there was performed a complementary bacteriological examination.

The bacteriological diagnosis evokes a streptococcal infection due to *Streptococcus equi*.

The cytological diagnosis is compatible with the chronic septic fibrin purulent peritonitis.

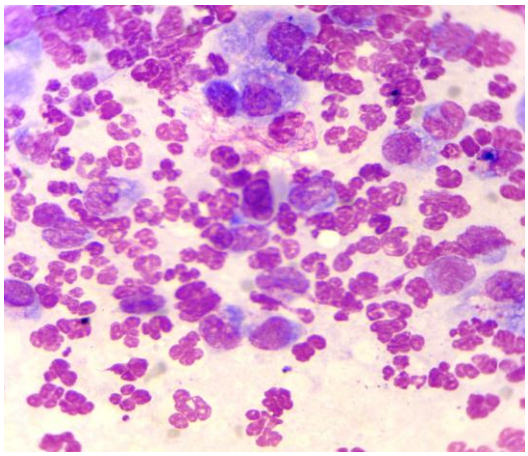


Fig. 7. Goat. Neutrophilic peritoneal exudate. Fibrin textures. Active macrophages. Septic chronic fibrin purulent peritonitis. Infection with *Streptococcus equi*. Col. MGG, x 400

Active effusions associated with parasitic infestations

The parasitic infestations are cytological represented by inflammatory exudative effusions (6 cases), 5 cases being peritoneal transudates and 1 case of pleural type.

Upon the microscopically examination, on an intense hemorrhagic background, there are identified macrophages, heavily vacuolated with large vacuoles, with foamy cytoplasm, especially perinuclear and with non-homogenous appearance. Inside the macrophages, there are identified hematoidine crystals of bright yellow color, representing the insoluble form of bilirubine crystals, chemically identical with bilirubine, usually presented in intense intracavitary hemorrhage (Fig. 8., 9).

The crystalline bilirubine represents the main end product of the degradation of red blood cells in most mammals, through the conversion of bilivertine to bilirubine by the process of bilivertine reductase (*Kathleen P. Freeman, 2007).*

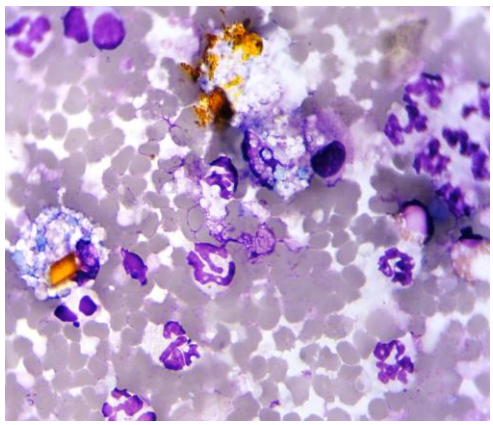


Fig. 8. Kid. Aseptic hemorrhagic pleural exudate. Intramacrophagic hematoidine crystals. Parasitic pleurisy.Col. MGG, x1000.

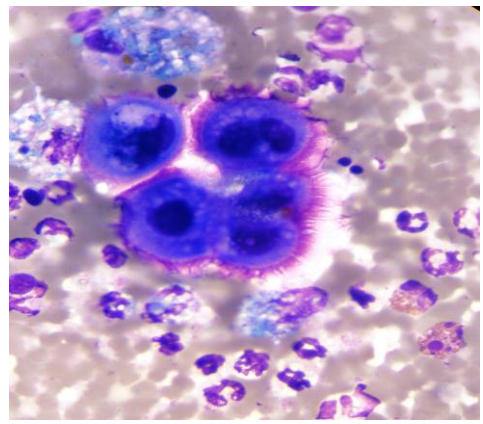


Fig. 9. Kid. Aseptic hemorrhagic exudate. Active mesothelial macrophages. Hypersegmented eosinophils. Parasitic infestation. Col. MGG x 1000

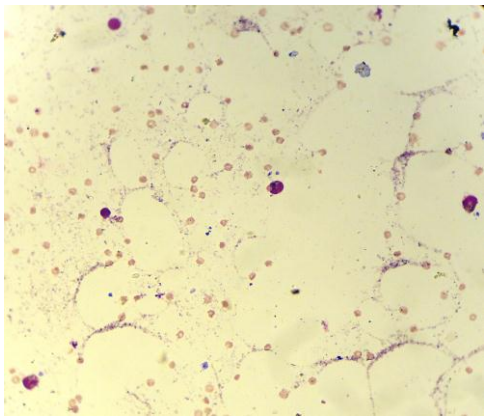


Fig. 10. Goat. Eosinophilic peritoneal exudate. Intensely eosinophilic background. Normal eosinophils. Col. MGG, x 400

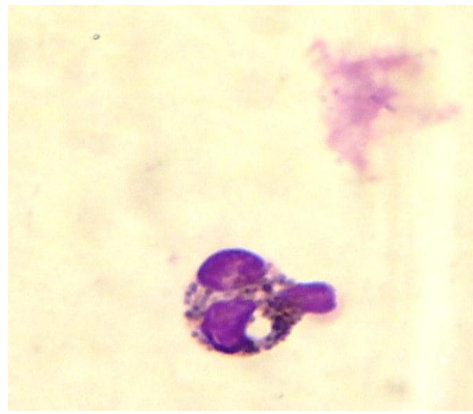


Fig.11. Goat. Eosinophilic peritoneal exudate. Normal eosinophils. Col. MGG, x 400

Almost in all fields, there have been identified images of erythro and/or citofagocytose. There are highlighted large mesothelial cells, 4-5 times the size of neutrophils, with oval nucleus, with weak highlighted or absent nucleus, with cytoplasm more or less basophilic. In addition to these cellular populations, there are identified, in a moderate amount, hypersegmented eosinophils and neutrophils granulocytes, young and segmented (Fig. 10, 11).

Cytological diagnosis: by corroborating the microscopically criteria–heavily bleeding background, macrophages and intensely active mesothelial basophils, hypersegmented eosinophils granulocytes and segmented neutrophils, we identify an aseptic exudate secondary to a non-specific pleurisy; we do not exclude the possibility of a parasitic infection, based on the presence of degranulate eosinophils.

In one case we identified an abdominal cloudy brown effusion, associated with an ovarian tumoral formation (information obtained from clinical observation data sheet).

At the cytological examination we found, on a hemorrhagic background, the presence of an ovoid, polygonal cellular population, arranged in small plates, presenting a central amorphous eosinophilic material (proteic secretion). The cells are then polarized with a moderate nucleo-cytoplasmic report and a net shape. The cytoplasm is basophilic, fine, granular, sometimes microvacuolated. The ovoid nucleus is fine reticular and it presents more voluminous basophils nucleolus (Fig. 12., 13).

The cytological diagnosis reveals a hemorrhagic neutrophilic inflammatory effusion, with the presence of neoplastic glandular epithelial cells - tumoral effusion. According to the anamnesis data it is likely that the origin to be of an ovarian nature (ovarian carcinoma).

An effusion is considered cancer when the tumoral cells are identified, in our case, these cells are difficult to identify because the reactive mesothelial cells mimics the classical criteria of cancer cells. To distinguish the tumor cells from the mesothelial ones, we observed numerous malignant characters: anisocytosis, anizonucleoză, large irregular nucleus, heterogeneous chromatin, etc.

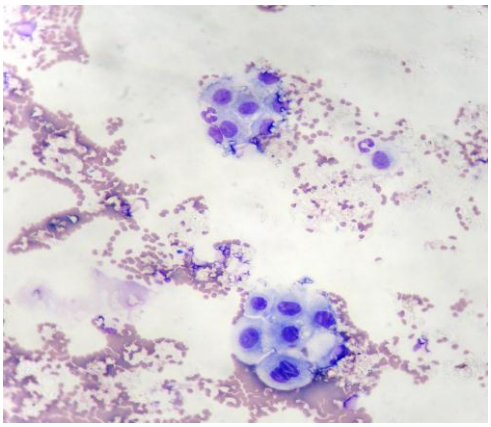


Fig.12. Goat. Tumor abdominal effusion. Posters of glandular epithelial cells posters. Ovarian carcinoma. Col. MGG, x 400

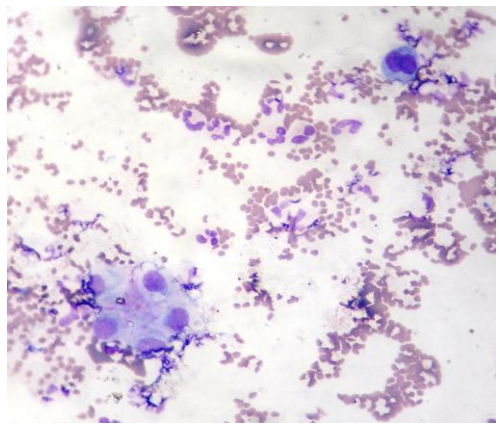


Fig. 13. Goat. Tumoral abdominal effusion. Blood elements. Posters of glandular epithelial cells. Ovarian carcinoma. Col. MGG, x 400

Conclusions

The cytological and bacteriological examination of cavitary transudates assessed in this study allowed the extraction of the following conclusions:

1. All samples assessed were classified based on microscopically biochemical and cytological examinations directly related to the bacteriological and clinical examination of the animal.
2. The identified effusions were then placed on the basis of morfocytological appearances, in the inflammatory lesion as follows:
3. Acute inflammations and effusions: nonsuppurative inflammations 4 cases, of which septic nonsuppurative 3 cases and aseptic nonsuppurative 1 case; septic suppurative and purulent inflammations 6 cases, parasitic type inflammations 6 cases and tumoral effusion 1.
4. In nonsuppurative inflammations there are cytological highlighted numerous normal neutrophils granulocytes, GN more hypersegmented, nondegenerate (no suppurative appearance), macrophages in an increased number, with signs of eritrophagocytose and small mature lymphocyte. No pathogens were visibly microsopic highlighted.
5. In aseptic nonsuppurative inflammations, there are identified mixed cellular populations, consisting of active macrophages, macrovacuolated, containing hemosiderine pigments, eritrofagocytose, large mesothelial, basophils with visible nucleolus, mature and segmented neutrophils granulocytes.
6. In septic nonsuppurative inflammations, cytological on an intense protein background, the preparations contain a polymorphous cell population consisting of piocytes, macrophages and mesothelial cells, it is observed, both intracytoplasmic and extracellular, the presence of pathogens represented by microorganisms rod-shaped, crowded, morphological structure typical for actinobacillus; bacteriological, there were isolated bacteria belonging to *Arcanobacter pyogenes* species.
7. Other cases of septic suppurative inflammations (2 cases) revealed us inflammatory transdates associated to the infection with *Pasteurella multocida.*

8. In the fibrin-purulent inflammation, the observed germs are microaerophilic bacteria, bacteriological represented by *Streptococcus equi.*
9. Parasitic infestations are cytological represented by exudative inflammatory effusions (6 cases), 5 cases being peritoneal transudates and 1 case of pleural type.
10. Microscopicallyally, there are identified macrophages, crystals of hematoidine of bright yellow color, large mesothelial cells, with oval nucleus, with weak highlighted or absent nucleolus, there are observed hypersegmented eosinophils and neutrophils granulocytes, young and segmented.

References

1. Cotea C., 2008 - Biologie celulară. Embriologie generală. Histologic generală, Editura Tehnopress, Iaşi.
2. Crespeau F., 2010 - Cytologie des epanchements cavitaires du chien et du chat, Cours de cytologie, Ecole Nationale Veterinaire d'Alfort.
3. Cowell R., Tyler R., Meinkoth J., Denicola D., 2008 - Diagnostic cytology and hematology of the dog and the cat. 3rd ed. Elsevier, 475p.
4. Kathleen P. Freeman, 2007 – Veterinary Cytology. Dog, cat, horse and cow, Ed. Manson, Londra.
5. Leib M.S., Monroe W.E., 1997 – Practical Small Animal Internal Medicine – W. B. Saunders Company, Philadelphia, USA.
6. Paul I., 2005 – Etiomorfopatologia bacteriozelor la animale, Ed. Pim, Iași.

FINE STRUCTURE OF THE THYMUS GLAND IN OSTRICH (*STRUTHIO CAMELUS L*)

El-Zoghby, I.M.A[1] and Attia, H.F[2]
[1]Histology & Cytology Dept. Faculty of Vet Med. Benha University
Faculty of applied medical science .Medical Lab. Dept. Taif Univ.KSA
[2]Histology & Cytology Dept. Faculty of Vet Med. Benha University
Faculty of applied medical science .Medical Lab. Dept. Taif Univ.KSA
drhossam222@hotmail.com

Abstract

Fifteen samples of the ostrich thymus glands were used to show the histological, histochemical and fine structure of the thymus glands. H&E, Gomori method and Crossman's trichrome; PAS and alcian blue stain were used for histological and histochemical demonstration respectively. The gland consisted of lobes of different size separated from each other by fine strands of connective tissues. These lobes surrounded by CT capsule of collagen and reticular CT fibers. Each lobe was divided by the septa into numerous thymic lobules. Each lobule was consisted of cortex (Darkly stained area) and medulla (Lightly stained area). The parenchyma mainly consisted of lymphocytes (Thymocytes) and epithelial-reticular cells which were few in the cortex and numerous in the medulla. Hassal's corpuscles were located in the center of the thymic lobules. It was consisted of concentric epithelial-reticular hyaline cells which appeared in different stages of degeneration. The ultrastructure of the thymic lobules showed numerous thymocytes of different size and epithelial-reticular cells between them.

Introduction

The thymus has attracted much attention in the recent literature, due in large part to its role in the immunological response. This role appears to be mediated by secretions (Trainin, 1974), the thymus gland of the ostrich lay at the base of the neck (Bezuidenhout, 1986). The ostrich are used principally for the production of meat of high protein value and low cholesterol level. Furthermore ostrich are used for the production of hides and feather (Horbanczuk, Sales, Cleeda, Konecka, zinab and Kawaka 1998).

Numerous papers were dealed with the histological structure of the thymus in poultry as, Hodges,1974, Kendall,1980 , Hashimoto et al.,1982 , Crivellato, Nico, Battistig, Beltrami and Ribatti ,2005 and Karaca , Yörük and Uslu, 2006 . The fine structure was discussed by Kendall and Frazier, 1979 and Chan, 1995.

Papers dealing with the structure of the thymus gland of ostrich were found to be rare from the anatomical point of view Bezuidenhout, 1986 and Wagner , Kirberger and Groenewald, 2001. Where, those discussed the histological structure were rare. (Elewa, 2005).

The aim of the current study is to throw focus spots of light on the histological, histochemical and fine structure of the ostrich's thymus gland.

Materials and methods

The thymus glands of 15 mature ostrich of both sex, their ages ranges from 6 months-2.5 years. The specimens were collected from different abattoirs, put in Susa fluid fixative, dehydrated in ascending grades of alcohols, cleared in xylene and embedded in paraffin then cut at 4-5 U.

The histological, histochemical and ultrastructure of the thymus glands were studied using different histological stains. Hematoxyline & Eosin for general character , Crossman's trichrome for identification of collagen fibers and Silver impregnation technique for identification of reticular fibers and combination of PAS and alcian blue for identification of both acid and neutral mucopolysacharides (Bancroft et al., 1994).

A very small pieces of 1x1x1 mm were fixed in 2.5% glutaraldehyde in 1M phosphate buffer (pH. 7.3) for 24 hours then post fixed in cold 1M phosphate buffered 1% osmium tetroxide (pH. 7.3) for 3 hours, rinsed in phosphate buffer for 30 minutes then dehydrated (Hayat,1986). Semi-thin sections were stain by Toluidine blue. Ultra thin sections were obtained and mounted on copper grids then stained with uranyl acetate and lead citrate (Reynolds,1965). For the TEM picture in (Faculty of science , Ain shams University)using Sumy Electron Optics SEO at 25 Kv.

Results

The thymus glands of the ostrich were large lobulated and consisted of numerous lobes. The thymus lobes were covered by CT capsule mainly consisted of collagen and fine reticular fibers (Fig. 1).

CT septa extended from the capsule divided it into thymic lobules. These septa contained the thymic blood vessels (Fig. 2).

The CT capsule and the thymic septa was contained dispersed single muscle cells (Fig. 3). The stroma of these lobules was consisted of fine collagen and reticular fibers and reticular cells.

The thymic lobules divided into two definite parts, the cortex which was dark in staining and located in the peripheral part of the thymic lobules, and the medulla which appeared light in staining and located in the core of the thymic lobules (Fig. 4).

The cortex was studded by numerous small size lymphocytes (Thymocytes) with darkly stained basophilic centrally located nuclei and peripheral rim of cytoplasm and numerous lymphoblasts with vesicular, centrally located nuclei (Fig. 5).

The medulla was appeared lightly stained in compared to the cortex due to it was occupied by the large size lymphocytes with centrally located nuclei and acidophilic cytoplasm. Epithelial-reticular cells were located between the lymphocytes. The epithelial reticular cells were numerous and characterized by distinct cell boundaries and faint basophilic cytoplasm and central to some what eccentric nuclei (Fig. 6). The small lymphocytes were few in comparison to that of the cortex.

The fine structure of the thymocytes consisted of large centrally located nuclei and condensed chromatin. Electron lucent non granular thin rim of cytoplasm (Fig. 7).While the lymphoblasts were characterized by large nuclei with numerous islets of heterochromatin, nucleolus and fine granular cytoplasm (Fig.8).

Epithelial-reticular cells were located between the lymphocytes but they were few. They were large cells with eccentric nuclei and faint acidophilic cytoplasm. The epithelial-reticular cells were large cells with oval nuclei with prominent nucleolus and fine granular dust like cytoplasm (Fig. 7 and 11).

The cortex of the young immature ostrich thymic lobules was studded with numerous eosinophilic cells (granular leucocytes), with shiny granular acidophilic cytoplasm and basophilic nuclei (Fig. 9).

The epithelial reticular cells might be arranged in the form of corpuscula thymica

(Hassal's corpuscles) which consisted of degenerated epitleilal -reticular cells and lymphocytes. (Fig. 10).

The epithelial-reticular cells gathered with the lymphocytes to form the Hassal's corpuscle which was characterized by degenerated structurless, hyalinized center and peripheral concentric arranged epithelial-reticular cells (Fig.11).

Few plasma and macrophage cells were located between the lymphocytes (Fig.12). Different stages of mitotic figures was noticed in the cellular elements of the thymus gland especially in the cortex .

The thymus gland of the adult ostrich was characterized by vacuolation in the cortex and the medulla, single and multiple vacuoles was located in the architecture of the thymic lobules (Fig. 13and 14 and 15).

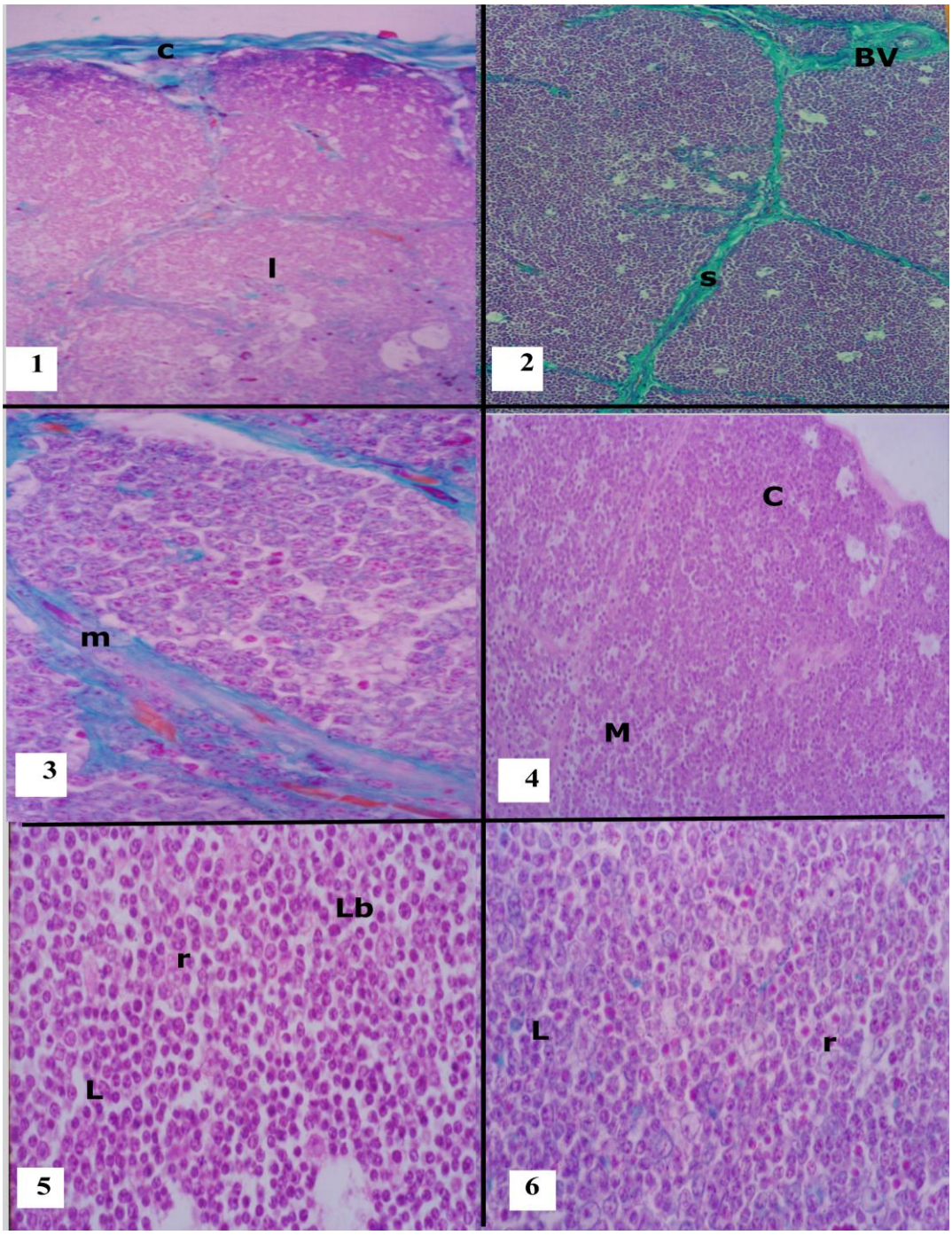
Cellular degeneration was recorded in the lymphocytes and the epithelial-reticular cells. The epithelial reticular cells were collected together as double cells or group of cells which appeared homogenous light eosinophilic masses (Fig.16) .

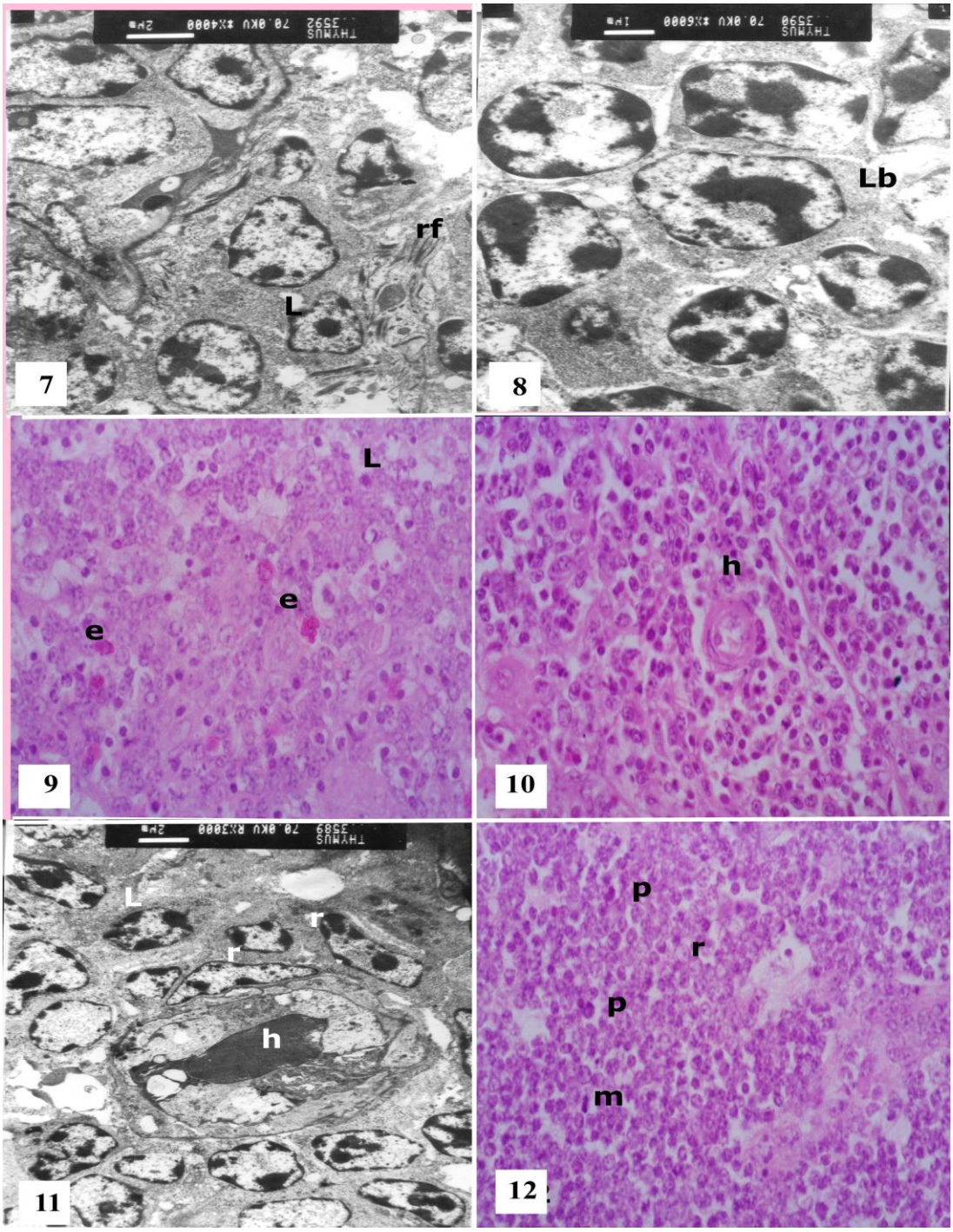
Large eosinophilic masses located in the medulla of the thymic lobules which appeared homogenous structurless, none nucleated (Fig.18).

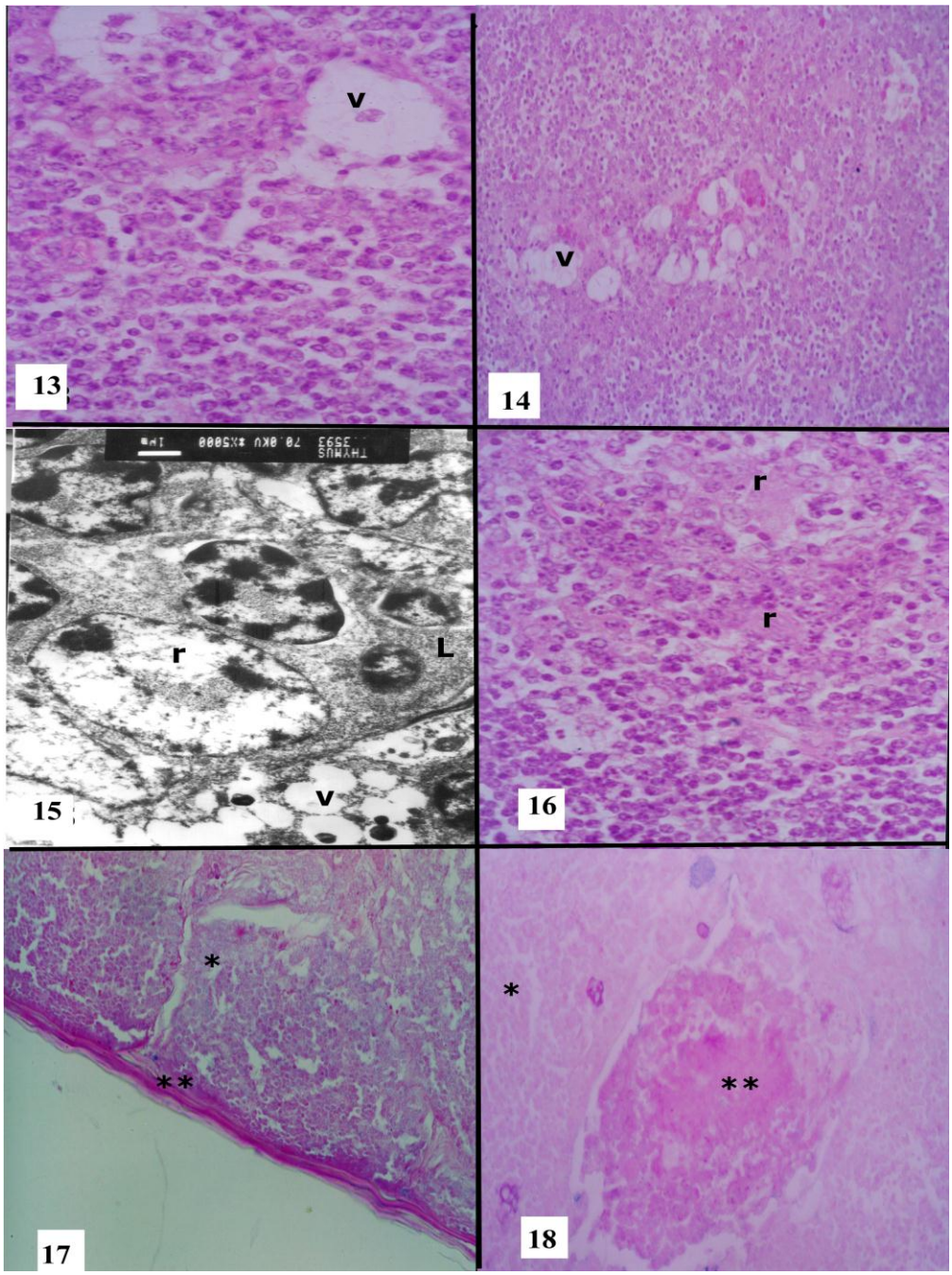
The CT capsule and the CT septa were strong PAS positive reaction and the cellular elements of the cortex were moderate PAS positive reaction (Fig.17). While the cellular elements of the medulla were very faint PAS reaction except the large eosinophilic mass which appeared moderate PAS positive reaction.(Fig.18).

List of figures

Fig.1: Photomicrograph of the thymic lobes showing the collagen fibers capsule (C) and the thymic lobules (l). Crossman's trichrome X20; **Fig.2:** Photomicrograph of the thymic lobes showing the blood vessels in the septa (Bv) and the CT septa (s). Crossman's trichrome X10; **Fig.3:** Photomicrograph of the thymic lobes showing the dispersed muscle cells in the CT septa (m). Crossman's trichrome X40; **Fig.4:** Ostrich thymic lobules to identify the dark staining cortex (c) and lightly staining medulla (M). H&E X10; **Fig.5:** The thymic cortex at 1 years old ostrich showing the numerous thymocytes (L) and lymphoblasts (Lb) with few epithelial reticular cells in between (r). H&E X100; **Fig.6:** The ostrich thymus cortex showing the thymocytes (L), Epithelio-reticular cells (r). X 40; **Fig.7:** TEM photography of the thymus cortex showing the thymocytes (L), and reticular fibers (rf) X4000; **Fig.8:** TEM photography of the thymus cortex showing the lymphoblast (Lb), X6000; **Fig.9:** Photomicrograph of the 6 months ostrich thymus gland cortex showing numerous eosinophils (e). H&E X100; **Fig.10:** Ostrich thymic medulla showing typical Hassal's corpuscle (h). H&E X100; **Fig.11:** TEM photography of the thymus medulla showing the typical Hassal's corpuscle (h) with degenerated center and epithelial reticular cells (r). X3000; **Fig.12:** Photomicrograph of the thymic cortex showing the plasma cells (p) and the thymocytes with different stages of mitotic figures (m). H&E X100; **Fig.13:** Photomicrograph of the 2 years ostrich thymus gland cortex showing the involuted thymic cortex with different size single vacuoles (v). H&E X40; **Fig.14:** Photomicrograph of the 2 years ostrich thymus gland medulla showing the involuted thymic medulla with grouped vacuoles (v). H&E X40; **Fig.15:** TEM photography of the thymus medulla showing the vacuolation (v) and the collected epithelial reticular cells (r).X5000; **Fig.16:** Photomicrograph of the thymus gland of mature ostrich showing collected epithelial reticular cells (r). H&E X100; **Fig.17:** Photomicrograph of the thymus gland cortex showing positive PAS in the capsule (**) and moderate PAS positive in the cortex thymocytes (*).Alcian blue-PAS combination. X40; **Fig.18:** Photomicrograph of the thymus gland medulla showing positive PAS in the large eosinophilic mass (**) and very faint PAS reaction in the medulla thymocytes (*).Alcian blue-PAS combination X40


c
l
1
BV
s
2
m
3
C
M
4
Lb
r
L
5
L
r
6


rf
L
7
Lb
8
L
e
e
9
h
10
L
r
r
h
11
p
r
p
m
12



Discussion

The thymus gland is necessary for normal development and function of gonads and thyroid gland as well as body growth (Azab et al., 2004).

The thymus gland of the ostrich located at the base of the neck as lobulated oval

structure (Elewa, 2005 and Bezuidenhout, 1999). The thymus gland was covered by CT capsule from which septa extended into the architecture of the gland dividing it into numerous thymic lobules. This statement was clarified in the ostrich and fowls thymus gland (Elewa, 2005 and Hodges, 1974) respectively. The lobulation is prounounced in the ostrich and chicken than that of the guinea fowl (Onyenanous et al., 1994).

The thymic lobules were divided into outer dark cortex and inner light medulla without any line of demarcation. The light and dark appearance due to the types of the lymphocytes colonized in the cortex (small and medium size lymphocytes) and in the medulla (few numbers, this finding was augmented by Kendall, 1981 and Mahmoud, 1987 in chicken and Elewa, 2005 in ostrich.

The epithelial-reticular cells were few in the cortex and numerous in the medulla, while the macrophage and the plasma cells were abundant in the cortex (Bradley and Grahame, 1960) than that of the medulla, while (Thorbecke et al., 1957) noted that the plasma cells only present in the outer part of the medulla. This finding may explain the suggestion of the microphagic role of these cells to remove the residual structure of the cortical dead cells as in the chicken (Mahmoud, 1987).

Eosinophilic masses of different size were formed as change in the reticular cells which started as small vacuoles appeared spherical in shape and later on filled with various amount of homogenous eosinophilic substances were located in the medulla of the thymic lobules (Payne, 1971). These masses are somewhat considered as a step of Hassle's corpuscle formation. (Oneynanous et al., 1994).

The typical structure was consisted of round-laminated cornified epithelial reticular cells with homogenous structurless mass center. The typical structure of the Hassle's corpuscle was augmented by the findings of Elewa, 2005; Firth, 1977 and Mahmoud, 1987 in ostrich, fowl and chicken respectively.

The thymus gland has defense mechanism action via the produced T-lymphocytes (Nickel et al., 1977 and Adkins et al., 1987). The thymus-dependent development is represented morphologically by the small lymphocytes of the circulation and the white pulp type of development in the tissues. As in mammals, the thymus-dependent tissues of the chicken are basic to the ontogenesis of cellular immunity Copper et al., 1966 and Aviles-Trigueros and Quesada, 1995).

The involutions start in the thymus gland of the ostrich and characterized by vacuolation of the epithelial-reticular cells with degree of cellular degeneration and appearance of some fatty vacuoles in the gland parenchyma (Elewa, 2005; Hodges, 1974 and Mahmoud, 1987). The involution start at the sexual maturity and characterized by loss of the cortical substance leaving the medullary tissues with few lymphocytes Karakoz et al., 1976 and Eliwa, 2005).

References

1. Adkins,B; Muller,C; Okada,C.V; Reichert,R.A; Weissman,I.L and Spangrude,G.J (1987): Early events in T-cell maturation . Ann.Rev. Immunol.,5:325-365.
2. Aviles-Triguueros, M and Quesada, J.A (1995): Myelopoiesis in the thymus of the Sea Bass, Dicentraarchuslabrax L. Anat.Rec.,242:83-90.
3. Azab, M.E; Fathalla, S.I and Emara, S.A (2004): Effect of thymoectomy on some physiological aspect in Japanese quails (Coturnix, coturnix japonica). Egypt.J.Basic Appl. Physiol., 3(1): 121-135.

4. Bancroft, J.D; Cook,H.C; Stirling, R.W and Turner,D.R(1994): " Manual of histological techniques and their diagnostic application' .2nd Ed.,Churchill Livingstone, Edinburgh, London, New York.
5. Bezuidenhout AJ. (1986): Anatomy of ostrich . In Deeming, D.C.(ed.): The ostrich biology, production and health. 1st Ed. CABI publishing , Wallingford, Oxon, pp.13-49.
6. Bezuidenhout AJ. (1999): The topography of the thoraco-abdominal viscera in the ostrich (Struthio camelus). Onderstepoort J Vet Res. 1986 Jun;53(2):111-117.
7. Bradley,O.C and Grahame,T (1960): The structure of the fowl.Oliver and Boyd, Edinburgh.
8. Chan AS (1995): Ultrastructure of myoid cells in the chick thymus. Br Poult Sci. 1995 May;36(2):197-203.
9. Cooper M D., RD. Peterson , MA South and R A. Good (1966): The functions of the thymus system and the bursa system in the chicken. Experimental Medicine, Vol 123, 75-102
10. Crivellato,E, B Nico, M Battistig, C A Beltrami, D Ribatti (2005): The thymus is a site of mast cell development in chicken embryos. Anat Embryol (Berl). 2005 Feb ;209 (3):243-249 .
11. Elewa,Y.H.A (2005): Some morphological studies on immunological organs in ostrich. M.V.Sc. Thesis. Fac. Of Vet. Med. Zagazig. Univ.
12. Firth, G.A (1977): The normal lymphatic system of the fowl.Vet.Bull.47:167-179.
13. Hashimoto Y, Kitagawa H, Kudo N, Sugimura M.(1982): Fine structural and immunohistochemical studies on the thymic cysts in ducks. Nippon Juigaku Zasshi. 1982 Aug;44(4):597-605.
14. Hayat, M. A. (1986):- Basic techniques for transmission electron microscopy. 1st Ed.Academic Press, Inc. Florida.
15. Hodges,R.D (1974): The histology of the fowl. Academic press. London., N.Y. Sanfrancisco.
16. Horbanczuk,J; Sale,J;Celeda, T; Konecka, A; Zinaba, G and Kawaka, P (1998): Cholesterol content and fatty acid composition of ostrich meat as influenced by subspecies. Meat Sci., (50): 385-388.
17. Karaca T, Yörük M, Uslu S.(2006): Age-related changes in the number of mast cells in the avian lymphoid organs. Anat Histol Embryol. 2006 Dec;35(6):375-379 .
18. Karakoz I, Hasek M, Kohoutová L. (1976): The role of bursa of Fabricius and thymus in antibody-mediated allograft rejection in ducks. Folia Biol (Praha). ;22(5):304-11.
19. Kendall, M.D. (1980): Avian thymus glands: A review. Dev. Comp. ImmunoL, 4 : 191-209.
20. Kendall, M.D. (1981): Cells of the thymus. In : The thymus gland, Kendall, M.D.(ed.), Academic Press, London, PP. 63-83.
21. Kendall, M.D. and Frazier, J.A. (1979): Ultrastructural studies on erythropoiesis in the avian thymus .1. Description of cell types. Cell Tissue Res., 199:37-61.
22. Mahmoud,M.H.M (1987): Some histological studies on the immune system of chickens. Ph.D . Thesis, Fac of Vet.Med, Cairo University.
23. Nickel.R; Schummer,A and Seiferle, E (1977): Anatomy of the domestic birds. 4th Ed. S.S. Verlag Paul Parey, Berlin, Hamburg.
24. Onyeanusi,B.I, J C Onyeanusi, A N Emma, C D Ezeokoli (1994): The thymus of the guinea fowl from the eighteenth day of incubation until maturity. Anat Histol Embryol. 1994 Dec ;23 (4):320-329
25. Payne, L.N(1971): the lymphoid system. In'' Physiology and biochemistry of domestic fowl''. 2nd Ed. Edited by Bell,D.J and Freeman, B.M. Academic press.London.PP.985-1037.
26. Reynolds, E. S. (1965): The use of lead citrate at high ph as an electron opaque in electron microscopy. J. Cell 26: 208-215.
27. Thorbecke , G.J; Gordor,H.A; Wostman, B.S ; Wagner, M and Reyniers, J.A (1957): Lymphoid tissue and serum gammaglobulins in young germ free chickens. J.Infect.Dis., 101:237-257.
28. Trainin, N. (1974). Thymic hormones and the immune response. Physiol. Rev. 54, 272-315.
29. Wagner WM, Kirberger RM, Groenewald HB.(2001): Radiographic anatomy of the thoraco-abdominal cavity of the ostrich (Struthio camelus). J S Afr Vet Assoc.Dec;72(4):203-8

EFFECT OF DIETARY PHYTOESTROGENS ON SOME GROWTH PERFORMANCE PARAMETERS IN RATS

Amal M. El-Nahla; Ismail, A.A; Abdoon, A.S* and Heba M.A. Abd EL-Razik
Department of physiology, Faculty Veterinary Medicine,Suez Canal University and
Department of animal reproduction and A.I*,National Research Center.
Faculty of Veterinary Medicine. Suez Canal University

Abstract

Phytoestrogens have been shown to possess many physiological actions on different body systems in female rats. This study investigated the effect of high dietary phytoestrogens (1320 µg/ g genistein and 704.7 µg/ g daidzein as determined by HPLC) versus low dietary phytoestrogens (45 µg/g genistein and 28 µg/g daidzein as determined by HPLC) on body weight gain (BWTG), feed intake (FI) and feed conversion ratio (FCR) in intact cyclic females. Also the effect of high dietary phytoestrogens on body weight (BWT) and feed intake in pregnant femal rats at the 7th gestation day (GD 7) and 19th gestation day (GD 19) was also investigated. The results showed significant (P<0.05) lower BWTG and FI in high phytoestrogens-fed cyclic females than the low phytoestrogens-fed females, while FCR was significantly (P<0.05) increased in treated females. In pregnant dams BWT and FI were significantly (P<0.05) lower in high phytoestrogens-fed group than the low phytoestrogens-fed one.

Introduction

Phytoestrogen is a general definition that has been applied to any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens usually by binding to oestrogen receptors (Sunita et al., 2008).

Interest in the physiological role of bioactive compounds present in plants has increased dramatically over the past decade. In relation to human health, phytoestrogens embody several groups of plant derived, nonsteroidal compounds with oestrogen like activity (Setchell and Cassidy, 1999). The major classes of dietary phytoestrogens are categorized into 3 classes, namely the isoflavones, lignans and coumestans (Cederroth and Nef, 2009).

Isoflavones, such as daidzein and genistein, are selective estrogen receptor modulators (SERM) or biochemical compounds that are able to agonize or antagonize estrogen receptors (ER) (Muthyala et al., 2004). Daidzein and genistein are able to bind both ERα and ERβ but demonstrate a higher affinity for ERβ (Kuiper et al., 1998 and Muthyala et al., 2004). Moreover, daidzein's intestinally produced metabolite, which equol, has a higher affinity for ERβ than daidzein or genistein (Setchell et al., 2005).

In a series of reports it was demonstrated that soy-derived isoflavones can significantly decreased body weight, adipose tissue deposition, skin tail temperature and produce anxiolytic effects (Lund & Lephart, 2001 and Bu & Lephart, 2006). Accordingly, this study was aimed to examine influences of soy-derived isoflavones via diet on feed intake, body weight gain, feed efficiency (FE) and feed conversion ratio (FCR).

Materials and methods

A total of 68 albino rats (60 females and 8 males) were purchased from Lab Animal House, National Research Center, Dokki, Cairo. Animals were kept in metallic cages, the females were housed separately five per cage. Animals were kept at room temperature and 12

hours light dark cycle (7.00: 19.00). Food and water were allowed *ad libitum*. Animals were kept for 2 weeks for acclimatization before starting the experiment. The females were divided into two groups each of 30 females: Group I, low phytoestrogens group fed on a casein based ration which was formulated to fulfill all the nutritional requirements of adult rat (Table 1). However, group II received high phytoestrogens diet. Both diets were formulated according to NRC, 1995. The diet was offered for 30 days.

Table 1. Composition of experimental diets

Ingredients in %	Low phytoestrogens group	High phytoestrogens group
Dried skimmed milk powder (25%)	5.00	5.00
Casein (82%)	6.00	-
Soybean seeds* (40%)	-	26.00
Corn gluten (62%)	7.00	1.00
Dried Alfa Alfa hey (15%)	-	1.00
Yellow corn	71.38	59.00
Cellulose	2.00	-
Corn oil	5.00	-
Soybean oil	-	5.00
Dicalcium phosphate	1.7	1.65
Ground limestone	1.00	0.50
Common salt	0.153	0.15
Premix**	0.25	0.25
Methionine	0.35	0.45
Lysine	0.17	-
Total	100.00	100.00

* Soybean was autoclaved at 110^0c for 30 minutes according to (Westfall and Hauge, 1948) to inactivate trypsin inhibitor, tannins, saponins, phytate, protease inhibitors, lectins and goitrogens.

**Premix produced by Muvco. Supplied per kilogram diet: 12.000 and 2.000 IU of vitamin A and D3 respectively; 10 g vitamin E, 1 g vitamin K , 0.005 g vitamin B_2, 0.0015 vitamin B_6, 10 g pantothenic acid, 0.02 niacin, 0.6 gm choline chloride, 0.03g iron, 0.06 g manganese, 0.004 g copper, 0.05 gm zinc, 1 mg vitamin B_1, 0.001 mg vitamin B_{12}, 1 mg folic acid, 0.05 mg biotin, 0.3 mg iodine, 0.1 mg cobalt and 0.01 mg selenium.

Isoflavones were extracted from the diet according to the method described by Thiagarajan et al., (1998).

Average weekly body weight gain and feed intake (gram/ week) were determined. Feed conversion ratio was determined by dividing the average weekly feed intake by the average weekly body gain (Fromageot, 1985).

$$\text{Feed conversion ratio} = \frac{\text{Average weekly feed intake}}{\text{Weekly body gain}}$$

Fifteen female from each group in proestrous phase (as determined by vaginal smear) were introduced to males in ratio of one male / two females / cage at the afternoon for overnight. Mating was confirmed by the presence of sperm in the vaginal smears or the presence of vaginal plug and this was designated as the zero day of pregnancy (Piesta et al., 2009). The pregnant females were removed from the mating cages and rehoused in separate cages, each contained 4 animals. The pregnant dams were weighed in GD 7 and GD 19. Feed intake was also determined as from 0 day till GD 7 and GD 19 as previously mentioned.

Statistical analysis

All data in the present study were expressed as mean ± SE. they were subjected to student "t" test using SPSS® software (Statistical Package for Social science, version 17.01, IIIinois, USA). The probability criterion for significance was $P < 0.05$.

Results and discussion

HPLC analysis to the experimental diet revealed that; low phytoestrogens diet contains 45 µg/g genistein and 28 µg/g daidzein respectively, while the high phytoestrogens diet contains 1320 µg/g genistein and 704.7 µg/g daidzein, respectively.

The obtained results revealed significant ($P<0.05$) decreased BWTG and FI while FCR was significantly ($P<0.05$) increased in high phytoestrogens fed group starting from the second week till 4th week. There was no significant difference between low and high phytoestrogens fed group after the first week (Table 2).

Genistein and daidzein are components among phytoestrogens which have estrogenic action; it is not surprising to ascertain that these effects are directly due to estrogenic mechanism. supplementation of mature cyclic female rats with dietary phytoestrogens decreased body weight gain and feed consumption significantly at ($P<0.05$). These results are generally consistent with those reported by other studies (Nagao et al., 2001 ; Naaz et al., 2003; Lephart et al., 2004; Bu et al., 2005 and Cederroth et al., 2007), and disagree with those of Weber et al., 1999 and Lewis et al., 2003. Reduction in feed intake may be due to the appetite repressing action of estrogen (Wade, 1975) as dietary phytostrogens decrease feed intake and hence decrease body weight. The decrease implies that the estrogenic hormone like action of phytoestrogens is beneficial to body fat regulation and the decreased level of leptin that is produced in adipose tissue that influences hypothalamic neuropeptide Y (NPY) levels which regulates feeding behaviour (Szkudelska et al., 2000 and Naaz et al., 2003).

Table 2. Effect of dietary phytoestrogens on body weight gain, feed intake (g/ week) and feed conversion ratio in cyclic female rats during 4 weeks

Duration/ group	BWTG		FI		FCR	
	Low phytoestrogens	High phytoestrogens	Low phytoestrogens	High phytoestrogens	low phytoestrogens	High
After one week	11.25±1.19	9.13±0.86	85.91±7.55	79.43±4.1	6.93±0.96	8.89±0.72
After two weeks	11.5±1.4	4.69±0.95*	73.28±4.41	62.32±2.09*	6.65±1.56	14.61±0.83*
After 3 weeks	4.63±1.3	1.7±0.50*	69.43±1.24	59.55±2.92*	16.31±2.04	30.98±6.47*
After 4 weeks	3.00±1.25	1.06±0.42*	70.1±0.39	59.58±2.73*	17.94±5.55	40.23±6.64*

*means significant at ($P<0.05$).

Similar findings were reported by Bu et al., (2005) who found that rodents fed a soy rich diet have significantly decreased adiposity compared with animals fed control diet. Lephart et al., (2004) contributed this finding to the effect of phytoestrogens on inhibition of lipogenesis and stimulation of Lipolysis. These results, concerning the effect of dietary phytoestrogens on reduction of body weight, are coincide with those recorded by Cederroth et al., (2007 & 2008) who found that mice fed dietary phytoestrogens were leaner because of increase their locomotor activity (as observed in this study) which may be due to preferential use of lipids as fuel source. Exercise is known to activate the fuel-sensing enzyme 5' adenosine monophosphate-activated protein kinase (AMPK) in both adipose tissue and skeletal muscles which in turn improves glucose uptake and fatty acid oxidation in peripheral tissue (Ruderman and Prentki, 2004 & Kahn et al., 2005) so the increase of FCR as demonstrated in this study is consequent due to loss of large amount of ingested food as energy lost during the increased locomotor activity. The publication of (Musatov et al., 2007) reported that a specific silencing of estradiol receptors alpha (ERα) in the hypothalamous lead to a phenotype resembling metabolic syndrome demonstrating the importance of central regulation by estrogen in the control of energy balance.

The BWT and FI significantly ($P<0.05$) decreased in high phytoestrogens fed dams than control one at GD 7 and GD 19. Pregnancy status of an organism is regulated by nutritional status, energy expenditure and hormonal signals. Ingestion of dietary phytoestrogens in this study causes significant ($P<0.05$) lower BWT associated with significant ($P<0.05$) lower FI in pregnant dams at GD 7 and GD 19. This may be attributed to the known anorectic effects of estrogen induced by dietary genistein and daidzein (Bonavera et al., 1994). These results are in agreement with those of Casanova et al., (1999), Flynn et al., (2000) and Delclos et al., (2001) who found a significant reduction in body weight gain at the high concentration of genistein administration. While they are disagree with Odum et al., (2001) and Soucy et al., (2006) who found that repeated administration of genistein and ingestion of dietary phytoestrogens by pregnant female rats has no effect on maternal body weight nor their feed intake.

Table 3. Effect of dietary phytoestrogens on body weight/ g and feed intake g phytoestrogens / week in pregnant female rats at GD 7 and GD 19

Duration/ group	BWT		FI	
	Low phytoestrogens	High phytoestrogens	Low phytoestrogens	High phytoestrogens
Gestation day 7	218.14±13.25	197.71±7.82*	85.78±15.20	73.25±10.10*
Gestation day 19	235.50±10.76	225.25±2.99*	232.80±13.50	211.65±12.71*

*means significant at ($P<0.05$)

References

1. Bonavera, J. J.; Dube, M. G.; Kalra, P. S. and Kalra S. P. (1994): Anorectic effects of estrogen may be mediated by decreased neuropeptide-Y release in the hypothalamic paraventricular nucleus. Endocrinology.; 134 (6): 2367-70.
2. Bu, L. and Lephart, E. D. (2006): Regulatory Behavior and Skin Temperature in Mid-Aged Male Rats on Three Different Isoflavone-Containing Diets. J. Med. Food; 9:567–571.
3. Bu, L.; Setchell, K. D. and Lephart, E. D. (2005): Influences of dietary soy isoflavones on metabolism but not nociception and stress hormone responses in ovariectomized female rats. Reprod. Biol. Endocrinol.; 3: 58.
4. Casanova, M.; You, L.; Gaido, K.W.; ArchibequeEngle, S.; Janszen, D. B.; Heck H. D. (1999): Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors α and β in vitro. Toxicol. Sci.; 51:236-244.
5. Cederroth, C. R. and Nef, S. (2009): Soy, phytoestrogens and metabolism: A review. Mol. Cell. Endocrinol.; 304(1-2):30-42.
6. Cederroth, C. R.; Vinciguerra, M.; Gjinovci, A.; Kühne, F.; Klein, M.; Cederroth, M.; Caille, D.; Suter, M.; Neumann, D.; James, R.W.; Doerge, D. R.; Wallimann, T.; Meda, P.; Foti, M.; Rohner-Jeanrenaud, F.; Vassalli, J. D. and Nef, S. (2008): Dietary phytoestrogens activate AMP-activated protein kinase with improvement in lipid and glucose metabolism. Diabetes; 57(5):1176-85.
7. Cederroth, C. R.; Vinciguerra, M.; Kühne, F.; Madani, R.; Doerge D. R.; Visser, T. J.; Foti, M.; Rohner-Jeanrenaud, F.; Vassalli JD and Nef, S. (2007): A phytoestrogen-rich diet increases energy expenditure and decreases adiposity in mice. Environ Health Perspect.; 115(10):1467-73.
8. Delclos, K. B.; Bucci, T. J.; Lomax, L. G.; Latendresse, J. R.; Warbritton, A.; Weis, C. C. and Newbold RR. (2001): Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. Reprod. Toxicol.; 15(6):647-63.
9. Flynn, K. M.; Ferguson, S. A.; Delclos, K. B. and Newbold, R. R. (2000): Effects of genistein exposure on sexually dimorphic behaviors in rats. Toxicol. Sci.; 55(2):311-9.
10. Fromageot, D. (1985): Le scure dan J'almientation dud poult de chair. Tose la faculte de medicine de paris. P. 17, 19, 38 and 65-71.
11. Kahn, B.B.; Alquier, T.; Carling, D. and Hardie, D.G. (2005): AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab.; 1:15–25.
12. Kuiper, G. G.; Lemmen, J. G.; Carosson, B. Orton, J. C., Safe, S. H.; vad der Saag, P. T.; van der Burg, B. and Gustafsson, J. A. (1998): Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinol.; 139: 4252–4263.
13. Lephart, E. D.; Setchell, K. R.; Handa, R. J. and Lund T. D. (2004): Behavioral Effects of Endocrine-disrupting Substances: Phytoestrogens. ILAR Journal; 45(4): 443- 454.
14. Lewis, R.W.; Brooks, N.; Milburn, J.; Soames, A.; Stone, S.; Hall, M. and Ashby, J. (2003): The effects of the phytoestrogens genestein on the postnatal development of the rat. Toxicol. Sci.; 71: 74- 83.

15. Lund, T. D. and Lephart, E. D. (2001): Dietary soy phytoestrogens produce anxiolytic effects in the elevated plus-maze. *Brain Res.*; 913:180–184.
16. Musatov, S.; Chen, W.; Pfaff, D. W.; Mobbs, C. V.; Yang, X. J.; Clegg, D. J.; Kaplitt, M. G. and Ogawa, S. (2007): Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proc. Natl. Acad. Sci. U S A; 104:2501–2506.
17. Muthyala, R. S.; Ju, Y. H.; Sheng, S.; Williams, L. D.; Doerge, D. R.; Katzenellenbogen; B. S.; Helferich, W. G. and Katzenellenbogen J. A. (2004): Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. Bioorg Med Chem.; 12:1559–1567.
18. Naaz, A.; Yellayi, S.; Zakroczymski, M. A.; Bunick, D.; Doerge D. R.; Lubahn, D. B.; Helferich, W. G. and Cooke, P. S. (2003): The soy isoflavone genistein decreases adipose deposition in mice. Endocrinol.;144 (8):3315-20.
19. Nagao, T.; Yoshimura, S.; Saito, Y.; Nakagomi, M.; Usumi, K. and Ono, H. (2001): Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod. Toxicol.; 15(4):399-411.
20. NRC, (1995): Nutrient Requirements of laboratory animals. National Academic Paris. Washington. D. C. 4th Revised Edition.
21. Odum, J.; Tinwell, H.; Jones, K.; Van Miller, J. P; Joiner, R. L.; Tobin, G.; Kawasaki, H.; Deghenghi, R. and Sabih, J. (2001): Effect of rodent diets on the sexual development of the rat. Toxicol. Sci.; 61, 115-127.
22. Piesta, A.; Maj, T. and Chełmońska-Soyta A. (2009): The influence of mating on estrogen receptor alpha protein level in spleen and uterine macrophages in female mice. Reprod. Biol., 9(3):225- 240.
23. Ruderman, N. and Prentki, M. (2004): AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. Nat. Rev. Drug Discov. 3:340 –351.
24. Setchell, K. D. and Cassidy A. (1999): Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129(1):758-67.
25. Setchell, K. D.; Clerici, C.; Lephart, E. D.; Cole, S. J.; Heenan, C.; Castellani, D.; Wolfe, B. E.; Nechemias-Zimmer, L.; Brown, N. M.; Lund, T. D.; Handa, R. J. and Heubi, J. E. (2005): S-Equol, a potent ligand for estrogen receptor {beta}, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. Am. J. Clin. Nutr.; 81:1072–1079.
26. Soucy, N. V.; Parkinson, H. D.; Sochaski, M. A. and Borghoff, S. J. (2006): Kinetics of Genistein and Its Conjugated Metabolites in Pregnant Sprague-Dawley Rats Following Single and Repeated Genistein Administration. Toxicol. Sci.; 90 (1): 230-240.
27. Sunita, P.; Jha, S. and Pattanayak, S.P. (2008): Health Benefits of Phyto-oestrogens – A Consensus Review. Pharmacognosy reviews; 2(3):35-42.
28. Szkudelska, K.; Nogowski, L. and Szkudelski, T. (2000): Genistein affects lipogenesis and lipolysis in isolated rat adipocytes. J. Steroid Biochem. Mol. Biol.; 75(4-5):265-71.
Thiagarajan, D. G.; Bennink, M. R.; Bourquin, L. D. and Kavaam, F. A. (1998): Prevention of precancerous colonic lesions in rats by soy flakes, soy flour, genistein, and calcium. J. Am. J. Clin. Nutr.; 68 (1): 1394S–9S.
29. Wade, G. N. (1975): Some effects of ovarian hormones on food intake and body weight in female rats. J. Comp. Physiol. Psychol.; 88: 183- 193.
30. Weber, K. S.; Jacobson, N. A.; Setchell K. D. R. and Lephart, E. D. (1999): Brain aromatase and 5α-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets. Proc. Soc. Exp. Biol. Med. 22: 131-135.
31. Westfall, R. L. and Hauge, S. M. (1948): The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. J. Nutr. 35: 379-389.

EFFECT OF AGE ON THE ANTIOXIDANT ENZYME ACTIVITY IN RAM SEMINAL PLASMA

V. Focsaneanu, L. Bogdan, Anamaria Blaga,Sidonia Bogdan, Simona Ciupe, Sanda Andrei
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
Faculty of Veterinary Medicine, Str. Mănăştur no. 3-5, Cluj-Napoca, Romania
anamariapetrean@yahoo.com

Abstract

The objective of this study was to determine relationships among age and antioxidant enzymatic activity in ram semen. The study was conducted on 15 rams grouped according to age in three groups. Freshly harvested sperm was processed by centrifugation and seminal plasma was separated. The activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) was analyzed using spectophotometric assays. In parallel, in seminal plasma total protein content was analyzed. In this study, we found a positive correlation between enzymatic activity of SOD and GPx with total proteins content in seminal plasma. In the present study, the highest levels of glutathione peroxidase, expressed in U/L SP were recorded in the case of 6 years old rams (average of 126.04 ± 3.74). In the case of rams of 3 years and 5 years recorded values do not show statistically significant changes. The highest values for SOD activity on seminal plasma volume (U/ml SP) are recorded from rams aged 5 years (average of 16.21 ± 5.48), followed by those aged 3 years and 6 years.

Key words: *antioxidants enzyme, proteins, seminal plasma, ram*

Introduction

For all species, the membrane structure of spermatozoa is very important for the viability, maturity, and functions of spermatozoa. In sperm, the lipid composition of the bilayer phospholipids membrane is unique within species with regard to the composition. Thus, it contains approximately 70% phospholipids (PL), 25% neutral lipids and 5% glycolipids. The physical properties of the sperm membrane, as membrane fluidity, is dependent on the ratio between the polyunsaturated (PUFA) and saturated (SFA) fatty acids components of membrane lipids and cholesterol concentration (1, 2). Ram sperm have a higher PUFA/SFA ratio and a lower cholesterol/PL molar ratio than other species, which which makes the sperm much more vulnerable to oxidative damage caused by reactive oxygen species (ROS) (3).

Reactive oxygen species are constantly produced in cells through normal metabolic processes. An increased level of ROS can lead to damage of molecules within the cell (lipids, proteins, and DNA) that can give rise to pathological consequences. For example, ROS generated by the cellular components of semen are considered to be among the factors contributing to poor quality semen (3, 4, 5).

On the other hand, semen cells contain a variety of antioxidants mechanisms that play a central role in the protection against reactive oxygen species. These antioxidants are classified depending on their structure and role in enzymatic and non-enzymatic systems with low-molecular weight. The most important low-molecular weight antioxidants exist in semen are: α-tocopherol, β-carotene, ascorbic acid, and some proteins with the role of fixing metal ions like transferrin, lactoferrin and caeruloplasmin (6). The antioxidant enzymatic system includes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase. The antioxidant enzymatic system

has been described as defense functioning mechanism against the lipids perioxidation in semen, and important in maintaining sperm motility and viability (7, 8).

To our knowledge, there is not any study which investigated the relationship between animal age and antioxidant activity in ram sperm. The novelty of this study was to determine the effect of age on the activities of the antioxidant enzymes (SOD and GPx) in ram seminal plasma (SP).

Materials and methods

Animals:

The study was conducted in a farm located in Cluj County, on 15 rams of the breed Turcana Alba, grouped according to age into three groups, each of 5 rams. Group 1 with rams of 3 years, group 2 with rams for 5 years and group 3 with rams for 6 years.

Semen collection and processing:

From each ram 3 ejaculates were collected, using an artificial vagina. A total number of 45 ejaculates were included in the study. Semen samples were diluted 1:1 with 0.85% (w:v) NaCl. Seminal plasma was separated from ejaculates by centrifugation at 5000 r.p.m. for 10 min. The recovered seminal plasma fraction was further centrifuged at 10.000 r.p.m. for 15 min., at 4°C and the supernatant was stored at –20°C until analysis.

Biochemical parameters:

The antioxidant enzymatic activity in seminal plasma was expressed in U/mL of seminal plasma. For a correct interpretation of the results, in samples total protein concentration was determined so that the enzymatic activity was expressed also in U / mg of protein.

Total protein content was determined using the Bradford method, by using a calibration curve obtained from bovine serum albumin (BSA) (9). The concentration was expressed in mg/ 100ml SP.

Determination of SOD and GPx activities was performed on using Randox commercial kits (Randox Laboratories Ltd.,U.K.) and semiautomatic biochemistry analyzer MasterPlus Screen. The GPx activity was expressed as in mU/L SP and mU/g proteins. The SOD activity was expressed U/mL SP and U/mg proteins.

Results and discussions

Seminal plasma is the natural medium for maturation of the spermatozoa through hormonal, enzymatic, and surface-modifying events. The addition and removal of a variety of proteins during epididymal maturation and at ejaculation play important roles in the capacitation of sperm and fertilization of the egg (10, 11).

The results obtained in the determination of total proteins concentration in SP are presented in Table 1. It is noted that there is a direct relationship between animals age and proteins level, the lowest values being recorded in young animals (average of 25.32 ± 11.61), values increasing to an average of 247.46 ± 89.13 to animals for 5 years and a average of 769.50 ± 60.85 for rams aged 6 years.

According to the study presented by Wenbin Yue et al. (12), seminal plasma composition in terms of proteins varies according to species. Moreover, in the case the same species and breeds, the protein content of seminal plasma differs between low-fertility and high-fertility sheep. Thus, the relative content of seminal plasma protein could be an essential index to evaluate ram fertility and semen quality.

Table 1. Average and standard deviations of seminal plasma total proteins according to the age

Rams age	Total protein mg/dl
3 years	13.3
	11.2
	26.7
	41
	34.4
	Average and standard deviation
	25.32 ± 11.61
5 years	117.1
	370.8
	261.4
	183.6
	304.4
	Average and standard deviation
	247.46 ± 89.13
6 years	820.1
	850.6
	704.02
	698.3
	774.5
	Average and standard deviation
	769.50 ± 60.85

Studies presented by Rodrigues et al. (13) have pointed to the actions of different proteins found in the semen, being related to the acquisition of fertilizing capacity of the spermatozoa in mammals, including sperm capacitation and the acrosome reaction and fertilization and embryonic development. There are also reports that seminal plasma proteins affect sperm motility.

Taking into account the large variations that occur in seminal plasma proteins concentration, we considered that it is correctly to express the activity of enzymes both in terms of volume of SP and depending on the proteins content. In Table 2 are presented the values of activity for all three enzymes studied, and the units of measurement used in each case.

Superoxiddismutase (EC 1.15.1.1.) is considered the most important enzyme characteristic for aerobic life, in terms of oxidative biochemical processes, and is present in all living cells. Superoxid dismutase (SOD) accelerates the dismutation of the toxic superoxide radical to hydrogen peroxide and is considered the first intracellular defense against reactive oxygen species. Determination of SOD is important in the evaluation of antioxidant status, under physiological or pathological conditions (14,15).

Table 2. Average and standard deviations of seminal plasma SOD and GPx activities, according to the age

Rams age	SOD		GPx	
	U/ml SP	U/mg proteins	U/L SP	U/g proteins
3 years	6.6	50.76	57.8	0.768
	7.5	68.18	70.3	0.787
	6.4	25	55.3	1.47
	8.4	20.48	28.3	1.16
	7.1	20.88	48.1	1.68
	Average and standard deviation			
	7.2 ± 0.71	**37.06 ± 19.17**	**51.96 ± 13.82**	**1.17 ± 0.36**
5 years	18.5	15.81	41.4	6.01
	9.25	2.5	53.3	19.76
	22.5	8.62	70.6	18.42
	20.7	11.31	60.8	11.12
	10.11	3.32	51.9	15.77
	Average and standard deviation			
	16.21 ± 5.48	**8.31± 4.99**	**55.6 ± 9.72**	**14.21 ± 5.05**
6 years	2.65	0.32	161.9	132.75
	5.3	0.62	141.1	119.9
	7.2	1.02	103.5	72.86
	3.01	0.43	97.8	68.26
	6.75	0.87	125.9	75.44
	Average and standard deviation			
	4.98 ± 1.86	**0.65 ± 0.26**	**126.04 ± 3.74**	**93.84 ± 26.93**

As can be seen from Table 2, if we report SOD activity on seminal plasma volume (U /ml SP), the highest values are recorded from rams aged 5 years, followed by those aged 3 years and 6 years. The variation is significantly different if enzymatic activity is related to the protein content of seminal plasma. Thus, in this case, the highest values occurring in rams of three years, followed by values about five times lower for rams for 5 years and more than 50 times lower on rams for 6 years.

Glutathione peroxidase, a selenocysteine enzyme, uses glutathione to reduce hydrogen peroxide to water and lipoperoxides to alkyl alcohols. In the present study, the highest levels of glutathione peroxidase, expressed in U/L SP were recorded in the case of 6 years old rams (average of 126.04 ± 3.74). In the case of rams of 3 years and 5 years recorded values do not show statistically significant changes.

Conclusions

The data presented in this paper provide some new insights into the effect of age on ram sperm composition and antioxidant capacity. In this study, we found a positive correlation between enzymatic activity of SOD and GPX with total proteins content in seminal plasma. We suggest that the activity of seminal antioxidant enzymes must be always correlated with proteins concentration of seminal plasma. In addition, our data showed the

significant correlation between activity of antioxidant enzymes, proteins concentration and age of rams. Thus, further studies are needed to clarify the role of age on activity of antioxidant enzymes of seminal plasma from rams.

Bibliography

1. Yilmaz A., Hulya A., Konca A., Aydın H.R., Asuman Ozkan, Sperm fatty acid composition in subfertile men, Prostaglandins, Leukotrienes and Essential Fatty Acids, 75:75–79 (2006)
2. Am-in N., Kirkwood R.N., Techakumphu M., Tantasuparuk W., Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility, Theriogenology, 75: 897–903 (2011)
3. Çoyan K., Baspınar N., Bucak M., Akalın P., Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters, Cryobiology, doi:10.1016/j.cryobiol.2011.04.001 (2011)
4. Martı J.I., Martı E., Cebrian-Perez J.A., Muino-Blanco T., Survival rate and antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure, Theriogenology 60:1025–1037, (2003)
5. Tavilani H., Goodarzi M., Doosti M., Vaisi-Raygani A., Hassanzadeh T., Salimi S., Joshaghani H., Relationship between seminal antioxidant enzymes and the phospholipid and fatty acid composition of spermatozoa, Reproductive BioMedicine Online, Vol 16 No 5., 649-656 (2008)
6. Sanocka D., Kurpisz M., Reactive oxygen species and sperm cells, Reproductive Biology and Endocrinology, http://www.rbej.com/content/2/1/12 (2004)
7. Bucak M.N. Atessahin A., Yuce A., Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process, Small Ruminant Research, 75: 128–134 (2008)
8. Andrei S, Pintea A., Bele C., Veterinary Biochemistry – Practical work, Editura AcademicPres Cluj (2012)
9. Barrios B., Perez R., Gallego M., Tato A., Osada J., Muino-Blanco T.,Cebrian-Perez J., Seminal Plasma Proteins Revert the Cold-Shock Damage on Ram Sperm Membrane, Biology of Reproduction 63, 1531–1537 (2000)
10. Bernardini A., Hozbor F., Sanchez E., Fornésc M.W., Alberio R.H., Cesari A., Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage, Theriogenology 76:436–447 (2011)
11. Wenbin Yue, Lei Shia, Zhiming Bai, Youshe Ren, Youying Zhao, Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of ram seminal plasma proteins and their correlation with semen characteristics, Animal Reproduction Science 116: 386–391 (2009)
12. Rodrigues M.A.M., Souza C.E.A., Martins J.A.M., Rego J.P.A., Oliveir J.T.A., Domont G., Nogueir F.C.S., Moura A.A., Seminal plasma proteins and their relationship with sperm motility in Santa Ines rams, Small Ruminant Research 109:94– 100 (2013)
13. Andrei S., Matei S., Zinveliu D., Pintea A., Bunea A., Ciupe S., Groza I., Correlation between antioxidant enzymes activity and lipids peroxidation level in blood and milk from cows with subclinical mastitis, Bulletin USAMV Cluj, Veterinary Medicine, 67(1): 5-12, 2010
14. Lei Shi, Chunxiang Zhang, Wenbin Yue, Liguang Shi, Xiaomin Zhu, Fulin Lei, Short-term effect of dietary selenium-enriched yeast on semen parameters, antioxidant status and Se concentration in goat seminal plasma, Animal Feed Science and Technology, 157: 104–108 (2010)

RENAL LESIONS CARRE'S DISEASE IN DOG

S. Pasca, M. Lazăr, Andreea Ancuta
FMV Iasi, Iasi, 8,M.Sadoveanu Alley
passorin@yahoo.com

Abstract

Carre's disease is immunosuppressive multisytemic disease at carnivores caused by a virus of the family Paramyxoviridae, genus Morbilivirus, canine morbilivirus. Immunosuppressive effect of the pathogen is given by inducing apoptosis and depletion of lymphocytes and macrophages in infected tissues, accompanied by severe immunosuppression in acute and subacute phases of the disease. In the context of this disease was followed severity of renal lesions, describing and quantifying them. Were observed membranous and membranoproliferative glomerulonephritis and dystrophic processes and degeneration of varying intensity in tubules.

Keywords*: Carre's disease, dog, glomerulonephritis*

Introduction

Canine distemper disease it is an immunosuppressive virosis encountered to unimmunized dogs, the most common form of the disease is the respiratory one. Dogs infection is done most of the time by respiratory tract and less by digestive system. In our study it was observed that at the renal glomeruli level is evolving concurrently the membranous and membranoproliferative glomerulitis. Membranous glomeruli are characterized by a particular aspect of the glomerular cappilary basal membranes. The immune complexes, regardless of their source produce glomerular injuries through a similar pathogenic mechanism. By electron microscopic studies performed on dogs with idiopathic membranous glomerulonephritis were able to deduce the evolutionary stages of this injuries.

In the first phase, immune deposits were noted, very discrete, electrono-dense on the epithelial surface of the glomerular basal membrane.

Subsequently, there is a slight thickening of the basal membrane and a fusion of the podocitary processes over the immune deposits present at the membrane level in diffuse and rarely appearance with localized character.

Podocitele appears tumefied and the podocitare processes also contain granular material. (1,2)

Subsequently basal membrane thickening is evident through the new synthesized membrane material, that is visible between membrane deposits and basal membrane itself.

It appears as small dots and spiculi on the epithelial surface of the glomerular basal membrane, giving it an pectinate aspect easily identifiable in silver impregnation (1, 3).

Membrano - proliferative glomerulonephritis (MPGN), also called mezangio-capillary glomerulonephritis is an morphoclinical entity characterized by mesangial cell proliferation and at the same time by glomerular basal membrane thickening, so have both aspects of membranous glomerulonephritis and mezangio-proliferative one. (4). These morphological aspects are considered intermediate injuries that ends with generalized chronic glomerulonephritis leading to chronic renal failure. Structural changes in this type of lesion justifies hematuria concurrently with proteinuria and nephrotic syndrome.

Results and discussions

The study material was represented by 4 dogs aged 1 to 3 years with clinical symptoms of respiratory, digestive, nervous specific distemper of dog. Confirmation of the disease was performed using rapid tests and on behalf highlight histology Lentz-Sinigaglia intracytoplasmic inclusions in lungs (Fig.1, Fig.2), renal pelvis epithelium (Fig. 3) and bile ducts epithelium (Fig. 4). In the spleen were observed syncytia giant cell specific of disease (Fig. 5).

The research aim was to reveal the renal lesions, particularly of glomerular one in this disease context.

The 4 cases with respiratory form present in the septic outbreaks in the lungs induced by likely superinfection with *Bordetella bronhiseptica* and other germs. In the context of these outbreaks septic lung, we studied the glomerul, corresponding changes of submitting antigen-antibody complexes preform, circulated.

Macroscopic, to the examination of the bodies was noted bilateral mucopurulent nasal catarrh, reddish liquid in the mouth with pulmonary origin, pale mucous membranes, severe dehydration, hyperplasia nose horn, muco-purulent conjunctivitis and ulcers with conjunctive - cutaneous limit.

- It was also observed hepatic steatosis, sero-fibrinous pericarditis (fine network of fibrin in the pericardial sac) catarrhal-purulent bronchopneumonia (a small case disseminated lung abscesses) sero-hemorrhagic pleural effusion (100-150ml), edema and acute pulmonary and congestion (in one case).
- All cases presented catarrhal gastritis (stomach contained a slightly translucent gelatinous material), hemorrhagic catarrhal duodenitis.

On histological examination of the lungs was identified diffuse interstitial bronchopneumonia with macrophage infiltrates and giant-cellular and neutrophilic infiltration areas, secondary intervention microbial association.

Our attention was directed especially to the kidneys. In all cases they were slightly bloodshot globose with cortico-medullary boundary deletion. In the kidney were observed varied injuries both the glomeruli and the urinary tubules and interstitium.

Membranoproliferative and membranous glomerulonephritis with diffuse character were noted histologically in all cases examined (Fig.6, Fig. 7, Fig. 8, Fig.9, Fig.10).

The explanation of the evolution of these types of glomerulo-disorders consist of abundance of circulating immune complexes and the corresponding free antigens both viral infection and secondary bacterial infection found in the lungs, which are submitted to the glomeruls.

These complexes may contain bacterial or viral antigens.

The pathogenicity and the mode of deposition of immune complexes in glomerular structures depends on their quantitative and qualitative aspects: the amount of Atg-Atc complex size complexes, molecular configuration, the affinity of the antibody to the antigen, electric charge, solubility.

Large and insoluble complexes and formed in excess blood is rapidly removed by the kidneys and phagocyted by the macrophage-monocyte system (EMS) or taken part of glomerular mesangial.

The immune complexes of intermediate size formed in the presence in excess of antigen remaining in solution and stored in the glomerular basement membrane of the capsule, of the vascular bundle or the mesangial vessel.

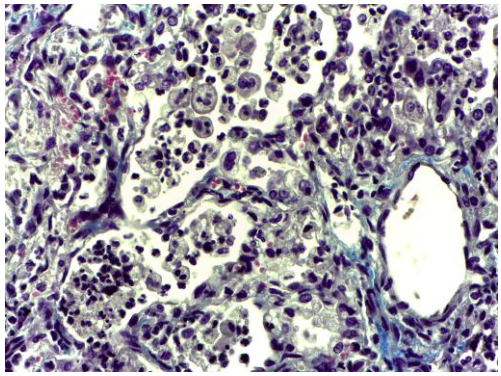


Fig.1. Dog. Lung. Giant cell with inclusions. Col. HEA, x1000;

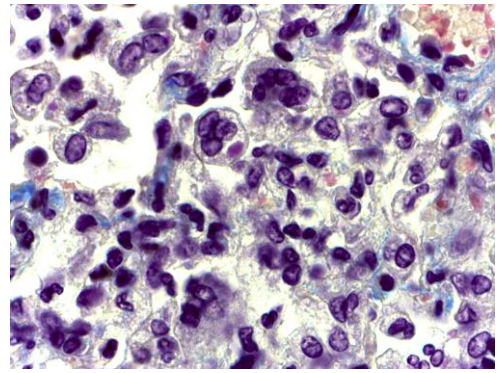


Fig.2. Dog. Lung. Giant cell with inclusions. Col. HEA, x1000;

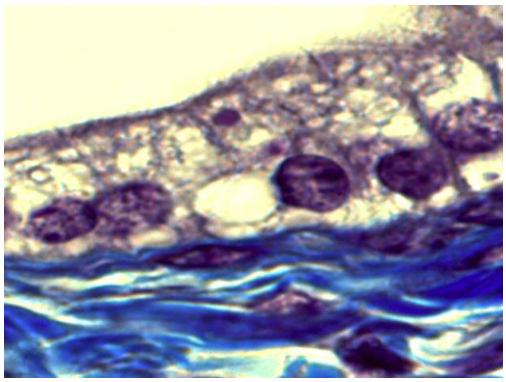


Fig.3. Dog. Liver. Acidophilic inclusions of epithelial cells in canaliculated biliary. Col. HEA, x1000;

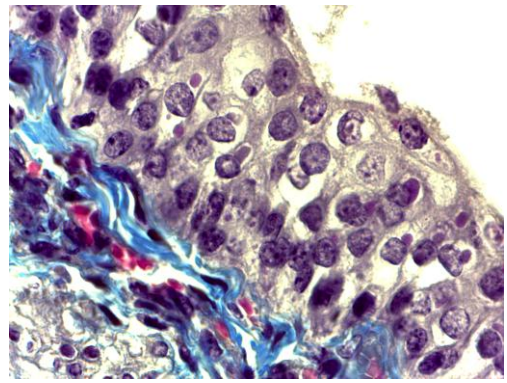


Fig.4. Dog. Kidney. Acidophilic inclusion in the basins epithelial cells. Col. HEA, x1000;

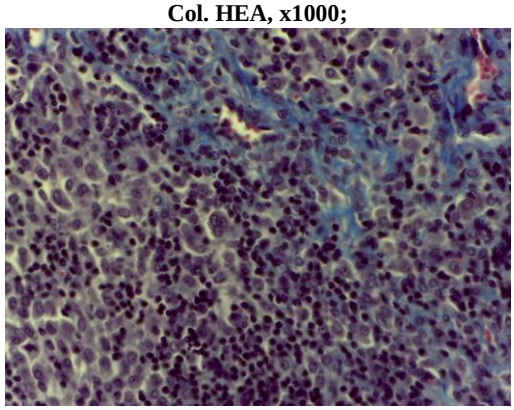


Fig.5. Dog. Tonsile. Gigantic-cell syncytia. Col. HEA, x200;

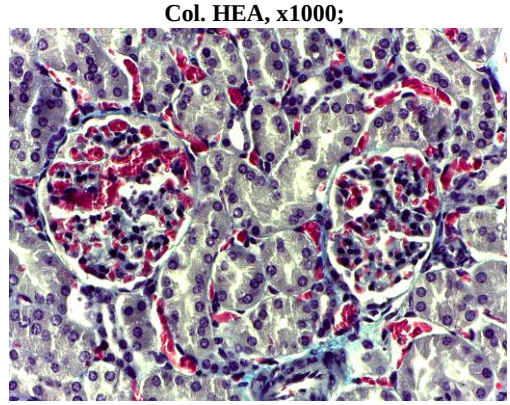


Fig.6. Glomerular and interstitial congestion, membranoproliferative glomerulonephritis. Col. HEA, x200;

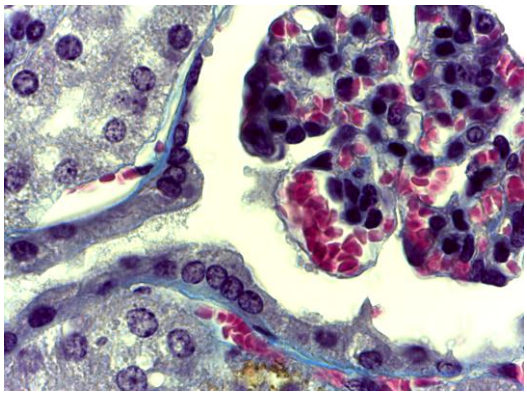


Fig. 7. Membranoproliferative glomerulonephritis. Parietal epithelial glomerular capsule cell proliferation. Col. HEA, x 1000;

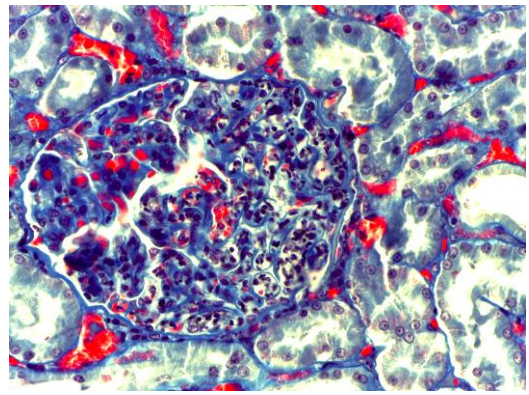


Fig.8. Dog. Membranoproliferative glomerulonephritis; neutrophilic infiltration. Col. HEA, x200;

The consequence of these glomerular changes is represented by the proteinuria secondary and intraepithelial hyalinosis followed by the formation of hyaline cylinders (Fig. 11).

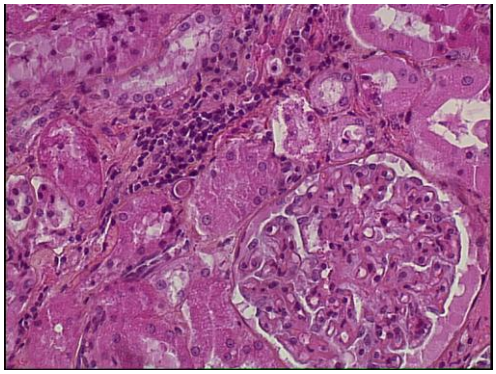


Fig.9. Dog. Membranous glomerulonephritis. Col. HES, x 400;

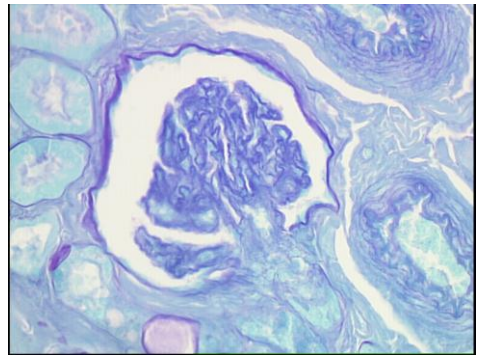


Fig.10. Dog. Membranous glomerulonephritis. Col. PAS-green luminous, x400;

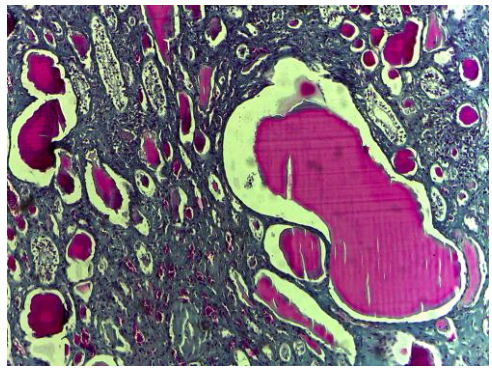


Fig.11. Dog. Hyaline cylinders in the urinary tubules. Col. HEA, x200;

Conclusions

It can be concluded that the membranoproliferative and membranous glomerulonephritis are present in lesional table of Carrie's disease in dogs.

The starting point of these two types of glomerulonephritis is the submission of pre-formed immune complexes containing both viruses (disease agent) and opportunistic bacteria antigens. Changes produced by depositing immune complexes in glomerular capillary structure evolves to severe glomerular sclerosis.

Acknowledgments:

The authors thank **UEFISCDI Romania** for the financial support (Research Program TE 112/2010).

References

1. Jergens A.E., 1987 – "Glomerulonephritis in Dogs and Cats", Compendium Small Animal, Vol. 9, No. 9;
2. Kluth D.C., Rees A., 1999 – New approaches to modify glomerular inflammation, Journal of Nephrology, nr.12, 265-270;
3. McGavin M., Carlton W., Zachary J., 2012 – Special Veterinary Pathology, Ed.V, Ed. Mosby, St. Louis, Missouri;
4. Osborne C.A., Finco, D.R., 1995 – Canine and Feline Nephrology and Urology, Ed. Williams and Wilkins, Baltimore;

MORPHOLOGICAL ASPECTS OF SOME ORGANOPATHIES IN WILD RABBITS *(LEPUS EUROPAEUS)* FROM NORTH MOLDAVIA

M. Lazăr[1*],Andreea Ancuţa[1], Roxana Lazăr[2], S. Paşca[1]
[1] UASVM of Iasi, Faculty of Veterinary Medicine, 8 Mihail Sadoveanu Alley, Iasi, Romania
[2] UASVM of Iasi, Faculty of Animal Science Iasi, 3 Mihail Sadoveanu Alley, Iasi, Romania
* Department of Pathology, Faculty of Veterinary Medicine, "Ion Ionescu de la Brad" University ; Iasi, 8, M. Sadoveanu Alley, 700490, Romania
lazar_mircea@yahoo.com

Abstract

Species of great cynegetic interest with a widespread population in the Northern Moldavia, the wild rabbit (Lepus europaeus) is already under mandatory monitoring for major sanitary impact diseases and pandemics, such as brucellosis, pasteurosis, leptospirosis, and borreliosis. The research that took place during 2011-2012 on 22 rabbits (Lepus europaeus) harvested in Northern Moldavia (the counties of Suceava, Neamt and Iasi) revealed a parasite invasion development (eimeriosis, fasciolosis) in the local population, as well as organopathies of unknown etiology. The blood sampling for haematological determinations was made from live hares caught by netting, then sedated, fed and accommodated in the dark to avoid stress, as well as from shot hares. The predominance of parasite invasions is commanding the implementation of prophylactic deparasitation programmes.

***Key words**: wild rabbit, hare, organopathies, parasite invasions, haematology*

Introduction

The pathology of wild fauna is among the primary concern topics in nowadays veterinary medicine, because of the sanitary and economical impact that various pathological conditions developing in sylvan fauna are creating by affecting the livestock and human health conditions, and, not in the last, the wild ecosystems' biodiversity. (Pikula, 2007)

The hare is a cynegetic interest species widespread in Northern Moldavia. The direct contact between sylvan and domesticated specimen has become increasingly frequent, and thus the role that the various vectors – of animal or otherwise origins – play in disease transmission is undeniable. (Oprean, 2009)

The complete hemoleucogram is a test able to provide diagnostics for numerous diseases. The results may indicate blood volume conditions (dehydration) or blood loss. They also may indicate dysfunctionality of blood cells generation, life cycle and destruction, as well as other acute or chronic infections.

Material and method

The studied material was constituted of blood and organs prelevated from 22 hare specimens, netted or shot during the hunting seasons of 2011 and 2012, on the hunting grounds of Iasi, Suceava and Neamt counties. The samples were analysed at the Pathological Anatomy Laboratory from the Faculty of Veterinary Medicine of Iasi.

The hare blood sampling was made using vacuum containers with EDTA anti-coagulant agent for haematological analyses.

The haematological determinations were made using an automated haematological analyser, ABX Micros VET ABC (fig. 1). This is a fully automated equipment, with internal

dilution system and printer for data recording and graphic presentation. The measuring function is based on impedance variation generated by the cells flux through the calibrated aperture. The sample is diluted in an electrical conductive diluent. The diluent's conductivity is very different compared to the blood cell conductivity. The analysed parameters are as follows: the white cells – leucocytes – score (WBC); the total haemoglobin content of the blood (HGB); the hematocrit levels in the blood (HCT); the mean corpuscular volume (MCV) – the size of red blood cells; the mean corpuscular haemoglobin (MCH); the mean corpuscular haemoglobin concentration (MCHC); the platelet count (PLT).

Macroscopical and histopathological tests of organs such as lung, liver, kidney, cord, as well as other organs obviously modified were conducted.

The organ specimens were fixed in formalin (1:4 concentrate), paraffin processed, sliced at 6 micrometers (µm), and coloured by usual methods: haematoxylin – eosin (HE), haematoxylin – eosin – methyl blue, aniline blue, or methyl green (trichromic HEA) and Schiff fuchsine acid (PAS).

Fig. 1. Haematological analyser – ABX Micros VET ABC

Results and discussions

Our observations were regarding 22 cases, out of which 8 were in normal parameters, 10 were carrying parasite invasions, and 4 cases presented various organopathies.

The total blood volume per bodyweight value for hare is 1/20 and 5-7ml/100g bodyweight. The total specific blood mass value is of 1.048-1.052, and the viscosity value is of 5.4. The blood composition varies depending of race, gender, age and maintenance conditions.

The mean erythrocyte value for rabbits varies greatly from author to author as follows: Marcu (1993) – 3.89 x 10^6, Costăchescu et al. (2000) – 6.4 to 8.9 x 10^6. They have biconvex shape with a diameter of 6.7-6.9 µ and a relatively short lifespan of 45-70 days. This observation is to be correlated with the very high reticulocytes value of 7.4 % for young hares (1-2 months of age), and the lower value of 3.1% for adult rabbits. The specimens' age is only dimly influencing the erythrocytes' parameters.

The leucocytes (WBC, white blood cells) may vary greatly (4x14) x103 and have lesser values for young hares. When over one year of age – as most authors are indicating - the specimens are experiencing a increase in the leucocytes' value of more than 2000 mm^3

compared to the youth under 6 months of age. The leucocytes' formula is also modifying according to age.

The mean heterophile value is extremely varied: 27.9-30.0% according to Nemi (1986); 8-50% according to Hoffman; 25.5 – 46.83% according to Marcu (1993). In the first year of life the value is lower, and is increasing by 100% after the first year of age. The main blood characteristic for rabbit is the heterophils' shape (neutrophils) with polymorphic nucleus and coloured in bright red blue.

The cytoplasm is of pale rose colour and contains numerous specific acidophilic bodies that may fusion with a variable number of basic lysozymes. The basic lysozymes are apparent on the smear as brightly red coloured large bodies (Lazăr, 2009).

The lymphocytes greatly vary in value (20 – 90%) and have the same morphological aspect as in other species. However, the monocytes, eosinophils and basophils have a peculiar morphology.

The monocytes are easily identifiable because they are the largest cells and their nucleus has an amoeba-like shape. The chromatin is lightly coloured, diffused. The cytoplasm contains vacuoles and granules.

The basophils are of different values and morphological aspect for the rabbit compared to other mammals. The rabbit is the only domesticated animal that has a high number of basophils, with a mean value of 3.8% - 12.5% according to Marcu (1993), 2.4% - 31% according to Nemi (1986), 6.5 - 30% according to Scermer (1967) - quoted by Costăchescu et al., 2000. The nucleus is purple coloured, while the cytoplasm presents red and black metachromatic granules.

The eosinophils have specific granules that are much larger than the heterophils. The eosinophils granules are three to four times larger than heterophils' granules and they are intensely acidophilic.

The trombocytes vary greatly (from numeric perspective) with a mean value of 0.29 - 0.35 x 10^6 according to Nemi (1986), 0.2 -1.0% according to Parvu (1992). They present a small cluster of azurophils granules encompassed by a light-blue cytoplasm. The granulocytes/ eosinophils ratio is of 1/1.

The present study aims in this context to offer an accurate image of the physiopathological status of the hare population in the studied area.

The highest mean leucocytes value (WBC) was observed for the females - 5.60 x $10^3/mm^3$, as well as the highest erythrocyte values (RBC) - 11.40x$10^6/mm^3$. The highest mean haemoglobin value was also recorded for females 22.05 (g/dL), compared to the males 20.00 (g/dL). The red cells level (HCT) recorded highest mean values for females - 68.1 %, while the lowest values were recorded for the males (62.18%). As for the mean red cells volume (MCV), relatively close values were recorded (62.18 μm^3 for males and 60.66 μm^3 for females). The mean haemoglobin concentration (MCHC) value was the most high for hare females (31,9 g/dL).

The trombocytes value (PLT) was the most high for hare females, 557.45 x $10^3/mm^3$.

The observed organopathies were chiefly caused by parasite invasions, such as parasitary Bronchopneumonia (6 cases), hepatic or intestinal eimeriosis (3 cases) and one case of hepatic fasciolosis.

Table 1. Average values of the haematologic parameters at the *Lepus Europaeus*

Haematological parameters	Male *Lepus Europaeus*	Female *Lepus Europaeus*
WBC	5.38	5.60
RBC (mil./mm^3)	10.56	11.4
HGB (g/100ml)	20	22.05
HCT (%)	62.60	68.1
MCV (μm^3)	62.18	60.66
MCH (pg)	18.84	19.2
MCHC (g/dL)	30.68	31.9
PLT ($10^3/mm^3$)	386.48	557.45

The parasitary bronchopneumonia is caused by the infestation with: *Protostrongylus myscens, P. cuniculum, P. pulmonalis , P.terminalis, P.oryctolagus,* and in some rare cases with *P.tauricus*. In our country, according to Nesterov (1984), 77.2% of the highlands wild rabbits, 20% of the hills wild rabbits and only 4% of the plain rabbits were found to be infected. The invasion frequency in our studied case mounts to almost 40%. From a histopathological perspective, there are to be observed: congested and haemorrhagic areas more or less extended with the destruction of the alveolar lumen (fig. 2), the interstitial presence of eggs and parasites in the alveolar lumen (fig. 3), or peribronchial lymphoid proliferation (fig. 4) both in the bronchial wall obliterated by parasite egg and larvae (fig. 5 and 6), and in the parasite free breathing tract with hyperplasia and mucous tissue and sub-epithelial tissue infiltrated by inflamated cells (fig. 7,8 and 9). The pulmonary agglomeration areas are constituted by egg and larvae nests or larvae only through a polymorphic inflamed cell mass (fig. 10 and 11).

The eimeriosis was observed in two cases of hepatic localisation and in one case of intestinal localisation.

The hepatic condition, or more accurately hepato-bile condition, is generated by *E. stiedae*, a protozoa with high affinity for intra-hepatic bile duct epithelium, where located produces cholangitis of varied intensities. There are well known cases of pseudo-tumoral hyperplasia of the infested bile epithelium and conjunctive tissue proliferation from the contiguous tissue (Owen, 1992; Dulceanu et al., 1994; Paul, 2001). In the cases we diagnosed the conjuctival proliferation has granular character, with numerous giant cells, some containing fagocitosed cysts. While the giant cells are mentioned by Dulceanu, the fagocitosis aspects seem to be a priority.

The intestinal eimeriosis in the wild sylvan rabbits is produced by *E. neoleporis* and is characterised by chronic enteritis with symptoms directly proportional with the intensity of the invasion. The condition manifests itself by destroying enterocytes, desquamation and occasional persistence of the chorionic skeleton of intestinal vilosities (fig. 12 and 13).

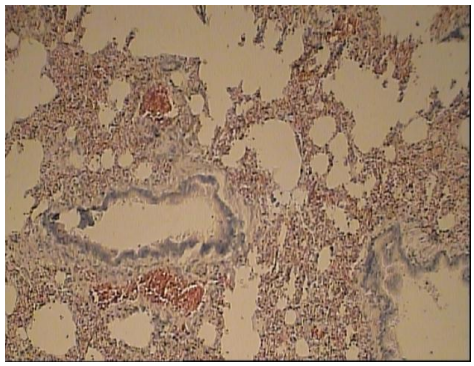


Fig. 2. Parasitary bronchopneumonia. Bronhopneumonie parazitară. Congested and haemorrhagic area at some distance from the parasitary core. HEA x 10.

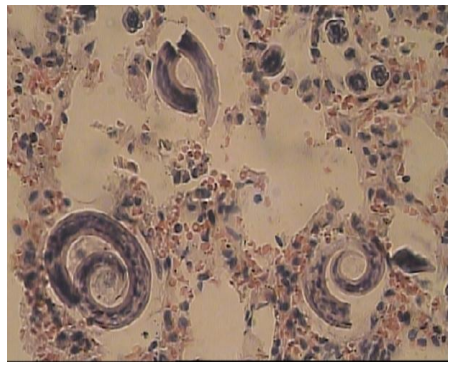


Fig. 3. Parasitary bronchopneumonia. Eggs and parasites in the pulmonary tissue. HEA x 100.

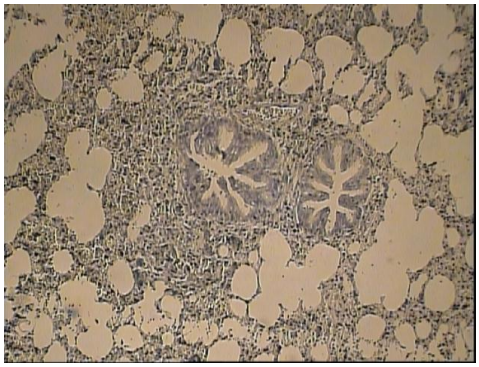


Fig. 4. Parasitary bronchopneumonia. Peribronchial lymphoid proliferation HEA x 10.

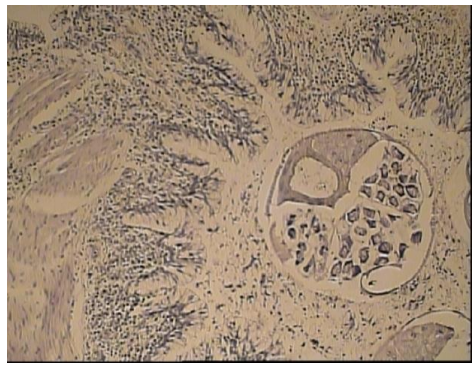


Fig. 5. Parasitary bronchopneumonia. Transversally sectioned parasite in the bronchial lumen. HEA x 20.

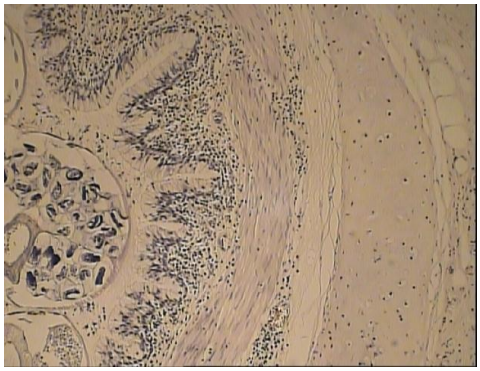


Fig. 6. Parasitary bronchopneumonia. Bronchial wall infiltrated with lymphoid cells. HEA x 20.

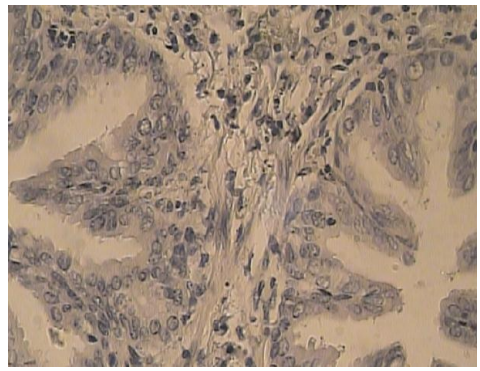


Fig. 7. Parasitary bronchopneumonia. Under endothelial infiltration. HEA x 40.

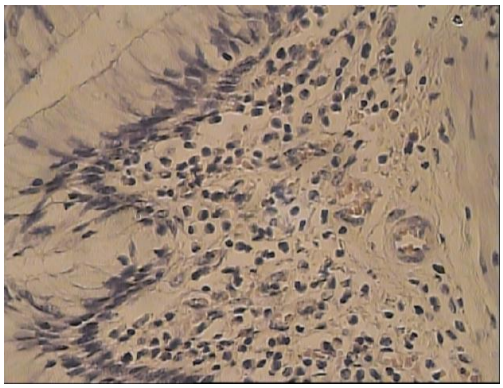


Fig. 8. Parasitary bronchopneumonia. Under epithelial proliferation rich in lymphocytes and plasmocytes. HEA x 40.

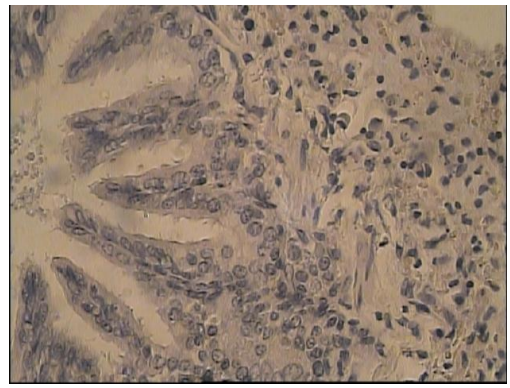


Fig. 9. Under epithelial proliferation rich in lymphocytes and plasmocytes HEA x 40.

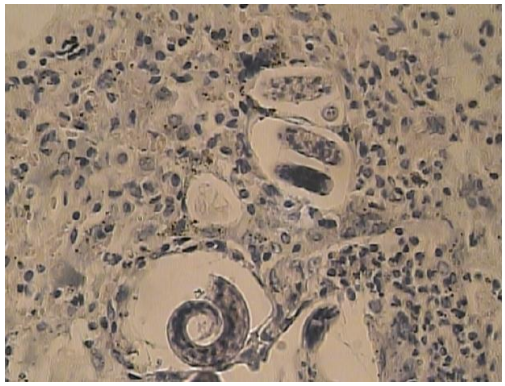


Fig. 10. Parasitary bronchopneumonia. Parasite nests engulfed in an inflamated cell mass HEA x 40.

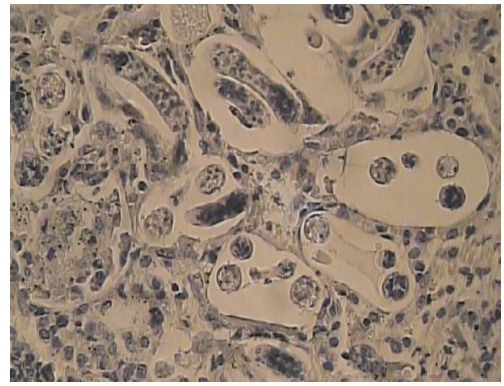


Fig. 11. Parasitary bronchopneumonia. A different field HEA x 40.

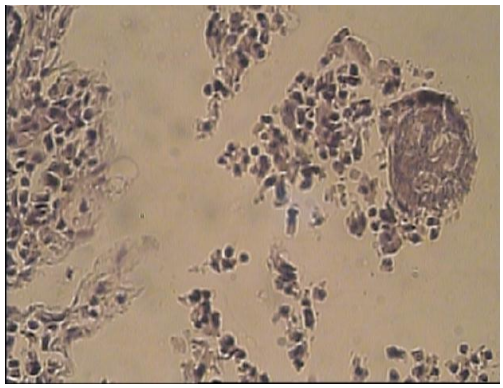


Fig. 12. Intestinal Eimeriosis. Cyst in the desquamated cells. HEA x 40.

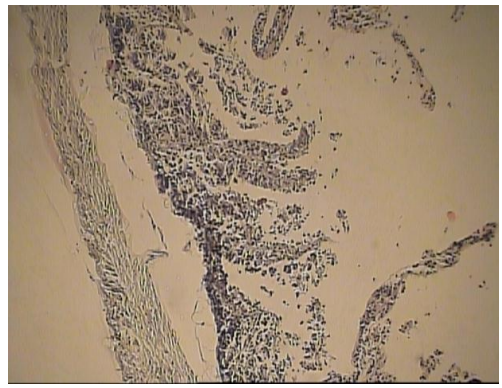


Fig. 13. Intestinal Eimeriosis De-epithelisation od intestinal vilosities. HEA x 10.

The hepatic fasciolosis was first noticed in the rabbits in our country by Cosoroaba in 1982 (quoted by Paul in 2001), Nesterov (1984, 10). We have discovered a single case, characterised by congestions and haemorrhages in liver mass and the presence of specific parasite eggs in the bile duct lumen. We confirm the deformed aspect of the eggs noticed by Nesterov (1984, 10) (fig. 14, 15 and 16).

In four additional cases there were observed only dystrophic lesions, of small intensity, respectively hepatic granular dystrophy (fig. 17 and 18) with edema (fig. 19) or renal (fig. 20).

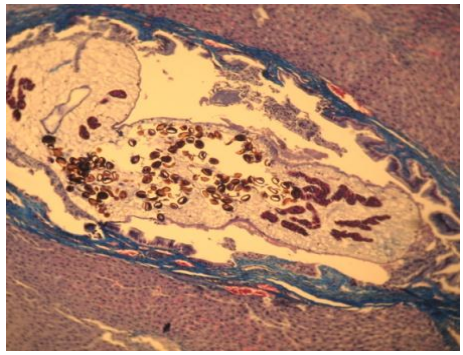


Fig. 14. Hepatic fasciolosis HEA x 40

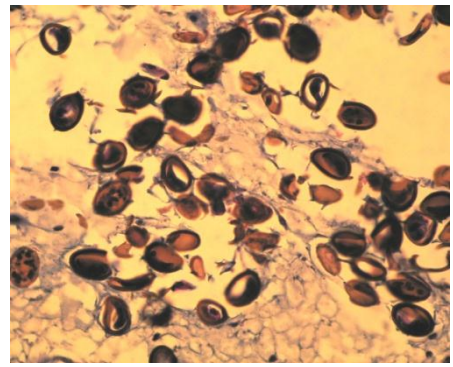


Fig. 15. Hepatic fasciolosis HEA x 100

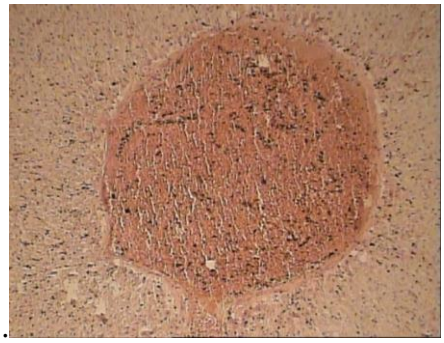


Fig. 16. Hepatic fasciolosis; arterial trombus HEA x 10.

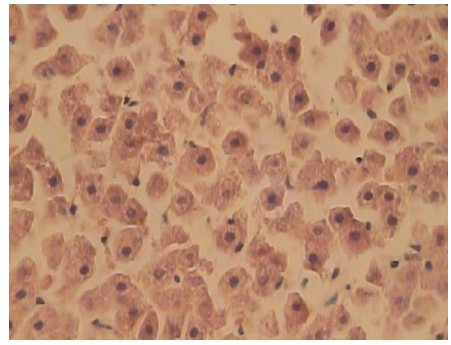


Fig. 17. Hepatic edema. HEA x 40.

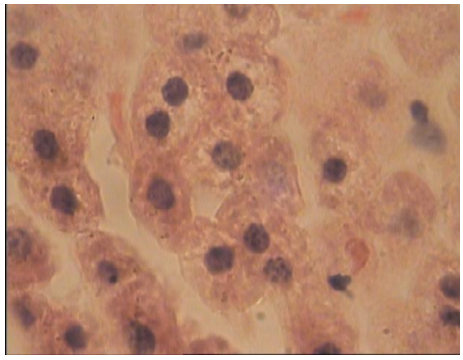


Fig. 18. Hepatic granular dystrophy HEA x 90.

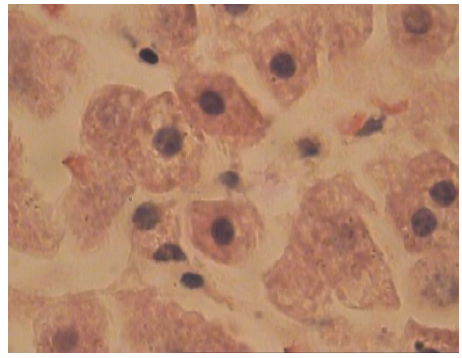


Fig. 19. Hepatic granular dystrophy, HEA x 90.

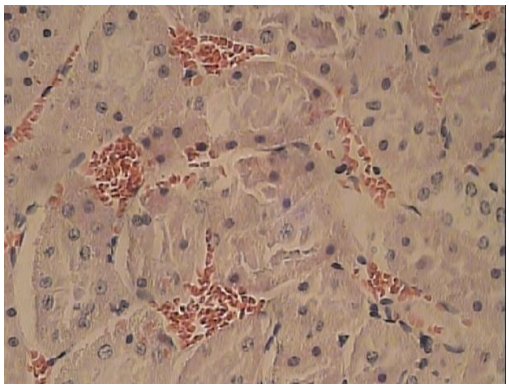


Fig. 20. Renal granular dystrophy HEA x 40.

Conclusions

There are to be noticed, regarding the haematological profile, that the recorder values are relatively close, between the species' variation limits, even though the environment and the physiological activity vary.

From an ethyologic perspective, eimeriosis and fasciolosis have been diagnosed as pathological entities of parasitary origins.

One can conclude from analysing the presented cases that the studied specimens, in their vast majority, did not presented pathological processes of great importance for veterinary pathology.

In future hunting seasons we shall increase the number of hunted and studied specimens, focusing on ethyologic diagnose determination.

References

1. Costăchescu Elena, Ciudin Elena, 2000 – *Iepurii. Creştere, valorificare şi patologie*, Ed. Moldogrup, Iaşi
2. Dulceanu N., Terinte Cristina, 1994 – *Parazitologie veterinară I.* Ed. Moldova, Iaşi
3. Lazăr Roxana, 2009 - *Corelaţiile profilului metabolic matern cu dezvoltarea intrauterină şi postnatală a produşilor de concepţie la iepuroaică.* Teză de doctorat. USAMV Iaşi
4. Marcu Elena, Pavel Geta, 1999 – *Fiziologie.* Ed. Vasiliana '98, Iaşi
5. Nesterov V., 1984 – *Bolile vânatului.* Ed. Ceres, Bucureşti
6. Oprean O.Z. 2009 - *Program de cooperare interinstituţională pentru diagnosticul, profilaxia şi combaterea stărilor patologice cu importanţă epidemiologică majoră la animalele sălbatice (ROPATOSILV)*, raportul ştiinţific şi tehnic: *Recoltări de probe, investigaţii morfologice, examene de laborator*, *identificarea agenților patogeni*, USAMV Ion Ionescu de la Brad, Iaşi
7. Owen D.G., 1992 - *Parasites of laboratory animals*. Laboratory Animal Handbooks,12, Royal Society of MedicineServices Ltd
8. Paul I., 2001 – *Etiomorfopatologie veterinară III,* Ed. Ion Ionescu de la Brad, Iaşi
9. Pârvu Gh., 1992– *Supravegherea nutriţional metabolică a animalelor*, Ed. Ceres, Bucureşti
10. Pikula J., Vojtech A., Bandouchova H., Beklova M., Horakova J., Horakova H, Kizek R., 2007 - *Blood Coagulation Times in the European Brown Hare (Lepus Europaeus)*. Veterinary Clinical Pathology 36

GREEN TEA AMELIORATES ADRIAMYCIN TOXICITY IN RATS

Heibashy, M.I.A.[1]; El-Nahla A.M.[2]; Ismail, T.A. [4,5] and A. A. H. Shallaby[3]
[1]Biological Applications Department, Nuclear Research Center, Atomic Energy Authority.
Physiology[2] and Biochemistry[3] Departments, FVM, Suez Canal University.
[4]Physiology Department, Faculty of Veterinary Medicine, Zagazig University.Egypt
[5]Medical Laboratory Department, Faculty of Applied Medical Sciences, Turabah, Taif University, KSA.
Corrseponding author : Tamer Ahmed Ismail , Medical Laboratory Department, Faculty of Applied Medical Sciences, Turabah, Taif University, KSA.
Tamersml77@yahoo.com

Abstract

The aim of the present study was to investigate the therapeutic effects of green tea in hypertensive rats induced by adriamycin (ADR). Male albino rats were divided into 2 groups; first group considered as a control (normotensive), the second group was injected intraperitoneally with ADR at a dose of 50 mg/kg body weight for 3 times day after day for one week (hypertensive). After last injection of ADR, the hypertensive group was divided into 2 subgroups, one of them was supplied with normal tap water (hypertensive recovered), the other group was supplied with 3% green tea as a drinking water for 30 days (hypertensive-green tea treated). Body weight and blood pressure were monitored in each group. Serum and cardiac tissue samples were collected from each group every 10 days. Serum creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST), nitric oxide (NO), malondialdehyde (MDA), triglyceride, cholesterol, HDL and LDL were measured. Asymmetric dimethylarginine (ADMA) was also assayed in plasma. As well a Glutathione (GSH) and glutathione peroxidase (G_{PX}) were estimated in heart homogenate. The results indicated that drinking green tea led to a significant improvement in the body weight and a pronounced a decline in the systolic blood pressure which induced by ADR in rats. Biochemical results noticed that green tea administration led to a significant decreased in the activities of serum enzymes (CK, AST and LDH) and the levels of cholesterol, triglyceride, LDL, MDA and ADMA as compared with recovered hypertensive rats. Moreover, NO, GSH and G_{PX} were restored after drinking green tea. These results suggested that green tea could ameliorate hypertensive and cardiac toxicity induced by ADR.

Introduction

Adriamycin (ADR named doxorubicin) is an anthracycline antibiotic that has been used for more than 30 years for the treatment of a wide variety of cancers. ADR is very important in the treatment of cancer patients as it causes DNA damage and stops cancer cells growing. Administration of ADR may be accompanied with acute and chronic side effects. Thus, cardiotoxicity and the risk of developing heart failure represent the major side effect limiting the clinical use of ADR in cancer therapy (Johansson et al., 2006).

It is widely accepted that oxidative stress and the production of free radicals are involved in doxorubicin action, both in terms of antitumor effects and cardiotoxicity (Singal et al., 2000). Thus, it has been reported that ADR leads to direct oxidative injury to DNA (Feinstein et al., 1993) and generates lipid peroxidation and their breakdown product like MDA (Quiles et al., 1999). These reactive oxygen species (ROS) are not only toxic to cardiac tissue but also to renal cells causing nephropathy and nephrosis which accompanied with hypertension, hyperlipidemia and proteinuria (Washio et al., 1994).

Hypertension (increased blood pressure) is the first cause of death world wide. Prevalence of hypertension is 26.3% of Egyptian adults (≥25 years). Yang and Ming (2006)

stated that decreased bioavailability of NO produced from endothelial NO synthase, referred to as endothelial dysfunction. NO plays a crucial role in the development and progression of hypertension. In addition to relaxation of blood vessels, NO contributes to a plethora of anti-atherogenic effects. One of the naturally occurring chemical substances in the blood, called asymmetric dimethylarginine (ADMA), plays an important role in hypertension as it interferes with L-arginine in the production of NO. Kielstein et al. (2004) recorded that increased blood concentrations of the endogenous nitric oxide synthase inhibitor ADMA have been linked to excess cardiovascular morbidity and mortality and might contribute to the hypertension associated with chronic renal failure in human.

The incidence of hypercholesterolemia, hypertension and cardiovascular diseases (CVD) have dramatically increased in recent years in men and women of developing countries such as United States, Europe, India, China and Japan (Crespy and Williamson, 2004). Having high serum cholesterol level, or hypercholesterolemia is closely correlated with risk of cardiovascular diseases, such as coronary heart disease (Martin et al., 1986). Therefore, reducing serum cholesterol levels, especially low density lipoprotein (LDL) cholesterol level in patients with hypercholesterolemia and high blood pressure is recommended.

Increasingly detailed understanding of the basic mechanisms involved in blood pressure regulation has led to identification of a large list of effective medications to reduce the risk of disability and death from cardiovascular disease. Many biologically active substances with medicinal properties have been identified in food (Fujita et al., 2001).

Tea is one of the most popular beverages consumed worldwide. it has been and is still being taken on a daily basis by the young and old in Japan and other African and Asian countries (Tsuchida et al., 2002). Tea, from the plant Camellia sinensis, is consumed in different parts of the world as green or black tea. Polyphenols present in green tea are flavonoids, flavanols, flavandiols and phenolic acids. Epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (ECG) and epigallocatechin-3-gallate (EGCG) are the main catechins that can be found in green tea (Brown, 1999). Fukushima et al. (2009) stated that coffee and green tea considered as a large source of antioxidant polyphenols which associated with reduction of lifestyle related diseases in the Japanese population. Catechins are known for their pharmacological properties including antioxidant activity, anticancer, anti-hypertension, anti-vascular diseases and anti-inflammatory action (Adrian & Bolwell, 2000).

Concerning the beneficial effects of green tea towards ADR, Kozluca et al. (1996) found that 20 mg/kg catechin reduced general and cardiac toxicity induced by ADR. Recently, Papparella et al. (2008) speculated that green tea attenuated angiotensin II-induced cardiac hypertrophy and hypertension in rats. It is now proven as future potential of becoming an important industrial and pharmaceutical raw material (Sharangi, 2009).

Previous reports studied the mechanism by which the green tea can be used as a protective from different diseases and attributed that to antioxidant effect. However, no study has yet been concluded to explore the beneficial effects of green tea after inducing hypertension and its accompanied hazards. So, the objective of this study was conducted to elucidate the possible therapeutic effects of green tea supplementation in amelioration of toxicity and hypertension induced by ADR in male albino rats. The current study investigates the blood pressure changes and extends to explore the cardiac enzymes activities, lipid profile and lipid peroxidation levels as well as plasma level of ADMA.

Materials and methods

Animals and experimental protocol

Experiments were performed on male albino rats Rattus Rattus (weighting 100-120 g), 6 weeks old. The animals were acclimated for 2 weeks before starting the experiment according to the guidelines of the Animal House, Faculty of Veterinary Medicine, Suez Canal University. During the experimental period, the animals were kept in wire-floored cages in a room under standard temperature condition 20-25°C and humidity 55 ±15 % on a 12hrs light-dark cycle, with a standard laboratory animal diet and water available ad libitum.

The study included two experiments; the first one was carried out to follow up the changes that could occur as a result of ADR treatment. To achieve this purpose, a comparison was done between a group of twenty control rats received intraperitoneally injections of normal saline (0.9% NaCl) for 3 times day after day for one week and served as normotensive control rats and other thirty five rats were injected intraperitoneally with ADR at a dose of 50 mg/kg body weight for 3 times day after day for one week to induce experimentally hypertension served as hypertensive animals group. This dose was equivalent to that used clinically in man as described by Rathaus et al. (1997) and Heibashy & Abdel Moniem (2005). ADR was purchased from Farmitalia-carlo (Milan, Italy) with commercial name "Ablastine".

After the end of the first experiment, five rats from each group were scarified by decapitation killing and two blood samples were taken from each rat for separation of both plasma and sera and stored frozen at -20°C till used. Heart was excised and kept at -40°C until measuring heart glutathione (GSH) content and glutathione peroxidase (G_{PX}) activity.

In the second experiment, ADR treated animals (n=30) were divided into 2 groups after last injection of ADR (15 rats for each). The first group was supplied with normal tap water for one month (hypertensive recovered group), the other group (15 rats) was supplied with green tea as a drinking water for 30 days according to Babu et al. (2007) (hypertensive green tea treated rats). Three comparisons were made between hypertensive recovered group (n=5) , hypertensive green tea treated group (n=5) and normotensive control group remaining from the first experiment without treatment (n=5).

After 10, 20 & 30 days from induction of hypertensive, 5 rats from each group were killed by decapitation. Blood samples were collected. Serum was obtained for analysis of cholesterol, triglyceride, HDH, LDL, NO, MDA, CK, LDH and AST. Plasma was obtained by using heparin as anticoagulant, centrifuged at 3000 rpm for 10 minutes for measuring ADMA. Hearts were excised and immersed immediately in cold physiological saline solution (0.9% NaCl) and kept at -40°C until measuring heart glutathione (GSH) content and glutathione peroxidase (G_{PX}) activity.

Green tea extract

The green tea solution was prepared freshly every day as follow: 5 g of commercial green tea (Arab Co. for Pharm. & Medicinal Plants – MEPACO, Inshas, Egypt) were boiled in 100 ml distilled water for five minutes according to Maity et al. (1998) and filtered to make 5% instant green tea solution. The filtrate was allowed to stand for one hour at room temperature before use and provided to rats as their sole source of drinking water.

Body weights and systolic blood pressures measurements

Body weights in grams of animals were recorded at the beginning of the experiment and after week, 10, 20 & 30 days from induction of the hypertension in all groups. Tail systolic blood pressures (TSBP) were monitored by using tail - cuff plethysmography in

unanaesthetized rats (LE Sool – Pressure Meter, Letica SA, Barcelona, Spain) in each group at the beginning of the experiment and after 10, 20 & 30 days from induction of hypertension.

Biochemical analysis

(1) Determination of the cardiac enzymes activities:-

In order to evaluate the extent of heart injury induced by ADR, the activities of serum aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK) were assayed kinetically using commercial kits (Randox Co., UK). When, animals were killed, blood sample was collected and centrifuged at 800 rpm for 5 min, and then the serum was collected. The enzymes activities AST (Reitman & Frankel, 1957), CK **(**Szasz et al., 1977) and LDH (Weisshaar et al., 1975) were detected by pyruvic acid method, N-broncholysin method and malate dehydrogenase method, respectively. All the assay kits were commercially available and all operations followed the instructions of the kits. The findings were detected on an ultraviolet/visible scanning spectrophotometer.

(2) Lipid profile tests:-

The measurements of serum triglycerides, cholesterol, HDL and LDL were performed spectrophotometrically with commercial kits obtained from Randox Co. (U.K) according to the methods of Fossati et al. (1982), Watson (1960), Gordon (1977) and Assman et al. (1984) respectively.

(3) Determination of the GSH, G_{px}, MDA, NO and ADMA:-

Glutathione (GSH) content was determined according to Baker et al. (1990) using a commercial ELISA kit (IBL-Hamburg, Germany). Glutathione peroxidase activity (G_{px}) was determined by measuring the oxidized NADPH in the presence of glutathione reductase after addition of H_2O_2 substrate at 25 (C for 1 min.). The commercial ELISA kit of G_{PX} was purchased from IBL-Hamburg, Germany. The results were expressed as nmol of NADPH oxidized / min / mg protein (Zhang et al., 1989).

Determination of malondialdehyde (MDA) in the serum was performed to estimate the extent of lipid peroxidation in the whole body. Analysis was performed with a colorimetric commercial ELISA kit (Lipid peroxidation assay kit, Calbiochem, La Jolla, CA, USA) according to Pedeson et al. (1990). Plasma ADMA (Kielstein et al., 1999) and serum total nitric oxide (Green et al., 1982) were assayed by ELISA (Sandwich Immunoassay Technique) using commercial kits (Assay Designs, Inc-Germany).

Statistical analysis

Student "t" test was used to test the difference in parameters tested herein between experimentally hypertensive rats and normal control (normotensive rats) in the preliminary experiment according to Milton et al. (1986). While, the comparison between the effects of antioxidant nutrient on blood parameters recorded herein were statistically analyzed using two ways classification analysis of variance (ANOVA) followed by Duncan's multiple range tests as described by Duncan (1955) and Snedecor & Cochran (1982).

Results and Discussion

Table 1. Comparison between normo and hypertensive rats in some blood variables related to heart function

Group / Parameters	Normotensive rats	Hypertensive rats
Body weight (g)	114.37 ± 2.63	139.71 ± 2.84 *
Systolic blood pressure (mm Hg)	107.86 ± 0.59	188.39 ± 1.77 *
Creatine kinase (U/L)	92.36± 0.41	185.61 ± 0.83 *
Lactate dehydrogenase (U/L)	233.19± 1.21	405.12 ± 1.93 *
Aspartate transaminase (U/L)	120.75± 0.44	197.52 ± 1.27 *
Cholesterol (mg/dl)	53.72± 0.94	113.29 ± 1.63 *
Triglycerides (mg/dl)	63.51± 0.33	152.17 ± 1.32 *
High density lipoprotein (mg/dl)	14.94± 0.23	26.11 ± 0.32 *
Low density lipoprotein (mg/dl)	26.08 ± 0.53	56.75 ± 0.81 *
Gutathione (mg/g heart tissue)	14.73± 0.61	8.18 ± 0.59 *
Gutathione peroxidase(µmol/min/g tissue)	72.23± 1.37	46.35 ± 0.87 *
Total nitric oxide (µmol / L)	57.32 ± 0.51	19.78 ± 0.24 *
Malondialdehyde (µM/dl)	37.18± 0.39	111.26± 1.07 *
Asymmetric dimethylaginine (µmol/L)	1.11± 0.19	3.24± 0.56 *

- Data are expressed as means ± standard error (SE).
- (*) significant different from normotensive group at (P<0.001).

The metabolic syndrome represents an emerging health burden for governments and health care providers. Metabolic syndrome refers to a clustering of several cardiometabolic risk factors including hyperglycemia, dyslipidemia and hypertension (Eckel et al., 2005). Particularly, relevant for prevention and early management of metabolic syndrome are lifestyle conditions including physical activity and the diet. It has been shown that green tea, when consumed on a daily basis, supports health (Thielecke and Boschmann, 2009).

Induction of hypertension with ADR, resulted in a significant increase in body weight compared with normotensive rats in table (1). Okuda et al. (1986) noticed that hypertensive rat where hypertension was induced with ADR led to a marked elevation in the body weight and a significant depletion in the kidney weight. They attributed these results to the abnormal retention of sodium and pharmacological nephropathy of ADR.

The current study demonstrated that drinking green tea attenuated the higher increase in body weight induced after hypertension (Table 2). Green tea has many mechanisms of action in stimulating weight loss. *In vitro* experiments as well as *in vivo* demonstrated that green tea inhibits lipogenesis (Mori and Hasegawa, 2003) and induces lipolysis (Chantre and Lairon, 2002). Green tea decreases the digestibility of dietary fat through inhibition of both gastric and pancreatic lipase. It also lowers intestinal fat absorption where catechin has a higher excretion of fat in the feces. Furthermore, Liao (2001) mentioned that green tea is also

a potent appetite suppressant by elevating cholecystokinin (Anorexigen hormone), a hormone which depresses food intake.

ADR induced hypertension (Table 1) when compared to normal control group. High blood pressure can also lead to an enlarged heart, kidney failure and an increased resistance in the peripheral arteries throughout the tissues of the body causing a were load on the heart muscle to pump the blood through these blood vessels. Adrenergic activation may favor the cardiac alterations leading to hypertension (Grassi, 2003).

Table 2. Effect of green tea on body weight and blood pressure of hypertensive rats

Intervals \ Groups		**Normotensive rats (Control)**	Hypertensive rats (Recovered)	**Hypertensive rats treated with Green tea**
Body Weight	10 days	120.65 ± 2.17 [a]	153.74 ± 3.29 [d]	148.66 ± 2.79 [d]
	20 days	128.93± 2.31 [b]	165.77± 3.42 [e]	156.38± 2.91 [d]
	30 days	136.91± 2.18 [c]	184.91± 3.38 [f]	163.92± 3.13 [e]
Blood Pressure	10 days	108.21± 0.62 [a]	170.18± 1.59 [b]	164.74± 1.49 [c]
	20 days	107.59± 0.57 [a]	161.67± 1.41 [c]	159.91± 1.37 [c]
	30 days	107.94± 0.58 [a]	152.28± 1.33 [d]	144.32± 1.28 [e]

- Data are expressed as means ± standard error (SE)
- Means with a different superscript within each parameter is significant at ($P<0.05$).

Green tea has long been believed to possess hypotension effects in popular Chinese medicine. However, conflicting results have been shown among trials and animal studies on the relation between tea consumption and blood pressure. The present study in table (2) declared that drinking green tea to hypertensive rats produced a partial restoration of hypertension; this restoration was much better after 30 days treatment with green tea. This result is parallel with previous findings of Negishi et al. (2004) who observed that black and green tea polyphenols attenuated blood pressures in male stroke-prone spontaneously hypertension rats through their antioxidative properties.

An increase in peripheral circulation is valuable for oxygenating tissue and is also associated with a relaxed mood. All four main catechins present in green tea were shown to have a dose-dependent vasodilating effect, with epigallocatechin gallate being the most potent. Green tea extract causes a dose dependent depressor action through blockade of adrenergic alpha-1 receptors (Lim et al., 2003). Catechins may act as calcium – channel blockers. Green tea catechins have been found to inhibit the proliferation of smooth muscle cells lining blood vessels *in vitro* (anti-proliferative action) through inhibition of protein tyrosine kinase activity.

Heibashy and Abdel-Moneim (2005) clarified that the increments in the levels of total nitric oxide and angiotensin II are responsible for an increase in the blood pressure and maintenance of hypertension through the stimulation of oxidative stress due to the injection with N-nitro-L-arginine methyl ester. Moreover, Napoli et al. (2006) denoted that impaired formation or function of NO in the vasculature is an important pathogenic factor in the development of vascular diseases such as atherosclerosis and hypertension. Nitric oxide

showed to be restored in the present study after supplementation with green tea, from this result we can conclude that restoration of hypertension by green tea attributed mainly to restoration of oxidative stress and NO level.

Table 3. Effect of green tea on serum AST, LDH & CK activities of hypertensive rats

Groups / Intervals		Normotensive rats (Control)	Hypertensive rats (Recovery)	Hypertensive rats treated with Green tea
AST (U/L)	10 days	121.19 ± 0.46 [a]	191.04 ± 1.16 [b]	184.29 ± 1.03 [c]
	20 days	120.46 ± 0.47 [a]	182.35 ± 1.07 [c]	171.55 ± 0.88 [d]
	30 days	121.38 ± 0.42 [a]	174.19 ± 0.93 [d]	158.74 ± 0.72 [e]
LDH (U/L)	10 days	231.17 ± 1.31 [a]	396.28 ± 2.07 [b]	374.07 ± 1.86 [c]
	20 days	234.59 ± 1.27 [a]	367.82 ± 1.76 [c]	342.19 ± 1.57 [e]
	30 days	232.42 ± 1.34 [a]	328.19 ± 1.53 [d]	297.23 ± 1.47 [f]
CK (U/L)	10 days	92.17 ± 0.39 [a]	180.76 ± 0.77 [b]	172.61 ± 0.67 [c]
	20 days	91.81 ± 0.42 [a]	171.31 ± 0.63 [c]	154.83 ± 0.58 [d]
	30 days	91.46 ± 0.41 [a]	157.93 ± 0.68 [d]	131.91 ± 0.46 [e]

- Data are expressed as means ± standard error (SE)
- Means with a different superscript within each parameter is significant at ($P<0.05$).

The ADR administration resulted in an increase in levels of cardiac markers enzymes such as AST, LDL and CK in serum which reflected the damage occurring in cardiac tissue (Table 1). Green tea administration in the present study ameliorated the cardiac damage induced by ADR through decreasing AST, LDH and CK activities as compared with recovered hypertensive rats which explained therapeutic role of green tea in hypertension (Table 3). This result is consistent with the result of Babu et al. (2007) who explored the cardioprotective effect of green tea in streptozotocin diabetic rats. They found that activities of AST, LDH and CK were significantly increased in serum of diabetic rats representing the cardiac damage, while administration of green tea to diabetic rats significantly ameliorated these enzyme activities.

It has been known for several decades that elevated blood cholesterol and triglycerides are promoted by hypertension. This clinical condition contributes to increased incidence of cardiovascular diseases. Among the risk factors of these diseases, hypercholesterolemia ranks first (Kannel et al., 1971). In this study, serum triglycerides, cholesterol and its distribution among different lipoprotein classes (LDL and HDL) were significantly ($p<0.001$) increased in hypertensive rats as compared with their relevant levels in control normotensive rats (Table 1). These results are in agreement with pervious trial by

Stathopoulos et al. (1996). The elevation in the lipid profile levels in hypertensive rats may be due to the disturbance in the hypothalamic-pituitary-thyroid axis (Heibashy, 2000). A mountain of researches was done and all of them pointed to marked elevation in all lipid fractions except HDL-cholesterol which showed negative correlation with hypertension (Kannel et al., 1971; Murakami et al., 1996 $_{a\&b}$ Rajagopalan et al., 1996 and Heibashy & Abdel Moniem, 2005).

Table 4. Effect of green tea on serum lipid profile of hypertensive rats

Groups / Intervals		Normotensive rats (Control)	Hypertensive rats (Recovery)	Hypertensive rats treated with Green tea
Cholesterol (mg/dl)	10 days	54.29 ± 0.88 ^{a}	107.36± 1.42 ^{b}	100.32± 1.29 ^{e}
	20 days	53.76 ± 0.91 ^{a}	98.86 ± 1.23 ^{c}	90.17 ± 0.91 ^{d}
	30 days	54.48 ± 0.93 ^{a}	90.19 ± 1.07 ^{d}	79.35 ± 0.95 ^{f}
Triglyceride (mg/dl)	10 days	62.39 ± 0.41 ^{a}	146.71 ± 1.24 ^{b}	138.59 ± 1.13 ^{c}
	20 days	63.17 ± 0.38 ^{a}	134.83 ± 1.19 ^{c}	121.73 ± 1.04 ^{d}
	30 days	61.83 ± 0.40 ^{a}	120.93 ± 0.95 ^{d}	102.66 ± 0.95 ^{e}
HDL (mg/dl)	10 days	14.96 ± 0.24 ^{a}	24.62 ± 0.34 ^{b}	23.17 ± 0.35 ^{b}
	20 days	15.31 ± 0.26 ^{a}	23.51 ± 0.36 ^{b}	21.22 ± 0.31 ^{c}
	30 days	15.22 ± 0.24 ^{a}	21.85 ± 0.33 ^{c}	19.41 ± 0.33 ^{d}
LDL (mg/dl)	10 days	26.85 ± 0.77 ^{a}	53.4 ± 0.89 ^{b}	49.43 ± 0.51 ^{c}
	20 days	25.82 ± 0.69 ^{a}	48.38 ± 0.72 ^{c}	44.6 ± 0.38 ^{d}
	30 days	26.89 ± 0.58 ^{a}	44.15 ± 0.68 ^{d}	39.41 ± 0.43 ^{e}

- Data are expressed as means ± standard error (SE)
- Means with a different superscript within each parameter is significant at ($P<0.05$).

Green tea consumption has also been inversely associated with the development and progression of atherosclerosis, which is consistent with the former observations. Green tea administration to hypertensive rats resulted in a significant improvement in the lipid profile as it produced a significant decrease in cholesterol, triglyceride and LDL levels when compared with recovered hypertensive rats as shown in table (4). The hypocholesterolemic effects of green tea have been confirmed by both animal (Yokozawa et al., 2002) and human (Tokunaga et al., 2002). Epidemiological studies of Hasegawa et al. (2003) indicated the hypocholesterolemic activity of powered green tea might be due to inhibition of synthesis of cholesterol in the liver or prevention the oxidation of VLDL and LDL cholesterol. Raederstorff et al. (2003) added that green tea intake decreases the absorption of triglycerides and cholesterol and these findings are in accordance with the fact that fat excretion increases.

Recently, Shrestha et al. (2009) observed that dietary green tea extract lowered plasma and hepatic triglyceride and cholesterol through decreased expression of sterol regulatory element-binding protein-1c m RNA, fatty acid synthase and stearoyl-CoA desaturase 1 mRNAs in the liver in fructose-fed ovariectomized rats. These results may suggest that green tea may have a lipolytic activity due to the mechanism by which the vitamin C contained in it inhibits triglyceride accumulation (Hasegawa et al., 2002

ADR injection produced a state of oxidative stress (decreased GSH and GP_X in heart while lipid peroxidation marker MDA showed an increase in serum) (Table 1). The result of the current study (Table 5) demonstrated that administration of green tea to hypertensive rats increased in the level of heart GSH and G_{PX} while MDA showed a decrease after green tea administration. Many investigations are in contaminant with the present results (Coimbra et al., 2006; Ounjaijean et al., 2008 and Burckhardt et al., 2008). The authors attributed these results to the powerful of green tea which acts as free radical scavenger and its antioxidant properties.

Recently, He et al. (2009) reported that subcutaneous injection of D-galactose induced learning and memory impairment in mice (decreased SOD, GSH-$_{PX}$ activities and increased MDA contents in the hippocampus). However, oral administration of green tea for 4 weeks significantly improved the congnitive deficits in mice and elevated SOD and GSH-$_{PX}$ activities and decreased MDA contents in the hippocampus. Moreover, Yeh et al., (2009) approved that antioxidant enzyme activities (G_{PX} and GSH) are induced by phenolic acids in rat liver and intestines. It was also reported that the change in the antioxidant enzyme activities was correlated with their respective mRNA expression in the phenolic acid supplemented rats. Green tea decrease lipid peroxidation indicated by decreased plasma MDA (Freese et al., 1999).

Nitric oxide (NO) is one of the major vasoprotective molecules produced by endothelial cells. In addition to relaxation of blood vessels, it has anti-atherogenic effects (Huang et al., 1998 and Heibashy et al., 2009). ADR injection induced downregulation of NO (vasorelaxant mediator) leading to hypertension (Table 1). This result may be due to the disturbance in the endothelial E-1 and endothelium nitric oxide synthase (eNOS). While, drinking green tea for 30 days in hypertensive rats restored NO level as compared with recovered hypertensive rats as obvious in the present work (Table 5). The up-regulatory effect of polyphenols on NO levels occurs through either activation of endothelium nitric oxide synthase (eNOS) or by removing free radicals and thereby inhibiting consumption of NO (Pechanova et al., 2004). Flavonoids may additionally induce an increase in eNOS expression (Hung et al., 2004).

Endothelial dysfunction may also importantly contribute to the development of hypertension. Impaired endothelial vasodilatation seems to be related to oxidative stress and reduced levels of NO likely as a result of accumulation of an inhibitor of NO synthase asymmetrical dimthylarginine (Nogueira and Weir, 2007).

Cook (2004) supposed that in the blood vessel, NO is impotant in vascular regeneration, mediating angiogenesis and the number of circulating endothelial progenitor cells. NO mediates the protective effects of the intact endothelium, acting as a vasodilator and endogenous anti-athergenic molecule (Boger, 2007).

ADMA is an endogenous competitive inhibitor of NO synthase (NOS) (Mac-Allister et al., 1993). This modified amino acid is derived from proteins that have been posttranslationally methylated and subsequently hydrolyzed (Kielstein et al., 1999). ADMA

is in part cleared by renal excretion. Reduced clearance of ADMA in renal failure is associated with endothelial vasodilator dysfunction, reversible by administration of L-arginine (Mac-Allister et al., 1993 and Kielstein et al., 1999) or by dialysis, which removes plasma ADMA (Mc-Dermott, 1976). However, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) accounts for most of the clearance of ADMA (Murray-Rust et al., 2001). Furthermore, DDAH metabolizes ADMA to L-citrulline and dimethylamine (Boger et al., 1998). ADMA is elevated to a level that can significantly inhibit NOS activity in individuals with hyperglycemia, hypercholesterolemia, hypertension, hyperhomocyst(e)inemia and tobacco exposure (Ito et al., 1999 and Stühlinger et al., 2001).

Table 5. Effect of green tea on heart GSH & G_{PX}, serum NO & MDA and plasma ADMA of hypertensive rats

Intervals / Groups			Normotensive rats(Control)	Hypertensive rats (Recovery)	Hypertensive rats treated with Green tea
Heart	**GSH** (mg/g heart tissue)	10 days	14.91 ± 0.58 [a]	8.39 ± 0.42 [b]	9.17 ± 0.53 [c]
		20 days	14.82 ± 0.53 [a]	8.92 ± 0.51 [c]	10.57 ± 0.61 [e]
		30 days	15.02 ± 0.62 [a]	9.87 ± 0.63 [d]	12.27 ± 0.55 [f]
	G_{PX} (µmol/min/g tissue)	10 days	72.71 ± 1.19 [a]	50.36 ± 0.67 [b]	51.01 ± 0.73 [b]
		20 days	73.85 ± 1.22 [a]	54.19 ± 0.78 [c]	58.37 ± 0.86 [d]
		30 days	71.93 ± 1.17 [a]	58.81 ± 0.79 [d]	63.78 ± 0.92 [e]
Serum	**NO** (µmol / L)	10 days	56.79 ± 0.54 [a]	22.71 ± 0.37 [b]	25.53 ± 0.42 [c]
		20 days	57.62 ± 0.51 [a]	26.56 ± 0.42 [c]	29.61 ± 0.52 [d]
		30 days	58.43 ± 0.52 [a]	29.84 ± 0.49 [d]	35.77 ± 0.59 [e]
	MDA (µM/dl)	10 days	37.49 ± 0.42 [a]	107.63 ± 0.94 [b]	94.48 ± 0.89 [e]
		20 days	37.27 ± 0.47 [a]	100.08 ± 1.01 [c]	81.17 ± 0.76 [f]
		30 days	37.56 ± 0.38 [a]	89.63 ± 0.82 [d]	72.29 ± 0.58 [g]
Plasma	**ADMA** (µmol/L)	10 days	1.09 ± 0.21 [a]	2.93 ± 0.52 [b]	2.74 ± 0.46 [e]
		20 days	1.12 ± 0.19 [a]	2.56 ± 0.49 [c]	2.25 ± 0.38 [f]
		30 days	1.11 ± 0.2 [a]	2.13 ± 0.51 [d]	1.61 ± 0.33 [g]

- Data are expressed as means ± standard error (SE)
- Means with a different superscript within each parameter is significant at ($P<0.05$).

Recently, Baylis (2008) deducted that increased circulating levels of endogenous NO synthase inhibitors, in particular ADMA causes of NO deficiency in chronic kidney disease. By assessing ADMA as a cardiovascular biomarker, Meinitzer et al. (2007) showed that plasma ADMA was an independent predictor of total and cardiovascular mortality. Because of involvement of ADMA in the mechanism of hypertension, Table 1 showed that ADMA level was increased after induction of hypertension. Also this study was interested to find out the effect of green tea on ADMA level after induction of hypertension. It was noticed that drinking green tea resulted in much more decrease in ADMA level compared with recovered hypertensive rats (Table 5). This result can be used to explain the restoration of NO level after giving green tea and lowering of blood pressure in hypertensive rats treated with green tea.

In conclusion, the present findings indicate that intake of green tea effectively blunts hypertension and it's accompanied hazards induced by infusion of ADR to rats. Also, the green tea attenuates the oxidative stress and cardiac toxicity induced by ADR by its ability to recover (GSH, G_{PX} and NO) and downregulate (AST, LDH, CK, MDA, ADMA in addition to lipid profile). So, the scientists take serious concern to propagate the message of medicinal properties and therapeutic values of tea to the consumers.

References

1. Adrian, J.P. and Bolwell, G.P. (2000): Phenols in the plant and in man: the potential for possible nutritional enhancement of the diet by modifying the phenols content or profile, J. Sci. Food. Agric. 80: 985–1012.
2. Assman, G.; Jabs, H.U.; Kohnert, U.; Note, W. and Schriewer, H. (1984): Enzymatic determination of LDL-cholesterol. Clin. Chem. Acta., 140: 77-83.
3. Babu, P.V.; Sabitha, K.E.; Srinivasan, P. and Shyamaladevi, C.S. (2007): Green tea attenuates diabetes induced Maillard-type fluorescence and collagen cross-linking in the heart of streptozotocin diabetic rats. Pharmacol. Res. 55(5):433-40.
4. Baker, M.A.; Cermigilia, G. J. and Zaman, A. (1990): Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large number of biological samples. Anal. Biochem., 190: 360 – 365.
5. Baylis, C. (
6. 2008): Nitric oxide deficiency in chronic kidney disease. Am. J. Physiol. Renal Physiol.; 294(1):F1-9.
7. Böger, R.H. (2007): The pharmacodynamics of L-arginine. J. Nutr.; 137(6 Suppl 2):1650S-1655S.
8. Boger, R.H.; Bode-Boger, S.M. and Szuba, A. (1998): Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation, 98: 1842–1847.
9. Böger, R.H.; Bode-Böger, S.M.; Tsao, P.S.; Lin, P.S.; Chan, J.R. and Cooke, J.P. (2000): An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes. J Am Coll Cardiol. ; 36(7):2287-95.
10. Brown, M.D. (1999): Green tea (Camellia sinensis) extract and its possible role in the prevention of cancer. Altern.Med.Rev. 4: 360-370.
11. Burckhardt, I.C.; Gozal, D.; Dayyat, E.; Cheng, Y.; Li, R.C.; Goldbart, A.D. and Row, B.W. (2008): Green tea catechin polyphenols attenuate behavioral and oxidative responses to intermittent hypoxia. Am. J. Respir. Crit. Care Med. 15; 177(10):1135-41.
12. Chantre, P. and Lairon, D. (2002): Phytomedicine. Recent findings of green tea extract AR25 (Exolise) and its activity for the treatment of obesity; 9(1):3-8.
13. Coimbra, S.; Castro, E.; Rocha-Pereira, P.; Rebelo, I.; Rocha, S. and Santos-Silva, A. (2006): The effect of green tea in oxidative stress. Clin Nutr.: 25(5):790-6.

14. Cook LN. (2004): Update on extracorporeal membrane oxygenation. Paediatr Respir 5 Suppl A:S329-37.
15. Crespy, V. and Williamson, G. (2004): A Review of the Health Effects of Green Tea Catechins in In Vivo Animal Models. J. Nutr. 134: 3431S–3440S.
16. Duncan, D.B. (1955): Multiple ranges and multiple F- test. Biometric. 11: 1 - 42.
17. Eckel, R.H.; Grundy, S.M. and Zimmet, P.Z. (2005): The metabolic syndrome. Lancet; 365: 1415–1428.
18. Feinstein, E.; Canaani, E. and Weiner, L.M. (1993): Dependence of nucleic acid degradation on in situ free-radical production by adriamycin. Biochemistry, 32: 13156- 13161.
19. Fossati, P.; Burghen, G.A.; Li, H.; Hudson, M.M. and Kun, L.E. (1982): Serum triglycerides determined calorimetrically with an enzyme that produce hydrogen peroxide. Clin. Chem., 8: 2078.
20. Freese,R;Basu,S; Hietanen, E. Nair, J; Nakachi, K, Bartsch, H and Mutanen,M. (1999): green tea extract decrease plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemostatic factors during a high-linoleic acid diet in healthy females agric food chem ;47(5):2020-5.
21. Fujita, H.; Yamagami, T. and Ohshima, K. (2001): Long-term ingestion of a fermented soybean-derived touchi-extract with a-glucosidase inhibitory activity is safe and effective in humans with borderline and mild type-2 diabetes. J. Nutr., 131: 2105-8.
22. Fukushima, Y.; Ohie, T.; Yonekawa, Y.; Yonemoto, K.; Aizawa, H.; Mori, Y.; Watanabe, M.; Takeuchi, M.; Hasegawa, M.; Taguchi, C. and Kondo, K. (2009): Coffee and Green Tea As a Large Source of Antioxidant Polyphenols in the Japanese Population. J. Agric. Food Chem.
23. Gordon, T. (1977): Colrimetric method for HDL-C determination. Am. J. Med., 62: 707.
24. Grassi G. (2003): Involvement of neuroadrenergic factors in hypertensive vascular remodelling: myth or reality? J Hypertens.21 (12):2239-41.
25. Green, L.C.; Wagner, D.A. and Glogowski, J. (1982): Analysis of nitrate, nitrite, and [N^{15}] nitrate in biological fluids. Anal. Biochem. 126, 131 – 138.
26. Hasegawa, N.; Niimi, N. and Odani, F. (2002): Vitamin C is one of the lipolytic substances in green tea. Phytother. Res. 16, S91–S92.
27. Hasegawa, N.; Yamda, N. and Mori, M. (2003): Powdered green tea has antilipogenic effect on Zucker rats fed a high-fat diet. Phytother Res.; 17(5):477-80.
28. He, M.; Zhao, L.; Wei, M.J.; Yao, W.F.; Zhao, H.S. and Chen, F.J. (2009): Neuroprotective effects of (-)-epigallocatechin-3-gallate on aging mice induced by D-galactose. Biol Pharm Bull. 32(1):55-60.
29. Heibashy, M.I.A. (2000): Hypolipidemic effect of taurine and L - carnitine on rats fed a high cholesterol diet. J. Union. Arab Biol., 14 (A): 11- 23
30. Heibashy, M.I.A. and Abdel-Moneim, A.E. (2005): Potential benefits of some antioxidant nutrients in reducing the high levels of some biochemical variables associated with induced hypertension in rats.
31. Heibashy, M.I.A.; Amal, M. El-Nahla and Shreif, Y.A. Saleh. (2009): Physiological and Biochemical Roles of Leptin and PYY in Pregnant Rats. Mansoura Conference.
32. Huang, Y.; Zhang, A.; Lau, C.W. and Chen, Z.Y. (1998): Vasorelaxant effects of purified green tea epicatechin derivatives in rat mesenteric artery. Life Sci.; 63:275–83
33. Hung, L.M.; Su, M.J. and Chen, J.K. (2004): Resveratrol protects myocardial ischemia–reperfusion injury through both NO-dependent and NO-independent mechanisms. Free Radic. Biol. Med.; 36:774–81.
34. Ito, A.; Tsao, P.S. and Adimoolam, S. (1999): Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylamino-hydrolase. Circulation, 24: 3092–3095.
35. Johansson, P.; Dahlström, U. and Broström, A. (2006): Factors and interventions influencing health-related quality of life in patients with heart failure: a review of the literature. Eur. J. Cardiovasc. Nurs. 5(1):5-15.
36. Kannel, W.B. ; Castelli, W.P. ; Gordon, T. and McNamara, P. (1971) : Serum cholesterol, lipoproteins and the risk of coronary heart disease. The Framingham study. Ann. Intern. Med., 74: 1 – 12.

37. Kielstein, J.; Boeger, R. and Bode-Boeger, S. (1999): Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: relationship to treatment method and atherosclerotic disease. J. Am. Soc. Nephrol., 10: 594–600.
38. Kielstein, J.T.; Simmel, S.; Bode-Böger, S.M.; Roth, H.J.; Schmidt-Gayk, H.; Haller, H. and Fliser, D. (2004): Suppressor dose asymmetric dimethylarginine modulates renal function in humans through nitric oxide synthase inhibition. Kidney Blood Press Res. 27(3): 143-7.
39. Kozluca, O.; Olcay, E.; Surcucu, S.; Guran, Z.; Kulaksiz, T. and Uskent, N. (1996): Prevention of doxorubicin induced cardiotoxicity by catechin. Cancer Lett., 99: 1- 6.
40. Liao S. (2001): The medicinal action of androgens and green tea epigallocatechin gallate. Hong Kong Med. J. ;7(4):369-74.
41. Lim,D.Y.;Lee,E.S.;Park,H.G;Kim,B.C;Hong, S.P. Lee,E.B.(2003): Comparison of green tea extract and epigallocatechin gallate on blood pressure and contractile responses of vascular smooth muscle of rats. Arch pharm Res. 26(3)214-23
42. Mac-Allister, R.J.; Parry, H. and Kimoto, M. (1993): Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. Endothelium, 1: 137–140.
43. Maity, S.; Vadasiromoni, J.R. and Ganguly, D.K. (1998): Role of glutathione in the antiulcer effect of hot water extract of black tea (Camellia sinensis). Jpn. J. Pharmacol.78:285–92.
44. Martin, M.J.; Hulley, S.B.; Browner, W.S.; Kuller, L.H. and Wentworth, D. (1986): Serum cholesterol, blood pressure and mortality: implications from a cohort of 361,662 men. Lancet, 2: 933-6.
45. Mc-Dermott, T. (1976): Studies on the catabolism of N^G-methylarginine, N^G,N'^G-dimethylarginine and N^G,N^G-dimethylarginine in the rabbit. Biochem. J., 154: 179–184.
46. Meinitzer, A.; Seelhorst, U.; Wellnitz, B.; Halwachs-Baumann, G.; Boehm, B.O.; Winkelmann, B.R. and März, W. (2007): Asymmetrical dimethylarginine independently predicts total and cardiovascular mortality in individuals with angiographic coronary artery disease the Ludwigshafen Risk and Cardiovascular Health study. Clin Chem.; 53(2):273-83.
47. Milton, J.S.; Cobert, J.J. and Mc-Teer, P.M. (1986): "Introduction to Statistic" 3rd ed. D.C. Health and company. Canada.
48. Mori M. and Hasegawa, N. (2003): Superoxide dismutase activity enhanced by green tea inhibits lipid accumulation in 3T3-L1 cells. Phytother Res.17 (5):566-7.
49. Murakami, S.; Nara, Y. and Yamori, Y. (1996 $_a$): Taurine accelerates the regression of hyperchloesterolemia in stroke-prone spontaneously hypertensive rats. Life. Science. 58: 1643-1651.
50. Murakami, S. ; Yamagishi, I. ; Asami, Y. ; Ohta, Y. ; Toda, S. ; Nara, Y. and Yamori, Y. (1996 $_b$) : Hyperlipidemic effect of taurine in stroke-prone spontaneously hypertensive rats. Pharmacol. 52(5): 303 -313.
51. Murray-Rust, J.; Leiper, J. and Mc-Alister, M. (2001): Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase. Nat. Struct. Biol., 8: 679–683.
52. Napoli, C.; de Nigris, F.; Williams-Ignarro, S.; Pignalosa, O.; Sica, V. and Ignarro Louis, J. (2006): Nitric oxide and atherosclerosis: an update. official journal of the Nitric Oxide Society ;15(4):265-79.
53. Negishi, H.; Xu, J.W.; Ikeda, K.; Njelekela, M.; Nara, Y. and Yamori, Y.(2004): Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats. J. Nutr.; 134(1):38-42.
54. Nogueira, J. and Weir, M. (2007): The Unique Character of Cardiovascular Disease in Chronic Kidney Disease and Its Implications for Treatment with Lipid-Lowering Drugs Clin. J. Am. Soc. Nephrol.; 2(4): 766 - 785.
55. Okuda, S.; Oh, Y.; Tsuruda, H.; Onoyama, K.; Fujimi, S. and Fujishima, M. (1986): Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. Kidney Int.; 29(2):502-10.
56. Ounjaijean, S.; Thephinlap, C.; Khansuwan, U.; Phisalapong, C.; Fucharoen, S.; Porter, J.B. and Srichairatanakool, S. (2008): Effect of green tea on iron status and oxidative stress in iron-loaded rats. Med. Chem.; 4(4):365-70
57. Papparella, I.; Ceolotto, G.; Montemurro, D.; Antonello, M.; Garbisa, S.; Rossi, G. and Semplicini, A. (2008) : Green Tea Attenuates Angiotensin II-Induced Cardiac Hypertrophy in

Rats by Modulating Reactive Oxygen Species Production and the Src/Epidermal Growth Factor Receptor/Akt Signaling Pathway. Am. Sc. Nutr.
58. Pechanova, O.; Bernatova, I.; Babal, P.; Martinez, M.C.; Kysela, S. and Stvrtina, S. (2004): Red wine polyphenols prevent cardiovascular alterations in L-NAME-induced hypertension. J Hypertens. ; 22:1551–9.
59. Pedeson, M.A.; Scott, C.S. and William, A. B. (1990): Evaluation of lipid peroxidation in inflammatory patients. Ann. NY Acad. Sci., 559, 45-62.
60. Quiles, J.L.; Ramı´rez-Tortosa, M.C.; Ibanez, S.; Gonza´lez, A.; Duthie, G.G.; Huertas, J.R. and Mataix, J. (1999): Vitamin E supplementation increases the stability and the in vivo antioxidant capacity of refined olive oil. Free Radic. Res., 31: 129-135.
61. Raederstorff, D. G.; Schlachter, M. F.; Elste, V. and Weber, P. (2003): Effect of EGCG on lipid absorption and plasma lipid levels in rats. J. Nutr. Biochem. 14: 326–332.
62. Rajagopalan, S.; Kurz, S.; Munzel, T.; Tarpey, M. ; Freeman, B.A. ; Griendling, K.K. and Harrison, D.G. (1996) : Angiotensen II – mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation : contribution to alterations of vasomotor tone. J. Clin. Invest., 97 : 1916 – 1923.
63. Rathaus, M.; Podjarny, E.; Benchetrit, S.; Green, J. and Bernheim, J.**(**1997): Nitric oxide and vascular reactivity in pregnant rats with adriamycin nephropathy. Clin. Sci. (Lond). ;93(3):227-34.
64. Rawal, N.; Rajpurohit, R.; Lischwe, M.A.; Williams, K.R.; Paik, W.K. and Kim, S. (1995): Structural specificity of substrate for S-adenosylmethionine:protein arginine N-methyltransferases. Biochim. Biophys. Acta.; 1248(1):11-8.
65. Reitman, S. and Frankel, S. (1957): A colorimetric methods for the glutamic pyruvate transaminase. Am. J. Clin. Path. 18: 56-63.
66. Sharangi, A.B. (2009): Medicinal and therapeutic potentialities of tea (Camellia sinensis L.). Food Research International. Article in press.
67. Shrestha, S.; Ehlers, S.J.; Lee, J.Y.; Fernandez, M.L. and Koo, S.I. (2009) : Dietary Green Tea Extract Lowers Plasma and Hepatic Triglyceride and Decreases the Expression of Sterol Regulatory Element-Binding Protein-1c mRNA and Its Responsive Genes in Fructose-Fed Ovariectomized Rats. J Nutr.
68. Singal, P.K.; Li, T.; Kumar, D.; Danelisen, I. and Iliskovic, N. (2000): Adriamycin-induced heart-failure: mechanism and modulation. Mol. Cell. Biochem. 207: 77- 85.
69. Snedecor, G.W. and Cochran, W.G. (1982): "Statistical Methods" 7th ed. Two state University press, Ames Iowa, U.S.A.
70. Stathopoulos, G.P.; Papadopoulos, N.G.; Stephanopoulou, A.; Dontas. I.; Kotsarelis, D. and Karayannacos, P.E. (1996): An increase of serum lipids after cumulative doses of doxorubicin and epirubicin in experimental animals. Anticancer Res.; 16(6B):3429-33.
71. Stühlinger, M.C.; Tsao, P.S. and Her, J.H. (2001): Homocysteine impairs the NO synthase pathway: role of ADMA. Circulation, 104: 2569–2575.
72. Szasz, G.; Gerhardt, W. and Gruber, W. (1977): Creatine kinase in serum: Further study of adenylate kinase inhibitors. Clin. Chem., 23: 1888-1892
73. Thielecke, F. and Boschmann, M. (2009): The potential role of green tea catechins in the prevention of the metabolic syndrome. Phytochemistry 70: 11–24.
74. Tokunaga, S.; White, I.R.; Frost, C.; Tanaka, K.; Kono, S.; Tokudome, S.; Akamatsu, T.; Moriyama, T. and Zakouji, H. (2002): Green tea consumption and serum lipids and lipoproteins in a population of healthy workers in Japan. Ann. Epidemiol.; 12(3):157-65.
75. Tsuchida, T.; Itakura, H. and Nakamura, H. (2002): Reduction of body fat in humans by long-term ingestion of catechins. Prog. Med. 22: 2189-203.
76. Vallance, P.; Leone, A.; Calver, A.; Collier, J. and Moncada, S. (1992): Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. J Cardiovasc Pharmacol.; 20 Suppl 12:S60-2.
77. Washio, M.; Nanishi, F.; Okuda, S.; Onoyama, K. and Fujishima, M. (1994): Alpha tocopherol improves focal glomerulosclerosis in rats with adriamycin-induced progressive renal failure. Nephron, 68: 347-352.
78. Watson, D. (1960): Calorimetric test for cholesterol lipid clearing factor. Clin. Chem. Acta., 5: 637.

79. Weisshaar H.D.; Prasad M.C. and Parker R.S. (1975): Estimation of Lactate Dehydrogenase in serum/plasma. Med. Welt. 26:387-391
80. Yang, Z. and Ming, X.F. (2006): Endothelial arginase: a new target in atherosclerosis. Curr. Hypertens. Rep., 8(1): 54-9.
81. Yeh, C.; Ching, L. and Yen, G. (2009): Inducing gene expression of cardiac antioxidant enzymes by dietary phenolic acids in rats. Journal of Nutritional Biochemistry 20: 163–171.
82. Yokozawa, T.; Nakagawa, T. and Kitani, K. (2002): Antioxidative activity of green tea polyphenol in cholesterol-fed rats. J. Agric. Food Chem. 50: 3549– 3552.
83. Zhang, L.; M. Maiorino and F. Urisini (1989): Phospholipid hydroperoxide glutathione peroxidase. Specific activity in tissues of rats of different age and comparison with other glutathione peroxidases. Biochem. Biophys. Acta, 1006: 140-143.

BIOCHEMICAL EFFECTS OF VITAMIN A AND E IN OBESE WISTAR RATS

Mohamed Mohamed Soliman[1,2*]; Omniya Mahmoud Abdel-Hamid[2]
[1]College of Applied Medical Sciences, Medical Laboratory Department, Taif University, KSA
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Benha University, Egypt

Abstract

Vitamins are important molecules that play critical roles in biochemical reactions as Vit. A and E. Vitamin A plays a role in body construction and its deficiency reflects the healthy state of body. Its active form named retinoic acid (carboxylic acid form). Vitamin E has 8 isoforms and is naturally present in eight isoforms. The most isoform known is □-tocopherol. Several studies performed in humans have reported a beneficial effect of vitamin A and E on improving insulin sensitivity. Vitamin effects occur without modulation of insulin secretion in insulin resistance and atherosclerosis. In this study we tested the effect of vitamin A and E on biochemical changes of liver, kidney metabolites in obese Wistar rats. The results showed that oral administration of Vit. A and E reduced the changes in body weight and food intake. Moreover, vitamin A and E induced significant changes in GPT and Urea levels as they increased in obese rats compared to normal fed rats and normalized by vitamin A and E for urea. Finally, changes in antioxidant parameters including catalase, MDA and nitrous oxide. In conclusion, vitamin A and E ameliorated the biochemical alterations accompanied obesity in Wistar rats.

Key words: *Antioxidant effects; Lipid profiles; Obesity; Vit. A& E; Wistar rats*

Introduction

Vitamins are so important molecules that affect all biochemical reactions inside the body and have peliotrophic effects on stability of human health. The active form of vitamin A is retinoic acid (RA), the carboxylic acid form of vitamin A, is a nutrient derivative with many remarkable effects on adipocytes biology and whole body adiposity (Bonet et al., 2003 and Villarroya et al., 2004). Previous studies have shown that, in mice, treatment with RA reduces body weight and adipose depot mass independent of changes in food intake (Berry and Noy 2009), and improves glucose tolerance and insulin sensitivity linked to effects on adipokines expression (Mercader et al., 2008). Chronic dietary vitamin A supplementation (as retinyl ester) also increases thermogenic potential in BAT and muscle reduces body fat content, and partly opposes the development of obesity in dietary and genetic models in mice and rats (Kumar et al., 1999; Felipe et al., 2003 and Jeyakumar et al., 2006).

Vitamin E mainly □-tocopherol isoform is the major fat-soluble dietary antioxidant. This compound is naturally present in eight isoforms (Raber 2007). Several studies performed in humans have reported a beneficial effect of vitamin E on insulin sensitivity (Manning et al., 2004; Montonen et al., 2004). Those effects occur without modulation of insulin secretion in insulin resistance and atherosclerosis. Mayer-Davis et al. 2002 reported an inverse relationship between plasmatic □-tocopherol concentration and incidence of diabetes. These data have also been supported by several studies in animal models (Laight et al., 1999).

Obesity has been reported to be associated with oxidative stress in humans and mice (Soltys et al., 2001) and oxidative stress can lead to pathogencity in obesity-associated metabolic syndrome (Furukawa et al., 2004). Moreover, disorders in carbohydrate and lipid

metabolism occur during obesity (Kumar et al., 1999). Adipose tissue is known to produce and secrete a variety of bioactive substances known as adipocytokines, among which is adiponectin and leptin. Leptin levels often correlate with adiposity, while adiponectin concentration is decreased in obesity (Rossi et al., 2005) and is associated with improving insulin sensitivity (Lara-Castro et al., 2007). White adipose tissue secrets adipokines that are increased during obesity are the main cause of metabolic related disorders (insulin resistance, atherosclerosis and Type II diabetes) that accompany obesity (Trayhurn *et al.,* 2001; Mohamed-Ali *et al.,* 1998 and Rossi et al, 2005).

The relationship between vitamins and oxidative stress together with antioxidant properties have not fully examined. So, this study hypothesized that obesity-induced systemic oxidative stress and induced oxidative stress in accumulated fat and modulated various changes in blood of obese Wistar rats. We therefore will examine whether treatment with the antioxidant vitamins as E and A could regulate body weight, liver, kidney and antioxidant parameters.

Materials and methods

Materials

Streptozotocin (STZ) was purchased from sigma Aldrich, USA. The Wistar albino rats were purchased from Egyptian Co for experimental animals import, Helwan, Egypt. Vehicles and related materials were from ADWIA pharmaceutical company, Egypt. Biochemical kits for lipids profiles, MDA, Catalase, NO and peroxidase were from *Clini Lab*, Cairo, Egypt.

Study design

Induction of obesity in Wistar rats and experimental design

Forty Wistar rats 3 week old, weighting 75–85 g were selected randomly and given high fat diet (HFD). The HFD rats (HFD, n = 30) were fed a high-fat diet (15.5% protein, 38.8 % fat and 45.7% carbohydrates, by calories) for 3 months. High fat diet components are shown in table 1. Obesity was confirmed by the increase in lipid parameters and body. Next, all rats (n=40) were subdivided into 4 subgroups. Control (normal fed rats n=10) and fed normal diet obese rats (n=30) which divided into other 3 subgroups. Obese group (obese group, n = 10) was given high fat diet and third group (Vit. E group) was given Vit. E (340 mg/kg/day) for 2 months. The fourth group (n= 10) was given Vit. A (129 mg/kg/day) for 2 months. After end of experimental procedures, all rats were decapitated after overnight fasting and blood was collected for serum extraction and liver tissue was taken on formalin for histopathology and in TriZol for RNA extraction and gene expression. During experimental procedures changes in body weight and food intake were measured as indicated time points in table 2 A and B.

Table 1. Components of diet induced obesity

Ingredients	Food (g/kg)
Normal pellet diet (NPD)	365
Lard	310
Casein	250
Cholesterol	10
Vitamins and Mineral Mix	60
dlMethionin	03
Yeast powder	01
Sodium chloride	01

Plasma chemistry analysis

Serum triglycerides (TG), total cholesterol (TC), VLDL and HDL were measured using commercial kits that based on spectrophotometric analysis. MDA, NO, peroxidase and catalase were measured using ELISA kits based on manufacture instruction manual.

Statistical analysis

Results are expressed as means ± S.E. of 10 independent rats. Statistical analysis was done using ANOVA and Fischer's post hoc test, with $p < 0.05$ being considered as statistically significant.

Results

Effect of vitamin A and E administration for 2 months on changes in body weight and food intake in obese Wistar rats

As seen in table 2, administration of vitamin A and E to obese rats for 2 months, decreased the increase in body weight relative to obese and control Wistar rats. The decrease in body weight was time dependent and was significantly from week 2 and continued to the end of experiment (Table 2 A). A parallel significant change in food intake was seen in table 2B. The changes in body weight and food intake were clear for Vit. E compared to Vit. A.

Effect of vitamin A and E administration for 2 months on changes in lipid profiles in obese Wistar rats

Induction of obesity in Wistar rats increased serum levels of TG, cholesterol, VLD and VLDL and decreased HDL levels. Administration of Vitamin A and E normalized the increase in lipid parameters in of TG, cholesterol, VLD and VLDL compared to control and obese rats (table 3). Regarding the changes in HDL levels, obesity decreased HDL levels and both Vit A and E administration re-increased its levels to nearly normal control levels confirming their action as hypolipidemic factors (table 3).

Effect of vitamin A and E administration for 2 months on antioxidant profiles and nitrous oxide (NO) levels in obese Wistar rats

MDA activity was increased in obese groups and Vit. A and E administration normalized it normal levels. Moreover, catalase activity showed a decrease in obese rats and normalization in Vit. A and E administered rats (table 4). Of note, obesity has no effect on

peroxidase activity and administration of either Vit. A or E has no effect on peroxidase levels. Finally as seen in table 4, obesity induced endothelial dysfunction through alteration in NO levels, as obesity decreased NO levels while both Vit. A and E administration normalized it (table 4).

Effect of vitamin A and E administration for 2 months on GPT, GOT, urea and creatinine levels in obese Wistar rats

Induction of obesity increased GPT and GOT activity in Wistar rats but administration of Vit. E failed to normalize such increase, while Vit. A normalized GPT but not GOT levels (table 5). Also, obesity increased urea levels but not affect creatinine. Administration of Vit. E and A decreased urea levels to normal levels compared to control and obese rats. Both have no effects on creatinine levels (table 5).

Table 2. Changes in body weight (A) and food intake (B) in obese Wistar rats.

(A) Changes in body weight

	0 week	2 weeks	4 weeks	6 weeks	8 weeks
Control	200 ±10	230 ± 12	255 ± 14	280 ±15	307 ±18
Obesity	300 ±28	330* ±30	355* ±20	380* ±35	400* ±18
Vit. E	290 ±20	270 ±31	242$^{*\#}$ ±23	220$^{*\#}$ ±19	215$^{*\#}$ ±25
Vit. A	289 ±12	280 ±13	258$^{*\#}$ ±10	250$^{*\#}$ ±9	240$^{*\#}$ ±7

(B) Changes in food intake

	0 week	2 weeks	4 weeks	6 weeks	8 weeks
Control	100 ±9	122 ± 12	145 ±7	175 ±8	181 ±11
Obese	120 ±9	140 ±10	175 ±14	199±14	210±13
Vit. E	122±8	115 ±6$^{\#}$	109 ±6.8$^{*\#}$	99 ±8.5$^{*\#}$	80 ±7.3$^{*\#}$
Vit. A	118.5±8	120 ±7.7$^{\#}$	110 ±10$^{\#}$	101 ±9.8$^{*\#}$	95$^{*\#}$ ±8.6

Data are presented as mean ± S.E for 10 rats per group. *p<0.05 *vs.* represented values at zero week control; $^{\#}$p<0.05 *vs.* represented same week obese group.

Table 3. Changes in lipid profiles after administration of Vit. A and E for 2 months in normal and obese Wistar rats

	Control	Obese	Vit. E	Vit. A
TG	45.9±5.5	122.3±11.8*	38.2±4.5$^{\#}$	62.38±4.36$^{\#}$
Cholesterol	103.06±7.7	326.78±12.2*	113.2±3.88$^{\#}$	110.56±2.04$^{\#}$
LDL	60.2±4.1	99.4±4.6*	58.6±5.2$^{\#}$	65.8±3.5$^{\#}$
VLDL	9.19±1.11	24.5±2.3*	5.8±0.8$^{\#}$	12.47±0.8$^{\#}$
HDL	35.4±2.5	20.7±1.9*	33.8±4.3$^{\#}$	31.7±3.2$^{\#}$

Data are presented as mean ± S.E for 10 rats per group. *p<0.05 *vs.* control; $^{\#}$p<0.05 *vs.* obese group.

Table 4. Changes in MDA, Catalase, peroxidase and NO levels after administration of Vit. A and E for 2 months in normal and obese Wistar rats

	Control	Obese	Vit. E	Vit. A
MDA	4.03±0.4	6.6±0.9*	3.6±0.58$^{\#}$	4.38±0.7$^{\#}$
Catalase	18. 6±2.15	10.4±1.1*	20.5±1.2$^{\#}$	16.83±2.04$^{\#}$
NO	26.4±3.8	13.7±3.1*	28.5±1.9$^{\#}$	26.9±3.4$^{\#}$
Peroxidase	73.6±8.5	73.8±7.7	67.9±5.4	73.9±5.7

Data are presented as mean ± S.E for 10 rats per group. *p<0.05 *vs.* control; $^{\#}$p<0.05 *vs.* obese group.

Table 5. Changes in GPT, GOT, Urea and creatinine levels after administration of Vit. A and E for 2 months in normal and obese Wistar rats

	Control	Obese	Vit. E	Vit. A
GPT	53.6±3.2	78.6±5.47*	90.1±7.2$^{\#}$	68.38±4.4$^{\#}$
GOT	81.4±7.9	185.4±10.8*	111.5±22.7$^{\#}$	122.54±21.04$^{\#}$
Urea	27.9±0.65	60.7±4.2*	42.82±4.78$^{\#}$	42.88±2.07$^{\#}$
Creatinine	2.3±0.15	3.42±0.168*	3.44±0.3	3.5±0.54

Data are presented as mean ± S.E for 10 rats per group. *p<0.05 *vs.* control; $^{\#}$p<0.05 *vs.* obese group.

Discussion

Retinoic acid, the carboxylic acid form of vitamin A, is a nutrient derivative with many remarkable effects on adipose tissue biology and the energy balance control system. Previous studies of our group have shown that, in mice, treatment with all-trans retinoic acid reduces body weight and adiposity independent of changes in food intake (Felipe *et al*., 2004 2005; Mercader et al., 2006; Ribot *et al*., 2001) and improves glucose tolerance (Berry and Noy 2009). Vitamin A induced body fat loss correlates with activation of brown adipose tissue (Bonet et al., 2000) and reduced adipogenic/lipogenic capabilities (Ribot et al., 2001) and increased capabilities for oxidative metabolism and thermogenesis in white adipose tissue (WAT) depots (Felipe et al., 2003), and is unaccompanied by increased circulating non esterifed fatty acids (Mercader et al., 2006), suggesting that fatty acids mobilized from fat stores actually undergo oxidation either within the adipocytes or in other tissues. In the same line of investigation, vitamin E is the best-researched fat soluble antioxidant known for its protective effects on lipid membranes and unsaturated fatty acids. Vitamin E is well documented to prevent atherosclerosis and may also help prevent Alzheimer's disease (Griffith, 2000). Its protective effects include the heart, brain, skin, eyes, liver, breasts, and prostate. It stabilizes blood fats so the blood vessels and heart are protected from free-radical induced injury. Selenium benefits treat or prevent some health conditions like, heart diseases, HIV and AIDS, miscarriages, arthritis, muscular degeneration, strokes, gray hair and different

types of cancer. Our body requires a very little amount of selenium. It also promotes antioxidant activity in the body. Selenium has also proved effective in fighting viruses that cause cold sores and shingles. Some studies have shown that consumption of selenium is helpful in making the blood "less sticky" which prevents heart strokes. (Dursun et al., 1998).Those already established dat are coincided with our findings for Vit. A and E.

Vitamins and mineral supplementation have been promoted as a strategy to prevent atherosclerosis. In vitro studies have shown that antioxidants such as vitamins E and C, carotene, and selenium reduce lipid peroxidation and free radical damage, which are important intermediaries in the pathogenesis of atherosclerosis (Bleys et al.,2006; Steinberg and Witztum 2002). Obesity is associated with oxidative stress in humans and mice (Soltys et al., 2001) which leads to obesity-associated metabolic syndrome (Furukawa et al., 2004) and disorders in carbohydrate and lipid metabolism (Kumar et al., 1999). Adipose tissue is known to produce and secrete a variety of bioactive substances known as adipocytokines, among which, adiponectin and leptin are considered to be among the most important. Leptin levels often correlate with adiposity, while adiponectin concentration is paradoxically decreased in obesity (Rossi et al., 2005) and is associated with improving insulin sensitivity (Lara-Castro et al., 2007). So probably, vitamins may act as local factor that may affect incidence of obesity through their alteration on oxidative stress. White adipose tissue together secrets inflammation and stress related proteins as serum amyloid A and C- reactive protein and haptoglobin (Trayhurn *et al.*, 2001 & Mohamed-Ali *et al.*, 1998). It increases in obesity and are the main cause of metabolic related disorders (insulin resistance, atherosclerosis and Type II diabetes) that accompany obesity (Trayhurn *et al.*, 2001& Mohamed-Ali *et al.*, 1998). As known, obesity is associated with endothelial dysfunction that resulted from hypercholesterolemia and leads to atherosclerosis. In this study obesity decreased NO levels while vitamin A and E normalized such changes.

The relationship between adipocytokines, especially adiponectin, and oxidative stress has not fully examined. However, adiponectin and leptin levels have been reported to be associated with oxidative stress levels (Nakanishi et al., 2005), although the cause-and-effect relationship between adipocytokines and oxidative stress remains unclear. In this study, we hypothesized that obesity-induced systemic oxidative stress and increased oxidative stress in accumulated fat is an underlying cause of adipocytokine mis-regulation. We therefore need to examine the effect vitamin E and A on adipokines expression in obese rats and possible signaling pathways involved in such regulation (under analysis). That suspicion is confirmed recently, as vitamin E has anti-inflammatory effect through its effect on IL-6 and 10 productions (Lira et al., 2011).

RA regulates gene expression in mammals through diverse mechanisms, notably by binding to and activation of two types of retinoid receptors that belong to the nuclear receptor superfamily of ligand-modulated transcription factors: the retinoic acid receptors (RARs), which respond to both all-trans RA (ATRA) and 9-cis RA, and the retinoid X receptors (RXRs), which respond specifically to 9-cis RA, both existing in different isoforms (Bastien and Rochette-Egly 2004).

Obesity is associated with type 2 diabetes mellitus, a disease frequently associated with abnormal lipid metabolism. Abnormal lipid levels may be present even when glycemic control is adequate and nephropathy is absent. Elevated triglyceride (TG) levels, reduced high-density lipoprotein (HDL), cholesterol, and a preponderance of small, dense low-density lipoprotein (LDL) particles are the key abnormalities that constitute diabetic dyslipidemia

(Csont et al., 2002). Here our results reported that Vit E and A supplementation corrected the changes induced in lipid profiles suggesting their improvement in insulin sensitivity. In conclusion, usage of Vit A and E ameliorated the biohazard effects associated with obesity incidence in obese Wistar rats.

References

1. Berry DC, Noy N (2009). All-trans-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor beta/delta and retinoic acid receptor. Mol Cell Biol 29:3286-3296.
2. Bonet ML, Ribot J, Felipe F, Palou A(2003). Vitamin a and the regulation of fat reserves. Cell Mol Life Sci 60:1311-1321.
3. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest, 114:1752–1761.
4. Felipe F, Bonet ML, Ribot J, Palou A (2003). Up-regulation of muscle uncoupling protein 3 gene expression in mice following high fat diet, dietary vitamin a supplementation and acute retinoic acidtreatment. Int J Obes Relat Metab Disord 27:60-69.
5. Jeyakumar SM, Vajreswari A, Giridharan NV (2006). Chronic dietary vitamin a supplementation regulates obesity in an obese mutant wnin/ob rat model. Obesity (Silver Spring) 14:52-59.
6. Kumar MV, Sunvold GD, Scarpace PJ. (1999). Dietary vitamin a supplementation in rats: Suppression of leptin and induction of ucp1 mrna. J Lipid Res 40:824-829.
7. Lira FS, Rosa JC, Cunha CA, Ribeiro EB, do Nascimento CO, Oyama LM, Mota JF. (2001). Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. Lipids Health Dis., 27; 10:37
8. Laight DW, Desai KM, Gopaul NK, Anggård EE, Carrier MJ. (1999). F2-isoprostane evidence of oxidant stress in the insulin resistant, obese Zucker rat: effects of vitamin E. Eur J Pharmacol 377:89–92.
9. Lara-Castro C, FuY,Chung BH,Garvey WT. (2007). Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease. Curr Opin Lipidol 18:263–270.
10. Manning PJ, Sutherland WH,WalkerRJ,Williams SM,De Jong SA, Ryalls AR, Berry EA. (2004). Effect of high-dose vitamin E on insulin resistance and associated parameters in overweight subjects. Diabetes Care 27:2166–2171.
11. Mayer-Davis EJ, Costacou T, King I, Zaccaro DJ, Bell RA. (2002). Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: the Insulin Resistance and Atherosclerosis Study (IRAS).Diabetes Care 25:2172–2177.
12. Mercader J, Granados N, Bonet ML,Palou A (2008). All-trans retinoic acid decreases murine adipose retinol binding protein 4 production. Cell Physiol Biochem 22:363-372.
13. Mohamed-Ali, V., .J. H. Pinkney, S.W. Coppack. (1998): Adipose tissue as an endocrine and paracrine organ. *Int. J. Obesity*, 22:1145–1158.
14. Montonen J, Knekt P, Javrvinen R, Reunanen A 2004. Dietary antioxidant intake and risk of type 2 diabetes. Diabetes Care 27:362–366.
15. Nakanishi S, Yamane K, Kamei N, Nojima H, Okubo M, Kohno N. (2005). A protective effect of adiponectin against oxidative stress in Japanese Americans: the association between adiponectin or leptin and urinary isoprostane. Metab Clin Exp;54:194–199.
16. Raber,M. G. (2007). Vitamin E regulatory mechanisms.Annu Rev Nutr, 27:347–362.
17. Rossi AR, Lombardo YB, Lacorte J, Chicco AG, Rouault C, Slama G, Rizkalla SW. (2005). Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats. Am J Physiol Regu Integr Comp Physiol, 289:486–494.
18. Soltys K, Dikdan G, Koneru B. (2001). Oxidative stress in fatty livers of obese Zucker rats: rapid amelioration and improved tolerance to warm ischemia with tocopherol. Hepatology, 34:13–18.
19. Trayhurn, P. J and H. Beattie. (2001): Physiological role of adipose tissue: white adipose

tissue as an endocrine and secretory organ. *Proc. Nutr. Soc.* 60: 329–339.
20. Villarroya F, Iglesias R, Giralt M (2004). Retinoids and retinoid receptors in the control of energy balance: Novel pharmacological strategies in obesity and diabetes. Curr. Med Chem11:795-805
21. Ribot J, Felipe F, Bonet ML, Palou A. 2001. Changes of adiposity in response to vitamin A status correlate with changes of PPAR gamma 2 expression. Obes. Res., 9:500-509.
22. Bonet ML, Oliver J, Picó C *et al.* 2000. Opposite effects of vitamin A deficient diet-feeding and retinoic acid treatment on brown adipose tissue UCP1, UCP2 and leptin expression. J. Endocrinol., 166: 511–517.
23. Felipe F, Bonet ML, Ribot J, Palou A. 2004 Modulation of resistin expression by retinoic acid and vitamin a status. Diabetes;53:882-889.
24. Felipe F, Mercader J, Ribot J, Palou A, Bonet ML: Effects of retinoic acid administration and dietary vitamin a supplementation on leptin expression in mice: Lack of correlation with changes of adipose tissue mass and food intake. Biochim. Biophys. Acta . 2005;1740:258-265.
25. Mercader J, Ribot J, Murano I, Felipe F, Cinti S, Bonet ML, Palou A: 2006. Remodeling of white adipose tissue after retinoic acid administration in mice. Endocrinology;147:5325-5332.
26. Bastien J, Rochette-Egly C. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene., 328:1-16.
27. Dursun, S.M; Oluboka, O.J ; Devarajan, S and S.P Kutcher (1998): Journal of Psychopharmacology, Vol 12 No 2 P: 220-222
28. Griffith, H. W.(2000): Minerals, Supplements & Vitamins: The Essential Guide. New York, NY: Perseus Books Group.
29. Csont T, Balogh G, Csonka C, Boros I, Horva´ I, Vigh L and Ferdinandy, P. Hyperlipidemia Induced by High Cholesterol Diet Inhibits Heat Shock Response in Rat Hearts. Biochem. Biophys. Res. Communications, 2002, 290:1535–1538

THE ROLE OF GALECTIN-3 IN THE RAT HEPATOCELLULAR CARCINOMA

Omar Mahmoud Mohamed Mohafez[1], Mohamed A. Abd EL-Aziz[2], Metwally kotb mahfouze[3], Hussein Abd El-Maksoud Ali[4], Gamal Kamel[5].

[1,2]Department of biochemistry, Faculty of Pharmacy, Al-Azhar University, Assiut branch,
[3]Department of biochemistry, Animal Health Research Institute,
[4]Department of biochemistry, Faculty of Veterinary Medicine, Benha University, Egypt
[5]Department of histology, Faculty of Veterinary, Assiut University
(omarmohafez@yahoo.com)

Abstract

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer death world wide, with an estimated mortality rate of about one million deaths annually. Galectin-3 is widely spread among different types of cells and tissues galectin-3 also found intracellularly in nucleus and cytoplasm. Galectin-3 affects numerous biological processes and seems to be involved in different physiological and pathophysiological conditions, such as development, immune reactions, and neoplastic transformation and metastasis. To investigate the role of expression pattern of galectin-3 in the rat hepatocellular carcinoma induced by diethylnitrosamine(DENA). In this study, we found over expression of galectin-3 protein and mRNA, in HCC tissue compared with normal liver tissue. In addition, we found expression of galectin-3 in the nuclear protein fraction. The overexpression of galectin-3 was accompanied with elevated levels of serum nitrite and aminotransferases (AST and ALT). ***Conclusion:*** *Galectin-3 might be a potential therapeutic target for the prevention and treatment of hepatocellular carcinoma. Moreover, the nucleo/cytoplasmic shuttling of gal-3 might play a role in inhibition and/or regulation of gene expression related to cancer.*

Key words : *HCC, Gal-3, AST/ ALT ratio*

Introduction

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of all primary liver malignancies. It is one of the most common cancers and the third leading cause of cancer mortality worldwide El-Serag , H. B. and Rudolph, K. L.(2007) . The development of HCC is related to chronic liver inflammation that leads to fibrosis and cirrhosis. However, HCC can be seen in non-cirrhotic patients with chronic viral hepatitis, toxin exposure, or other chronic liver diseases such as hemochromatosis Seeff, L. B. (2007).

Galectin-3, a 31-kDa unique trimeric gene product, is an intracellular and extracellular lectin which is presumed to interact with glycoproteins of the cell surface matrix. It consists of three structural domains: (a) an NH2-terminal domain; (b) a repeated collagen-like sequence which is rich in glycine, proline, and tyrosine; and (c) a COOH-terminal carbohydrate recognition Gong, H. C. et al (1999) . Galectin-3 is expressed widely in cancer cells as well as epithelial and immune cells Pacis, R.et al (2000) and Takenaka, Y. et al (2004). Galectin-3 is shown to be involved in cell growth, cell proliferation, cell differentiation, cell adhesion, angiogenesis, apoptosis, and tumor progression and metastasis mainly through binding to glycoproteins. The expression of galectin-3 in tumor cells is correlated with tumor invasion and metastatic potential of several types of cancer Irimura, T. et al (1999). Clinical evidences have shown that the expression of galectin-3 is associated with the carcinogenesis and malignant potential in melanoma, head and neck, thyroid, gastric,

colon, uterine, and renal cancers Kopper, L. and Timar, J. (2006) and Prieto, V. G. et al (2006).

Although galectin-3 is predominantly localized in the cytoplasm of several types of tumor cells, it has also been detected in the nucleus, suggesting that galectin-3 is a shuttling protein between the nuclear and cytoplasm cellular compartments and accordingly may have multiple functions Davidson, P. et al (2002).. In this study, we aimed to investigate the expression pattern of galectin-3 in hepatocellular carcinoma in a rat model.

Material and methods

Chemicals:

RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.). RT using RevertAid^{TM} First strand cDNA synthesis kit (Fermentas). PCR using Tag master/high yield (Jena Bioscience, Germany). DNA ladder using low range DNA ladder 50-1kbp linear scale (Jena Bioscience). Protein marker using page Ruler^{TM} prestained protein ladder (Fermentas). PVDF membrane (Amersham Hybond^{TM}-P GE Healthcare) was used. All the other chemicals and solvents used in the study were of analytical grade and were obtained either from Sigma Chemical Company or commercial suppliers, unless otherwise mentioned.

Treatment and sampling:

Male Albino rats (n = 20) (150±25g body wt) were purchased from animal house of Assiut University. Rats were divided into two groups; first group (control group n = 5) received normal saline. Second group (n =15) received diethylnitosamine (DEN) 200mg/kg intraperitoneal. Five rats were sacrificed after 16 weeks and the remaining eight survived rats were sacrificed after 20 weeks. Blood samples were collected and livers were excised rapidly and used for RNA preparation or homogenization in 20mM Tris, 100mM NaCl, 1mM EDTA and 0.5% Triton X100 buffer. Protein content of liver homogenate was determined using Biuret reagent and bovine serum albumin as standard. The protease inhibitors mix was added, aliquot and stored at -80°C tell use.

Histopathology:

Formalin-fixed liver specimens were transferred to 70% ethanol and embedded in paraffin. Tissue sections (5µm) were stained with haematoxylin and eosin (HE). At least three slides were prepared from each specimen and examined under Optica B-82 microscope for detection of pathological changes.

Assessment of serum liver function tests and serum nitrite:

Blood samples were collected by cardiac puncturing method, centrifuged and sera were isolated for serological studies. Serum ALT, AST and nitrite were estimated by using commercially available kits according to the manufacturers instructions (Biodiagnostic, Egypt)

Subcellular fractionation:

One gram of fresh liver was washed with ice cold PBS and homogenized in a Dounce homogenizer with 9 volumes of 0.25M sucrose containing 2µg/ml each of leupeptin and pepstatin-A and 0.1mM PMSF. The homogenate was centrifuged at 800xg for 10 min. The supernatant is the cytosolic fraction and the pellet is the nuclear fraction. The pellet was washed to remove the contaminant from the cytosol then re-suspended in appropriate volume of the homogenization. The protein content in all fractions was determined, denatured and stored at -70C0 until use.

Oligonucleotides used for amplifications:

NCBI reference sequence: NM_031832.1 and NM_031144.2 were used for design primers for rat Gal-3, and β-actin respectively. The coding sequences were used to design the primer pairs and the distance between the two primers was 407 bases for the Gal-3 and 500 bases for the β -actin.

The primer sets as following:

Gal-3 upper: 5′- GGC AGA CGG CTT CTC ACT T-3′
Gal-3 lower: 5′- GGG CAT ATC GTA GGG CAC T-3′
β-actin upper: 5′- CAT GGA TGA CGA TAT CGC TG-3′
β-actin lower: 5′- CAT AGA TGG GCA CAG TGT GG-3′

RNA preparation and RT-PCR:

Total RNA fractions were prepared using total RNA extraction kit from BioFlux, Bioer Technology Co., Ltd. according to the instruction manual. The first strand cDNA was synthesized according to the instruction manual of RevertAidTM First strand cDNA synthesis kit (Fermentas) from rat liver total RNA. The PCR performed using Tag master/high yield (Jena Bioscience) as following condition; pre-denaturing for 5 minute at 94°C then denaturing at 94°C for 30sec, annealing at 55°C, and extension at 72°C for one minute. The amplification was carried out in 28 cycles using Biometra cycler (Germany).

SDS–PAGE and Western blotting:

50µg from each protein homogenate were denatured by boiling for 5min in 2% SDS and 5% 2-mercaptoethanol and loaded in each lane. SDS–PAGE was done at 100 volts for 2 hours using 12% gel. The electro-transfer was done using T-77 ECL semidry transfer unit (Amersham Biosciences) for 2 hours. The membrane was blocked in TBS buffer that contains 0.05 Tween and 5% non-fat milk for one hour. The primary antibodies that used were rabbit polyclonal anti-galectin-3 and β-actin (SANTA CRUZ Biotechnology, INC). Polyclonal goat anti-rabbit immunoglobulin, conjugated to alkaline phosphatase (Sigma–Aldrich, Schelldorf, Germany), diluted 1:5000 served as a secondary antibody.

D- Statistics analysis:

Statistical differences between groups were determined by analysis of variance with multiple comparisons. All calculations were done using Microsoft Excel from the Office 2007 suite. Statistical analysis was done using 1-way ANOVA using Prism software version 5.03 (Graph Pad). The T-test was used for comparisons when only two parameters were evaluated. $P < 0.05$ was considered significant.

Results and discussion

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related mortalities. The liver, unique in its capacity for regeneration following injury, also gives rise to this malignancy commonly associated with the inflammatory state of advanced fibrosis, or cirrhosis men Newell, P. et al. (2008). Several hepatotoxic agents have been used in the induction of HCC in animal model. One of these agents is Diethylnitrosamine (DEN); DEN induces pericentral foci of small dysplastic hepatocytes and acts by ethylating nucleophilic sites in DNA Koen, H. et al. (1983)causing cirrhosis and multifocal HCC within 18 weeks Pascale, R. M. et al. (1993).

Galectins are members of a growing family of animal lectins of which galectin-3 is the most extensively studied Kasai, K. and Hirabayashi, J. (1996). This lectin is composed of 2 domains: a carboxyl terminal domain that contains the carbohydrate-binding region and an

amino-terminal domain consisting primarily of tandem repeats of 9 amino acids Liu, F. T.(1990).

Serum levels of aminotransferases:

A significant ($p<0.01$) increase in rat serum AST and ALT levels in all time points in comparison to the controls. The mean ± SD were obtained from each group are shown in table-1. The AST concentration order was 19±3.3, 102.1±14.7, and 120.1±11.9 u/L (for control, 16 and 20 weeks) groups respectively. The ALT activity order was 18±7.9, 81.5±12.5 and 90.3±12.03 u/L for control, 16 and 20 weeks respectively. The AST: ALT ratio was 1.25 for the 16 weeks group and 1.33 for the 20 weeks group in comparison to 1.05 for the control group

Serum nitrite levels:

Table-2 shows a significant increase ($p<0.05$ and $p<0.01$) in rats mean serum levels of nitrite at 16 and 20 weeks respectively after receiving DEN in comparison to the control group. The level order was 1.27±0.58, 2.3±0.64 and 3.3±0.7μM (for control, 16 and 20 weeks) respectively.

Histopathological study:

To asses the changes in parenchymal cells of the liver after DEN administration, HE stained slides were examined 16 and 20 weeks after DEN administration, as shown in figure-1. Administration of DEN induced morphological deformations in the liver pronounced with chronic hepatitis with hydropic degeneration and macroregenerative nodules. These nodules show focal prominence of bile ductular proliferation and after 20 weeks of DEN administration induced severe morphological and histopathological deformations in the liver pronounced by focal large cell changes in a trabecular hepatocellular carcinoma (Figure-1).

Galectin-3 expression in hepatocellular carcinoma:

The results exhibited over expression of Gal-3 protein synthesized by liver cells at all time points in all rats. Figure-2A shows the expression levels of Gal-3 protein in total liver homogenate for five rats from the control, 16 and 20 weeks groups. Figure-2B shows expression of Gal-3 protein in total protein homogenates (T), cytosolic fractions(C) and nuclear fractions (N) for a representative rat from each group.

Galectin-3 mRNA expression:

The over expression of Gal-3 was found associate with the over expression of mRNA in all rats as shown in figure-3 and the highest expression was found after 20 weeks.

In the current study, we found over expression of galectin-3 protein as indicated by the very intense bands in both 16 and 20 weeks groups after DEN injection in the total homogenate. These results are in agreement with another model of cancer made by Hsu, D. et al. (1999) who found; Firstly, expression of galectin-3 was observed to be up-regulated in proliferating fibroblasts with pronounced nuclear localization. Secondly, while present in low or undetectable levels in certain normal cells, galectin-3 was highly expressed when these cells were neoplastically transformed.

The mechanism by which galectin-3 affects cell morphology and growth properties through the interaction with certain intracellular proteins was explained by Hsu, D. et al. (1999). Galectin-3 has been shown to have a role in pre-mRNA splicing; in addition, while galectin-3 is diffusely present in the cytoplasm, it becomes localized in the nuclei of proliferating cells, suggesting that this protein may be involved in the regulation of cell growth.

The expression pattern of galectin-3 in both the cytosolic and nuclear fractions is referred to what is known as "nucleocytoplasmic shuttling" that was defined by Borer et al., (1989) as the repeated bidirectional movement of protein across the nuclear membrane. Also, Nakahara et al., (2006), reported that the shuttling is very significant as it might be developed as a therapeutic modality to inhibit and/or regulate gene expression related to cancer. The mechanism of nuclear import of galectin-3 is explained by Gong et al., (1999) who reported that the NH2-terminal region of Gal-3 is responsible for its nuclear localization, whereas others have argued that Gaudin, J. C. et al., (2000), and stated that the last 10 amino acids of the COOH-terminal region of Gal-3 are the responsible terminus for its nuclear transport properties Davidson, P.J. et al., (2006)

Many authors clarified the role of expression pattern of galectin-3 in different tumor cells where; cytoplasmic galectin-3 has an important role in its anti-apoptotic activity but has an opposite effect when localized in the nucleus Califice, S. et al., (2004). In human prostate cancer, over-expression of galectin-3 in the cytoplasm can promote its anti-apoptotic activity as well as increase cell proliferation, tumor growth, invasion, and angiogenesis while galectin-3 expression in the nucleus decreases cell proliferation Califice, S. et al., (2004).

Serum AST and ALT activity levels have been used to evaluate the extent of the damage in the liver, and their ratio (AST/ALT) is another good indicator of the state of the liver and our ruslts are in agreement with Sherman, K. E. (2000).

Table 1. Serum levels of AST, ALT (U/L) and their ratio in control, and in the hepatocellular carcinoma groups

	AST (U/L)	**ALT (U/L)**	**AST/ALT**
Control (n=5)	19 ± 3.3	18 ± 7.9	1.055
16 weeks (n=5)	102.1 ± 14.7**	81.5 ± 12.5**	1.25
20 weeks (n=8)	120.1 ± 11.9**	90.3 ± 12.03**	1.33

Values are means ± SD. *P* values are shown as **P*< 0.05 ***P*<0.01 vs Control

Table 2. Serum level of nitrite (µM) in control, and in the hepatocellular carcinoma groups

	Nitrite (µM)
Controls (n=5)	**1.26 ± 0.58**
16 weeks (n=5)	**2.3 ± 0.45***
20 weeks (n=8)	**3.3 ± 0.73****

Values are means ± SD. *P< 0.05 **P<0.01 vs Control

Our results are in agreement with Moriyama et al., (2000) who found that the plasma concentrations of nitrite increased in patients with HCC that are correlated with tumor volume. The role of nitrite in tumoristatic and tumoricidal effect through the mitochondrial injury of tumor cells was explained by Brown et al., (2000), Kupffer cells, the largest population of fixed tissue macrophages, form an important component of the immune system in the liver Brown, G.C. (1995). It has been reported that the cytotoxicity of Kupffer cells against hepatoma cells is related to NO muntané, j. and lamata, m.d. (2010).

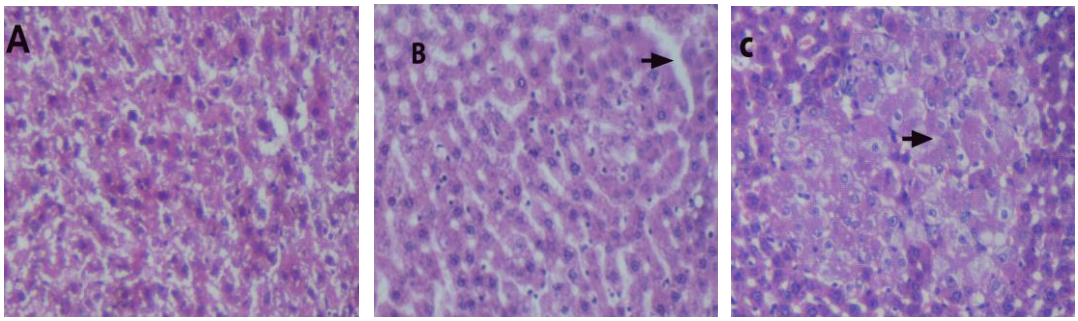


Fig. 1. Histopathological changes in hepatocellular carcinoma

A)Normal histology of liver with polygonal hepatocytes with prominent nucleus and maintained sinusoidal space B) 16 weeks after DEN administration induced morphological deformations in the liver pronounced with chronic hepatitis with hydropic degeneration and macroregenerative nodule. This nodule shows a focal prominence of bile ductular proliferation. C) 20 weeks after DEN administration induced severe morphological and histopathological deformations in the liver pronounced by focal large cell change in a trabecular hepatocellular carcinoma. Some tumor cells contain intranuclear cytoplasmic inclusions (HE, 400×).

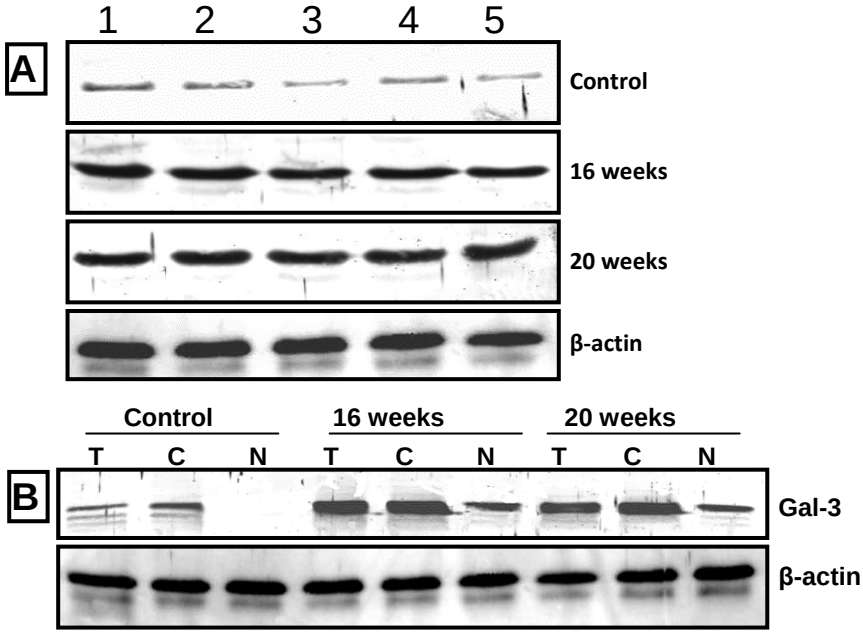


Fig. 2. Galectin-3 expression in hepatocellular carcinoma

A) Galectin-3 expression of 50µg of total liver homogenate were used from control group (n = 5 rats) 16 weeks group, and 20 weeks group. B) 50µg of total liver homogenate (T), cytosolic fraction (C) and nuclear fraction (N) were used from control group, 16 weeks group, and 20 weeks group. Rabbit polyclonal antibodies for Gal-3 were used in 1:200 dilution. Anti-rabbit secondary antibody conjugated to alkaline phospatase was used in dilution 1:3000. Data are representative of three separate experiments. β-actin re-probed on the same immunoblot to sure the identity of loading.

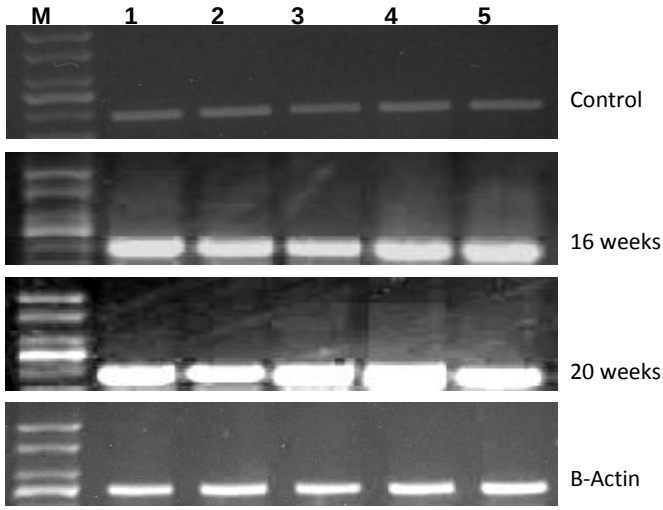


Fig. 3. RT-PCR of Gal-3 Expression

mRNA expression of Gal-3 in the control and after 16 weeks and 20 weeks of receiving DEN. B-actin used as internal control gene to show the integrity of the RNA. M: DNA marker and 1-5 represent five rats from each group.

Acknowledgments

The authors wish to thank professor Gamal Kamel. For her excellent assist in histological work

References

1. Borer, R. A. Lehner, C. F. Eppenberger, H. M. and Nigg, E. A. 1989. (Major nucleolar proteins shuttle between nucleus and cytoplasm), Cell, 56, 379.
2. Brown, G. C. 1995. (Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase), *FEBS Lett.,* 369, 136.
3. Califice, S. Castronovo, V. Bracke, M. and van den Brule, F. 2004 (Dual activities of galectin-3 in human prostate cancer: tumor suppression of nuclear galectin-3 vs tumor promotion of cytoplasmic galectin-3), *Oncogene*, 23, 7527.
4. Davidson, P. J. Davis, M. J. Patterson, R. J. Ripoche, M. A. Poirier, F. and Wang, J. L. 2002. (Shuttling of galectin-3 between the nucleus and cytoplasm), *Glycobiology*, 12, 329.
5. Davidson, P. J. Li, S. Y. Lohse, A. G. Vandergaast, R. Verde, E. Pearson, A. Patterson, R. J. Wang, J. L. and Arnoys, E. J. 2006. (Transport of galectin-3 between the nucleus and cytoplasm. I. Conditions and signals for nuclear import), *Glycobiology*, 16, 602.
6. El-Serag , H. B. and Rudolph , K. L., 2007. (Hepatocellular carcinoma: epidemiology and molecular carcinogenesis), *Gastroenterol*., 132, 2557
7. Gaudin, J. C. Mehul, B. and Hughes, R. C. 2000. (Nuclear localisation of wild type and mutant galectin-3 in transfected cells), *Biol. Cell,* 92, 49.
8. Gong, H. C. Honjo, Y. Nangia-Makker, P. Hogan, V. Mazurak, N. Bresalier, R. S. and Raz, A. 1999. (The NH2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells), *Cancer Res.,* 59, 6239.
9. Hsu, D. K. Dowling, C. A. Jeng, K. C. Chen, J. T. Yang, R. Y. and Liu, F. T. 1999. (Galectin-3 expression is induced in cirrhotic liver and hepatocellular carcinoma), *Int. J. Cancer*, 81, 519.
10. Irimura, T. Matsushita, Y. Sutton, R. C. Carralero, D. Ohannesian, D. W. Cleary, K. R. Ota, D. M. Nicolson, G. L. and Lotan, R. 1999. (Increased content of an endogenous lactose-binding lectin in human colorectal carcinoma progressed to metastatic stages), *Cancer Res.,* 51, 387.

11. Kasai, K. and Hirabayashi, J. 1996. (Galectins: a family of animal lectins that decipher glycocodes), *J. Biochem.,* 119, 1.
12. Koen, H. Pugh, T. D. and Goldfarb, S. 1983. (Centrilobular distribution of diethylnitrosamine-induced hepatocellular foci in the mouse*), Lab. Invest*., 49, 78.
13. Kopper, L. and Timar, J. 2006. (Genomics of renal cell cancer-- does it provide breakthrough?) *Pathol. Oncol. Res.,* 12, 5.
14. Liu, F. T. 1990. (Molecular biology of IgE-binding protein, IgE-binding factors, and IgE receptors), *Crit. Rev. Immunol*., 10, 289.
15. Moriyama, A. Tabaru, A. Unoki, H. Abe, S. Masumoto, A. and Otsuki, M. 2000. (Plasma nitrite/nitrate concentrations as a tumor marker for hepatocellular carcinoma), *Clin. Chim. Acta.,* 296, 181.
16. muntané, j. and lamata, m.d. 2010. (nitric oxide and cancer), *world j. hepatol*.,27;2(9):337.
17. Nakahara, S. Oka, N. Wang, Y. Hogan, V. Inohara, H. and Raz, A. 2006. (Characterization of the nuclear import pathways of galectin-3), *Cancer Res*., 66, 9995.
18. Newell, P. Villanueva, A. Friedman, S. L. Koike, K. and Llovet, J. M. 2008. (Experimental models of hepatocellular carcinoma), *J. Hepatol., 48*, 858.
19. Pacis, R. A. Pilat, M. J. Pienta, K. J. Wojno, K. Raz, A. Hogan, V. and Cooper, C. R. 2000. (Decreased galectin-3 expression in prostate cancer), *Prostate*, 44, 118.
20. Pascale, R. M. Simile, M. M. and Feo, F. 1993. (Genomic abnormalities in hepatocarcinogenesis. Implications for a chemopreventive strategy), *Anticancer Res*., 13, 1341.
21. Prieto, V. G. Mourad-Zeidan, A. A. Melnikova, V. Johnson, M. M. Lopez, A. Diwan, A. H. Lazar, A. J. Shen, S. S. Zhang, P. S. Reed, J. A. Gershenwald, J. E. Raz, A. and Bar-Eli, M. 2006. (Galectin-3 expression is associated with tumor progression and pattern of sun exposure in melanoma), *Clin. Cancer Res.,* 12, 6709.
22. Seeff, L. B. 2007. (Introduction: The burden of hepatocellular carcinoma), *Gastroenterol*., 127, S1.
23. Sherman, K. E. 1991. (Alanine aminotransferase in clinical practice), *A rev. Arch. Intern. Med.,* 151, 260.
24. Takenaka, Y. Fukumori, T. Yoshii, T. Oka, N. Inohara, H. Kim, H. R. Bresalier, R. S. and Raz, A. 2004. (Nuclear export of phosphorylated galectin-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs), *Mol. Cell Biol.,* 24, 4395.

HISTOPATHOLOGICAL FINDINGS OF THE GINGIVAL MUCOSA IN DOGS WITH PERIODONTAL DISEASE

Silviu Grămadă, Eusebiu-Viorel Şindilar, Sorin-Aurelian Paşca
Facultatea de Medicină Veterinară Iaşi
silviu_gramada@yahoo.com

Abstract
The aim of this study was to observe the histopathological changes of the gingival mucosa and to examine the lesions associated with the periodontal disease in dogs. The gingival mucosa was obtained from dog corpses. The found lesions include active and passive hyperemia, edema, cellular vacuolization and necrosis, epithelial hyperplasia, abscesses in the gingival mucosa, lympho-histiocytic inflammation.

Keywords: *dog, periodontal disease, histological examination*

Introduction

The periodontal disease results from the interactions between the localized infection of the tissues surrounding the tooth, mostly produced by anaerobic Gram-negative bacteria, and the immune response of the host. Periodontal disease is a major cause of tooth loss. The determining factor of the periodontal disease is the plaque, which starts the immune response of the host. The immune response is in part responsible for the periodontal destruction and for the progression of the periodontitis (Igna C., 2008, Wiggs R.B., Lobprise Heidi, 1997).

The periodontal disease affects a large number of dogs (93%), but is most of the time neglected by the veterinary practitioners and the pet owners. It is responsible for a lower well-being of the dogs and also causes pain and dysphagia. Systemically it can cause endocarditis, atrial fibrillation, kidney and liver failure, diabetes, etc. (Glickman L., Glickman Nita, 2011, Grămadă S., Sindilar E.V., 2012 (a), Grămadă S., Sindilar E.V., 2012 (b), Guang Y., Yang Y., 2010).

Materials and Methods

The study was realized in the Pathology Department of the Faculty of Veterinary Medicine Iasi.

The mucosa samples were taken from euthanized dogs after a thorough dental examination and periodontal disease. The staining of the samples was done with Hematoxylin-Eosin-Toluidin blue.

Results and Discussion

The histological lesions found include active and passive hyperemia, edema, cellular vacuolation, cellular necrosis, cellular hyperplasia, abscesses in the gingival mucosa, lympho-histiocytic inflammation.

The active hyperemia represents the accumulation of blood in the capillaries as a result of a reactive vasodilation. Histologically, the capillaries and arterioles are full with eritrocites (Fig. 1). The diagnostic value of the active hyperemia is low because it has a common occurrence, preceding the inflammation.

The passive hyperemia represents the accumulation of blood in the venous system as a result of the dilatation of the small vessels which occurs in the inflammatory process. Because of the lack of flow and the increased permeability, the erythrocytes leave the blood vessels and invade the interstitium (Fig. 2).

Edema is an abnormal accumulation of fluid in the interstitium. Histologically, edema is seen as clear areas of exudate that dilacerate the cellular and fibrillar components of the gingival mucosa (Fig. 3).

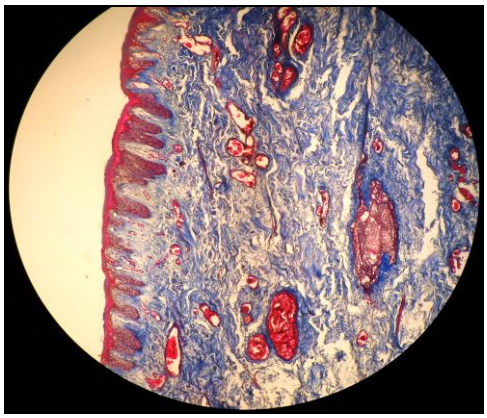


Fig. 1 Active hyperemia of the gingival mucosa. HEA x100

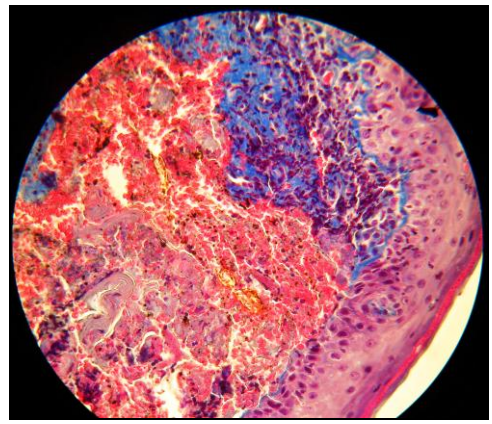


Fig. 2 Passive hyperemia of the gingival mucosa. HEA x100

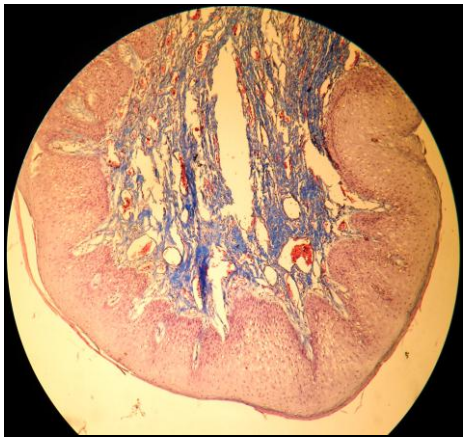


Fig. 3 Edema in the gingival mucosa. HEA x100

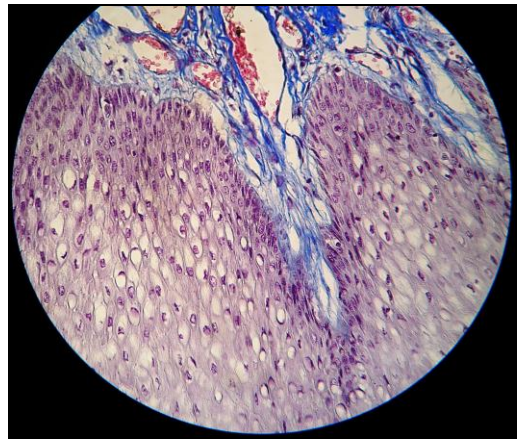


Fig. 4 Cellular necrosis. Vacuolation. Pyknotic nuclei. HEA x 100

Cellular necrosis (Fig. 4) is determined by various pathological agents, especially by toxins that lead to the alteration of the cellular membrane. This alteration determines the

increase of the cellular membrane permeability, leading to the degradation of the sodium/potassium/calcium balance. The excess of sodium and calcium ions inside the cell determine the destruction of the lysosomes` membrane and the release of their content, thus leading to the cellular death.

The hyperplasia of the keratinized stratified squamous epithelium can occur as a reactive process following the inflammation. Histologically there are rows of epithelial cells that proliferate in the connective tissue like some islands (Fig. 5).

An abscess represents a localized for of the purulent infection (Fig. 6). Histologically the abscess wall has three concentric zones:

- internal zone – which envelops the abscess` cavity; is made of macrophage cells;
- middle zone – is made of lymphocytes, plasmocytes and many newly formed capillaries;
- external zone – is very developed in old abscesses; is mainly fibrous, made of fibroblasts, reticulin and collagen fibers.

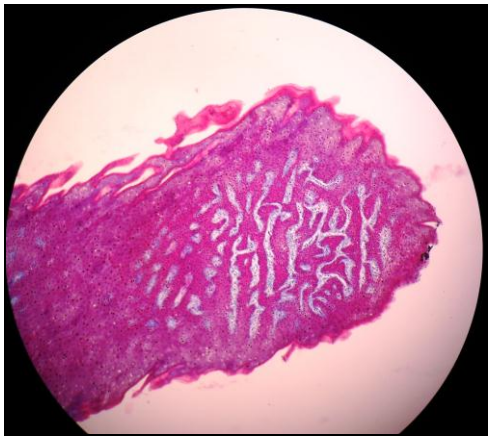


Fig. 5 Epithelial hyperplasia. Islands of epithelial cells in the connective tissue. HEA x100

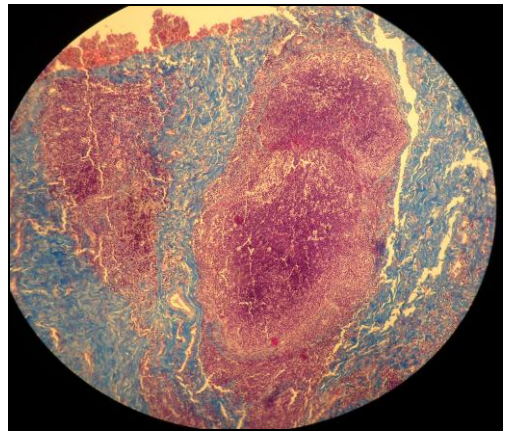


Fig. 6 Abscesses in the gingival mucosa. HEA x100.

Lymphohistiocytic inflammation. Under the influence of various biologically active substances (interleukins), released in the initial stages of the inflammatory process, takes place the hyperplasia and differentiation of the local mesenchymal cells into lymphocytes and histiocytes. Histologically the lymphocytes have strong purple stained nuclei in the HEA staining. The nucleus- cytoplasm ratio of the lymphocytes is very high (8/1 – 9/1). Their cytoplasm is seen as a narrow basophilic perinuclear strip Histiocytes have big nuclei and a ramified cytoplasm that is poorly differentiated (Fig. 7 a, b).

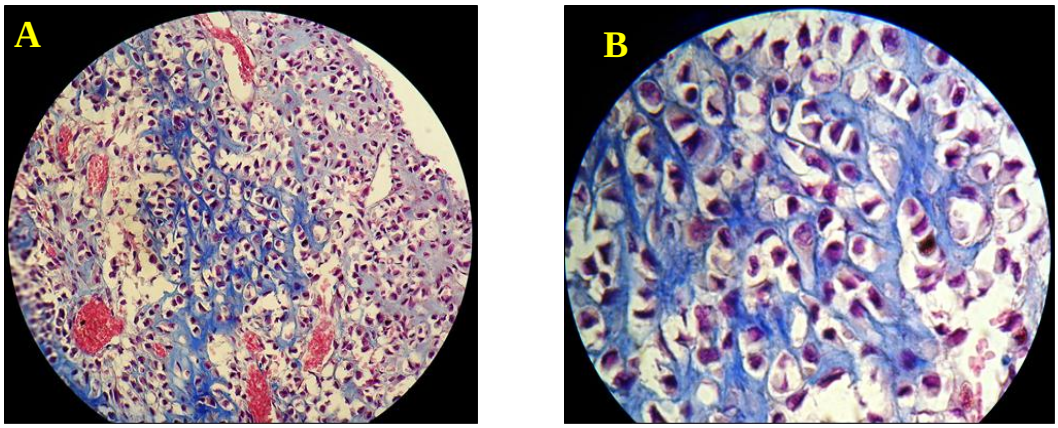


Fig. 7 Lymphohistiocytic inflammation.
A: HEA x100; B: HEA x400

Conclusions

1. In the first stage of periodontal disease (gingivitis) the inflammation is predominantly exudative, with lesions of congestion and edema. The histological lesions include active and passive hyperemia, edema in the connective tissues and abscesses in the gingival mucosa and epithelial hyperplasia.

2. In the second stage of periodontal disease (mild periodontitis) the inflammation is predominantly destructive with lesions of gingival retraction. The histological lesions include ulcerations and lymphohistiocytic inflammation.

3. In the advanced stages of periodontal disease (stage III and IV) the destruction of the periodontal tissues is severe, with the resorption of the alveolar bone and the formation of periodontal pockets.

Acknowledgments

This work was co-financed from the Social Fund through Sectorial Operational Program Human Resource Development 2009-2012, number POSDRU/88/1.5/S/52176 „Supporting the participation of doctoral students in doctoral programs".

References

1. Glickman L., Glickman Nita, 2011 – Association between chronic azotemic kidney disease and the severity of periodontal disease in dogs, Preventive Veterinary Medicine 99 (2011) 193–200
2. Grămadă S., Sindilar E.V., 2012 (a) – Periodontal disease: clinical findings on a population of dogs in Berlin, Germany, Lucrări Stiintifice, Seria Medicină Veterinară, Vol. 55 (nr. 1-2) USAMV Iasi, 311-312
3. Grămadă S., Sindilar E.V., 2012 (b)– The influence of the type of food and the oral hygiene to the periodontal disease in dogs, Lucrări Stiintifice, Seria Medicină Veterinară, Vol. 55 (nr. 1-2) USAMV Iasi, 316-318
4. Guang Y., Yang Y., 2010 - Effect of periodontitis on susceptibility to atrial fibrillation in an animal model, Journal of Electrocardiology 43, 359-366
5. Igna C., 2008.- Stomatologie veterinară, ed. Brumar, Timisoara
6. Wiggs R.B., Lobprise Heidi, 1997 – Veterinary dentistry – Principles & Practice, ed. Lippincott-Raven, Philadelphia

EFFECT OF SELENIUM SUPPLEMENTATION ON SERUM IRON, CALCIUM AND MAGNESIUM LEVELS IN RATS EXPOSED TO CADMIUM OR LEAD

B.G. Şlencu[1,2,*], C. Ciobanu[1,2], Carmen Solcan[3], Alina Anton[3], S. Ciobanu[3], Gh. Solcan[3], Rodica Cuciureanu[2]

"Grigore T. Popa" University of Medicine and Pharmacy - Iasi, Universităţii Street No. 16, 700115, Iasi, Romania, [1] – PhD student; [2] - School of Pharmacy;
"Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine, M. Sadoveanu Alley No. 3, 700490, Iasi, Romania, [3] – School of Veterinary Medicine;
[*] bogdan.slencu[at]d.umfiasi.ro

Abstract

Selenium is an essential trace element which possesses antioxidant, immunomodulatory and endocrine regulatory properties. Lead and cadmium are toxic metals which can affect various organs. One of their main toxicodynamic mechanisms is the generation of reactive oxygen species. Both metals interfere with iron and calcium metabolism. Aim: To assess the effect of selenium supplementation on serum iron, calcium and magnesium levels during cadmium or lead intoxication through the drinking water. Materials and methods: The rats were divided in 6 groups (n=6): Control, Se (Se^{+4}: 0,2 mg/l), Cd (Cd^{+2}: 150 mg/l, Pb (Pb^{+2}: 300 mg/l), Cd+Se (Cd^{+2}: 150 mg/l; Se^{+4}: 0,2 mg/l) and Pb+Se (Pb^{+2}: 300 mg/l; Se^{+4}: 0,2 mg/l). The rats were sacrificed after 56 days of exposure to the elements and blood was collected by cardiac puncture. Results and discussion: Cadmium and lead produced a decrease in serum iron levels compared to Control; selenium supplementation to intoxicated rats produced a further decrease. Serum calcium levels were decreased in Cd and Pb groups compared to Control; practically no difference was observed between Cd and Cd+Se groups, whereas in the Pb+Se group a decrease was observed, compared to Pb group. Individual exposure to cadmium, lead or selenium produced a marked and significant increase in serum magnesium levels compared to Control; selenium supplementation in cadmium or lead intoxicated rats alleviated this change, but the levels were still higher than in the Control group. Conclusions: Selenium had a synergistic effect in relation to cadmium or lead in altering serum iron levels. Selenium supplementation had no effect on cadmium-induced changes in serum calcium levels and had a minor synergistic effect in relation to lead. Cadmium or lead-induced alterations in serum magnesium levels were alleviated by selenium.

Keywords: *selenium, cadmium, lead, subacute toxicity, drinking water, biochemical markers;*

Introduction

Selenium is an essential micronutrient and is indispensable for the functioning of several enzymatic systems (Hatfield et al., 2006). Selenium has an antioxidant role (Tinggi, 2008), modulates the immune response (Hoffmann et al., 2008) and is involved in the regulation of some specific endocrine pathways (Hatfield et al., 2006). In high doses it can be toxic, clinical and metabolic manifestations depending on the dose, the chemical form, the period of exposure, the intake of other trace elements and several other factors (Reilly, 2006).

Lead is a toxic metal which can induce multiple behavioral, biochemical, haematological and histological disorders in humans and animals. Lead can affect the peripheral and central nervous system, the gastrointestinal tract, the haemopoietic system, muscles and kidneys (Ciobanu et al., 2012). One of the main mechanisms of lead-induced pathogenesis is the generation or release of reactive oxygen species (ROS) (Gurer et al, 2000).

Cadmium is a ubiquitous toxic metal which affects animal organisms in various ways. Acute or chronic exposure to cadmium produces tissue injuries and can damage various organs, especially the liver, kidneys, thyroid, bone and testis in humans and animals (Hammouda et al., 2008). Its molecular toxicodynamic mechanisms are diverse. Studies have shown that cadmium stimulates the formation of ROS, including oxygen free anyon radical, hydrogen peroxide and hydroxyl radical (Stajn et al., 1997).

Cadmium and lead interfere with iron and calcium homeostasis. The relation is bidirectional, since iron and calcium status also affects cadmium and lead absorbtion and metabolism. Iron or calcium deficiency increases lead absorption; iron deficiency increases cadmium absorption (Goyer, 1995).

Selenium has protective effects in cadmium or lead intoxication (Nehru et al., 1997; Reilly, 2006; Newairy et al., 2007), but there is insufficient and inconclusive data regarding the specific effect of selenium on iron, calcium and magnesium levels in blood.

The aim of this study was to assess the effect of selenium supplementation on serum iron, calcium and magnesium levels in rats during subacute exposure to toxic doses of cadmium or lead through the drinking water.

Materials and methods

The study was conducted in agreement with 86/609/EEC directive and all other applicable regulations. The study protocol was approved by The Ethics Committee for Research of the "Grigore T. Popa" University of Medicine and Pharmacy.

A number of 36 male Wistar rats were used in the experiment (initial body weight: 250-400 g; source: "Cantacuzino" National Institute for Research and Development in Microbiology and Immunology - CNIRDMI, Bucharest, Romania). The rats were housed in collective cages, in relative constant environmental conditions (temperature 18-25^{o} C, light/dark cycle: 12 h/12h) and had free access to water and food (dry chow for rats, mice and hamsters; CNIRDMI, Romania). The rats were randomly divided into 6 groups of 6 rats. Each group was housed in a single collective cage.

Selenium (Se^{+4}), cadmium (Cd^{+2}) and lead (Pb^{+2}) were added in the drinking water by disolving sodium selenite pentahydrate (Sigma Aldrich), lead acetate trihydrate (Chemical Company, Romania) and respectively cadmium chloride hemipentahydrate (Chemical Company, Romania). The water from the dispensers was changed every day and the unconsumed volumes were measured.

The experimental groups were: Control, Se (Se^{+4}: 0,2 mg/l), Cd (Cd^{+2}: 150 mg/l), Pb (Pb^{+2}: 300 mg/l), Cd+Se (Cd^{+2}: 150 mg/l; Se^{+4}: 0,2 mg/l) and Pb+Se (Pb^{+2}: 300 mg/l; Se^{+4}: 0,2 mg/l). The elements were added in the water for 56 days. The animals were weighed every 7 days. The mean approximate intake of elements (per kg body weight; Table I) was estimated based on the volumes of water consumed by each group during each day, the cumulative body weights and on the concentrations of the elements in drinking water.

Table 1. Estimative mean approximate intake of elements throughout the experiment

Element	Group					
	Control	**Se**	**Cd**	**Pb**	**Cd+Se**	**Pb+Se**
Se (µg/kg bw)	-	15,48±0,42	-	-	9,81±0,30	15,90±0,43
Cd (mg/kg bw)	-	-	10,48±0,41	-	7,36±0,23	-
Pb (mg/kg bw)	-	-	-	25,34±0,68	-	23,84±0,64
Values are expressed as mean ± Standard deviation						

The animals were anesthetized and sacrificed after 56 days. Ketamine (injectable solution for veterinary use) was administered intraperitoneally in a dose of 100 mg/kg bw to induce anesthesia. Blood was collected in vacutainers without anticoagulant by cardiac puncture. After coagulation, serum was obtained by centrifugation. Serum iron, calcium and magnesium levels were determined using an Accent 200 biochemical analyzer (Cormay, Poland) and specific assay kits.

Microsoft Office Excel 2007 with Data Analysis add-on was used for statistical interpretation. A paired Student's t-test was used ($p < 0,05$ was considered significant).

Results and discussion

Both cadmium and lead produced a decrease in serum iron levels compared to Control; selenium supplementation to cadmium or lead exposed rats led to a further decrease (Fig. 1).

Apparently none of the mean values were significantly different one from another from a pure statistically point of view so the results could be inconclusive.

Moshtaghie et al. (2013) also observed a marked decrease in serum iron levels in rats after exposure to lead and suggested that it could be caused by the interference of lead with iron binding to transferrin.

In one study dietary cadmium loading reduced packed cell volume and plasma iron, as well as iron levels in the liver and kidneys. Dietary iron supplementation reversed these effects, suggesting that the decrease in iron levels was caused by cadmium, which impaired iron intestinal absorption (Crowe et al., 1997).

Chareonpong-Kawamoto et al. (1993) have shown that selenium deficiency causes iron overload in serum and in the tissues of rats. Besides the increase in serum iron levels, the same researchers found that selenium deficient rats had significantly lower transferrin concentrations in serum in comparison to selenium-adequate rats, (Chareonpong et al., 1991). Christensen et al. (2000) found that in Se-deficient rat livers the expression of transferrin, transferrin receptor and an iron regulatory protein was increased compared to Se-adequate rat livers. By extrapolation to the present study, it could be possible that the same type of mechanisms could be involved in the slight decrease in serum iron level observed in the Se group, considering that selenium status was higher in the Se group than in Control. The same supposition could explain the decrease observed in the Cd+Se and Pb+Se groups compared to Cd, respectively Pb groups, suggesting that selenium and cadmium, respectively selenium and lead effects are additive.

Cadmium and lead decreased serum calcium levels compared to Control. Selenium supplementation to cadmium intoxicated rats did not have a marked effect, the mean serum calcium level being approximately the same as in the Cd group. A statistically significant decrease was observed in the Pb+Se group, compared to Pb (Fig. 2). Selenium supplementation only produced a slight decrease in serum calcium levels compared to Control. The same modification was observed by Saleh (2007).

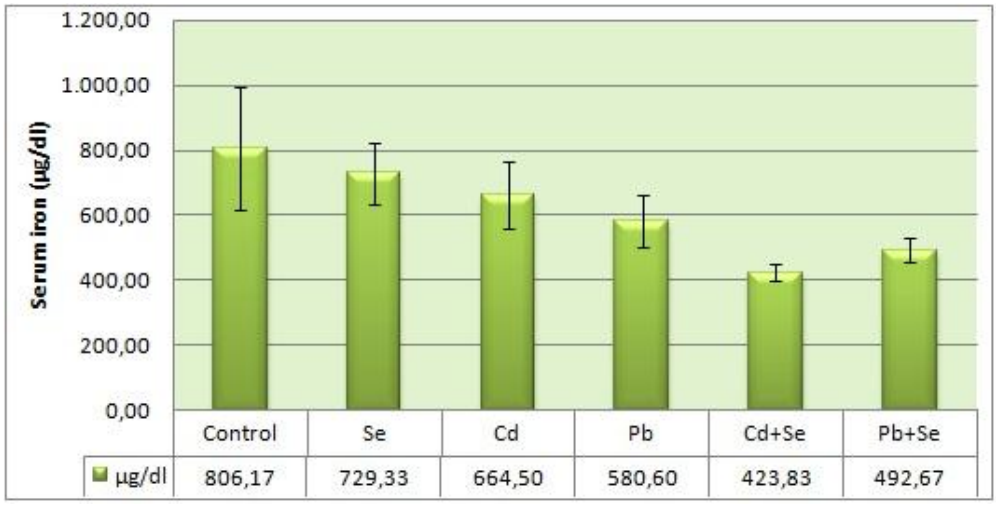


Fig. 1. Serum iron levels. Error bars are SEM; ■ = significantly different compared to Control ($p < 0,05$); ▲ = significantly different compared to Cd ($p < 0,05$); ● = significantly different compared to Pb ($p < 0,05$);

Fig. 2. Serum calcium levels. Error bars are SEM; ■ = significantly different compared to Control ($p < 0,05$); ▲ = significantly different compared to Cd ($p < 0,05$); ● = significantly different compared to Pb ($p < 0,05$)

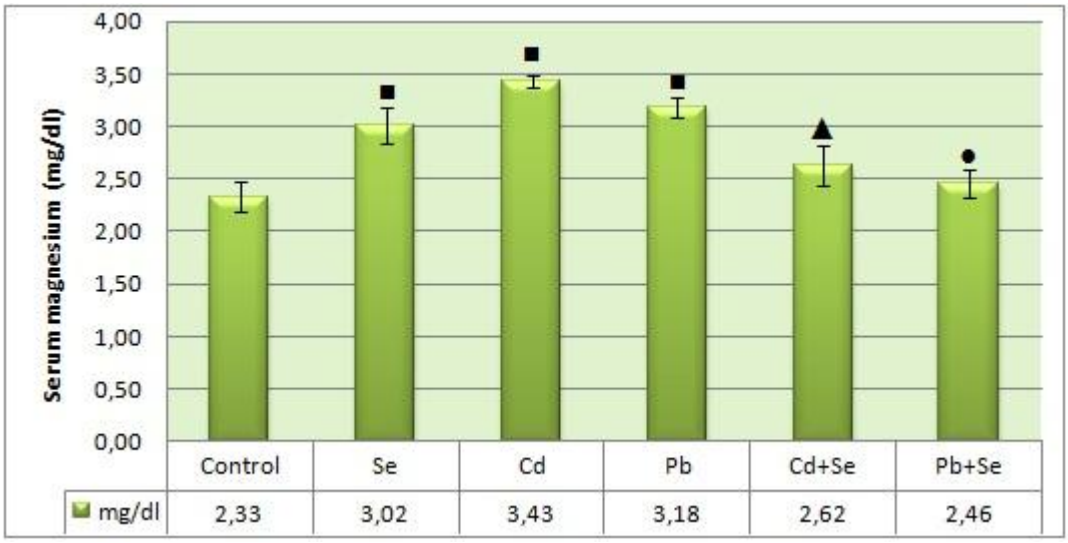


Fig. 3. Serum magnesium levels. Error bars are SEM; ■ = significantly different compared to Control ($p < 0,05$); ▲ = significantly different compared to Cd ($p < 0,05$); ● = significantly different compared to Pb ($p < 0,05$)

Chertok et al. (1981) established that acute or chronic exposure to cadmium reduces calcium absorption. Cadmium interferes with the calcium transport mechanisms in the intestine (Goyer, 1995). Due to the relative long experimental period of the present study (56 days), probably the decrease in serum calcium levels in the groups which received cadmium in the drinking water is due to a moderate cadmium-induced calcium deficiency.

Lead blocks calcium efflux from cells by substituting calcium in calcium-sodium-ATP pumps, this being one of the mechanisms by which lead interacts with calcium in the intestine. Another mechanism by which lead reduces calcium absorption is the competition between calcium and lead for binding sites on calcium-binding proteins (Goyer, 1995). Xue-Qin et al. (2007) observed that lead exposure decreased serum calcium and osteocalcin levels in rats and also decreased the length and the diameter of the femur. Apparently in the present study lead and selenium had a synergistic effect on reducing serum calcium levels.

A statistically significant increase in serum magnesium levels was observed in the Se, Cd and Pb groups, compared to Control. In the Cd+Se and Pb+Se groups the increase was alleviated, but the values were higher than in the Control group (Fig. 3).

Sivrikaya et al. (2013) observed an increase in magnesium levels in the livers of rats supplemented with selenium.

There is little data regarding the effect of lead or cadmium on the serum magnesium levels. Todorovic T et al. (2008) found that lead exposure leads to a decrease in magnesium levels in hard tissues of rats. In a similar way, Itokawci Y et al. (1973) have found that cadmium exposure leads to a decrease in magnesium levels in bones. It is a known fact that lead and cadmium interfere with bone metabolism (Goyer, 1995). In the context of the present study it is possible that the high serum magnesium levels observed in the lead and

cadmium exposed groups could be the result of magnesium release from bones. Selenium apparently had a protective effect against lead or cadmium toxicity, in relation to this aspect.

Conclusions

Selenium supplementation did not alleviate cadmium and lead-induced changes in serum iron levels, but intensified them. Practically selenium had no effect on cadmium-induced change in serum calcium level and had a slight synergistic effect in relation to lead-induced change. Cadmium and lead induced disturbances in serum magnesium levels were alleviated by selenium supplementation.

Acknowledgements

This article was developed within the project "Doctoral scholarships for increasing the competitiveness in the medical and pharmaceutical field", contract POSDRU/88/1.5/S/58965.

References

1. Chareonpong N, Yasumoto K. Iron and magnesium levels and electrocardiogram in rats fed selenium deficient diet. *Trace Nutr Res* 1991; 8:61-66.
2. Chareonpong-Kawamoto N, Yasumoto K. Iron overload secondary to selenium deficiency in rat. *Trace Nutr Res* 1993; 10:87-92.
3. Chertok RJ, Sasser LB, Callaham MF, Jarboe GE. Influence of cadmium on the intestinal uptake and absorption of calcium in the rat. *J Nutr* 1981; 111:631-638.
4. Christensen MJ, Olsen CA, Hansen DV, Ballif BC. Selenium regulates expression in rat liver of genes for proteins involved in iron metabolism. *Biol Trace Elem Res* 2000; 74: 55-70.
5. Ciobanu C, Şlencu BG, Cuciureanu R. *Rev Med Chir Soc Med Nat Iași* 2012; 116:617-623.
6. Crowe A, Morgan EH. Effect of dietary cadmium on iron metabolism in growing rats. *Toxicol Appl Pharmacol* 1997; 145:136-146.
7. Goyer RA. Nutrition and metal toxicity. *Am J Clin Nutr* 1995; 61: 646S - 650S.
8. Gurer H, Ercal N. Can antioxidants be beneficial in the treatment of lead poisoning ?. *Free Radic Biol Med* 2000; 29:927-945.
9. Hatfield DL, Berry MJ, Gladyshev VN editors. *Selenium - Its molecular biology and role in human health, 2nd ed*. New York: Springer, 2006.
10. Hoffmann PR, Berry MJ. The influence of selenium on immune responses. *Mol Nutr Food Res* 2008; 52:1273-1280.
11. Hammouda F, Messaoudi I, El Hani J, Baati T, Said K, Kerkeni A. Reversal of cadmium-induced thyroid dysfunction by selenium, zinc or their combination in rat. *Biol Trace Elem Res* 2008; 126:194-203.
12. Itokawci Y, Abe T, Tanaka S. Bone changes in experimental chronic cadmium poisoning. *Arch Environ Health* 1973; 26: 241-244.
13. Moshtaghie M, Malekpouri P, Dinko MR, Moshtaghie AA. Changes in serum parameters associated with iron metabolism in male rat exposed to lead. *J Physiol Biochem* 2013; 69:297-304.
14. Nehru B, Dua R, Iyer A. Effect of selenium on lead-induced alterations in rat brain. *Biol Trace Elem Res* 1997; 57:251-258.
15. Newairy AA, El-Sharaky AS, Badreldeen MM, Eweds SM, Sheweita SA. The hepatoprotective effects of selenium against cadmium toxicity in rats. *Toxicology* 2007; 242:23-30.
16. Reilly C. *Selenium in food and health, 2nd ed*. New York: Springer, 2006.
17. Saleh HA. Effects of selenium supplementation on the structure of the testis of adult albino rat jubjected to experimentally-induced chronic renal failure - histological and biochemical study. *Egypt J Histol* 2007; 30:383-396.

18. Sivrikaya A, Akil M, Bicer M, Kilic M, Baltaci AK, Mogulkoc R. The effect of selenium supplementation on elements distribution in liver of rats subject to strenuous swimming. *Bratisl Lek Listy* 2013; 114:12-14.
19. Stajn A, Zikic RV, Ognjanovic B et al. Effect of cadmium and selenium on the antioxidant defense system in rat kidneys. *Comp Biochem Physiol* 1997; 117:167-172.
20. Tinggi U. Selenium: its role as antioxidant in human health. *Environ Health Prev Med* 2008; 13:102-108.
21. Todorovic T, Vujanovic D, Dozic I, Petkovic-Curcin A. Calcium and magnesium content in hard tissues of rats under condition of subchronic lead intoxication. *Magnesium Res* 2008; 21:43-50.
22. Xue-Qin H, Man-Tian M. Effect of lead on calcium absorption and bone development in weanling rats. *Acta Academiae Medicinae Militaris Tertiae* 2007-05.

CADMIUM AND LEAD CONTENT IN SOME FOODSTUFFS FROM ROMANIAN MARKET

Constantin Ciobanu[1]*, Bogdan Gabriel Slencu[1], Stelian Ciobanu[2], Rodica Cuciureanu[1]

"Grigore T. Popa" University of Medicine and Pharmacy - Iasi, Universităţii Street No. 16, 700115, Iasi, Romania, [1] - School of Pharmacy;

"Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine, M. Sadoveanu Alley No. 3, 700490, Iasi, Romania, [2] – School of Veterinary Medicine;

* - correspondence: costy_pharm@yahoo.com

Abstract

The existence of toxic metals in the enviromnent and food chain represents a serious problem for most of the countries around the world. As part of their diet, beside essential metals, humans also ingest toxic elements. Cadmium and lead are associated with various health risks such as: cardiovascular diseases, impaired neurobehavioural functioning in children, decrements in intelligence quotient, renal tubular dysfunction, etc. In this study we determined the levels of cadmium and lead in 135 samples of different foodstuffs collected from the northeastern romanian markets between 2010 and 2011. The samples were analysed using an atomic absorption spectrometer with an air-acetylene flame. The chosen food items represent the most commonly consumed foodstuffs in Romania. The highest concentrations for the two metals were obtained in offal sausage (0.116 mg/kg for lead and 0.049 mg/kg for cadmium) and in pork pâté (0.094 mg/kg for lead and 0.054 mg/kg for cadmium). On the other hand, the lowest concentrations of cadmium were found in hard cheese samples (less than the detection limit).

Keywords: *cadmium, lead, foodstuffs*

Introduction

Environmental pollution with toxic metals, such as cadmium and lead represents a worldwide problem for the human health (Rajaganapathy et al., 2011).These toxic metals are neither physiologically nor biochemically essential to the human or animal organism, however they have a significant impact on living organisms (Nordberg et al., 2007; Skerfving and Bergdahl, 2007).

The toxic elements of chemical nature present in food are a heterogeneous group of compounds that derive from various sources. The most common toxic metals from the environment that are found everywhere include lead and cadmium (Munoz et al., 2005). The emergence of ecosystem components contaminated with these toxic metals results from the rapid industrial growth, the progress in the chemical industry or from other human activities. Although they are constituents that are naturally found in earth's crust, these metals can originate from various anthropogenic activities such as: burning fossil fuels, paint industry, mining and siderurgical activities, the use of fertilizers and metal-based pesticides, industrial emissions, harvesting and storage processes and irrigation of crops with contaminated water (EFSA, 2009, 2010).

The amount of metal ingested by humans is closely related to their food habits and of course to the foodstuffs' metal content. The concentration of metals in food depends on the soil characteristics such as the pH, the content of organic matter, etc., which can influence the elements' bioavailability (Wasserman, 1998). Heavy metal pollution becomes an unavoidable problem of our days. Air, soil and water pollution contribute to the emergence of harmful elements such as cadmium and lead in the feed. All these factors have led to the spread of

toxic metals in the environment and consequently to impaired health of the population through consumption of polluted foodstuffs (Zukowska et al., 2008).

Fresh vegetables and fruits represent a very important part of a human's diet because of their vitamins and esential trace elements content. However, these plants as well as other foodstuffs contain both essential and toxic metals in different concentrations (Radwan and Salama, 2006). Toxic metals released from various mining activities could penetrate the soils where crops are grown. Therefore vegetables, cereals and fruits cultivated on contaminated soil can take up cadmium and lead, thus contamining the food chain and this can cause hazardous effects on human health (Luo et al., 2006).

Nutritional deficiencies increase the risk of exposure to lead and cadmium from the consumption of food by increasing the absorption and toxicity of the two metals. Metals that can influence plasma concentrations of lead and cadmium are calcium, iron or zinc. Although the basic role of nutritionally essential metals is to provide the necessary elements in a vital biochemical or enzymatic reaction, some metabolic interactions between essential and toxic metals can reduce the toxicity of these nonessential metals (Goyer, 1997).

In non-occupationally exposed population, diet is the main source of exposure to cadmium and lead (Bocio et al., 2005; Jarup and Akesson, 2009), over-accumulation of these metals in food being associated with various diseases, especially with renal effects, bone damage, neuropsychiatric disorders, etc. (Jarup et al., 1998; Skerfving and Bergdahl, 2007). The kidney is the most affected organ in long term oral exposure to cadmium resulting in various histopathological changes of the renal tissue evidenced by low molecular weight proteinuria, glucosuria and aminoaciduria (Jarup et al., 1998; WHO, 2010). Beside the renal damage, cadmium is also able to induce bone damage. Itai-Itai disease is caused by long-term oral exposure to cadmium and manifests through multiple fractures and distortion of the long bones in the skeleton causing severe pain (Jarup et al., 1998). Cadmium's toxicological properties result from its chemical similarity to zinc, which is an essential metal for both human and animals. Moreover, zinc may be displaced by cadmium from its enzymatic structures therefore interfering with these enzymatic mechanisms (Nordberg et al., 2007).

Cadmium has been classified by IARC (International Agency for Research on Cancer) as "carcinogen to humans (group 1)" (ATSDR, 2008). IARC has also classified lead as being "probably carcinogenic to humans (group 2A)" (ATSDR, 2007).

Apart from its carcinogenity, lead is known to interfere with the haem biosynthesis, thus leading to anemia and most important lead has been associated with impaired neurobehavioural functioning in children and the decrease of intelligence quotient (IQ) (WHO, 2010). Moreover, recent studies sustain the association between low blood lead levels (2-10μg/dL) and neuropsychiatric disorders such as attention deficit hyperactivity disorder and antisocial behavior (Bellinger, 2008; Nicolescu et al., 2010). Because of the persistent and cumulative properties, as well as the potential toxic effects of cadmium and lead as a result of consumption of foodstuffs, there is a need to analyse these food items and determine their content in toxic metals.

The purpose of the present study was to determine the levels of cadmium and lead in various foodstuffs romanian markets.

Materials and methods

Sampling

A total of 135 food samples consisting of different fruits, vegetables and other foodstuffs were purchased from various markets across the northeastern Romania in 2010 and 2011. In order to quantify the content of cadmium and lead in each purchased foodstuffs, multiple samples (3 to 10) were taken from different locations. The final sample consisted of three subsamples which were homogenized prior to analysis. The parts of the samples that were mechanically damaged or rotten, were removed and only the edible parts of the samples were used for analysis. In order for the determination to be more accurate, the samples were finely chopped into smaller pieces to create the same conditions as the ingestion.

Reagents

The reagents used for the digestion of the samples were of analytical grade. Purified water was used for all dilutions. All the glassware was cleaned using a 10% nitric acid solution and then rinsing them with purified water to prevent contamination. In order to prepare the calibration curves, element stock solutions (1000 μg/ml) were provided by Inorganic Ventures (Madrid, Spain).

Apparatus

A high resolution continuum source atomic absorbtion spectrometer (ContrAA 300, Analytic Jena, Germany) was used to carry out the determinations. The content of cadmium and lead was determined using an air-acetylene flame. The wavelenghts for cadmium and lead were 228.8018 nm and 217.005 nm respectively. The length of the flame burner was 100 mm and the gas mixture flow was 50 L/h for cadmium and 65 L/h for lead. All the instrumental parameters were optimized according with the manufacturer's recommendations.

Sample preparation

In order to mineralise the samples, the dry ashing method was used. 20 to 30 grams of homogenized samples were left for 12 hours at a temperature of 105 ºC using a muffle furnace (Nabertherm, Germany). After that the temperature was increased with 50 ºC/h up to 450 º C until a white or grey ash residue was obtained. 5 ml HNO_3 65% were added over the obtained residue and maintained on a sand bath at a temperature of approximately 150 ºC in order to dissolve the remaining ash. The solution was filtered and brought up to a volume of 25 ml with purified water. A blank digest was carried out in the same way.

Statistical analysis

The statistical analysis of the results was performed using Microsoft Excel 2007. Values were expressed as mean ± standard deviation.

Results and discussion

European Commission issued Regulation 1881/2006 (EC, 2006) that sets maximum levels for different pollutants including lead and cadmium. This was done to ensure the food safety and of course the consumer's health.

The main objective of this study was to determine the levels of cadmium and lead in commonly ingested foodstuffs in northeastern part of Romania. Cadmium and lead average concentrations in the selected foodstuff samples are presented in Table 1 and Table 2. The

results are expresed as mean concentrations (mg/kg) ± standard deviation, the minimum and maximum values of cadmium and lead. In analysed foodstuffs the levels of lead were found to be the highest while cadmium levels were the lowest (fig. 3).

Cadmium was found in 90% of the analysed samples. The distribution of cadmium in the positive samples was: 58% in samples of plant origin foodstuffs, 18% in animal origin foodstuffs and 14% in sweets samples (fig. 1). With respect to the lead content, 89% of the analysed food items were positive: 53% in samples of plant origin foodstuffs, 20% in animal origin foodstuffs and 16% in sweets samples (fig 2).

The mean cadmium content in the analysed food items varied from concentrations lower than the method's detection limit in the case of hard cheese and concentrations of 0.054 mg/kg in pork pâté, 0.049 mg/kg in offal sausage and 0.040 mg/kg in mushrooms (*Agaricus bisporus*) and beetroot.

Table 1. Cadmium concentrations in some foodstuffs from the romanian market

Nº.	**Foodstuff**	**Nº. of analised samples / nº. of positive samples**	**Minimum/ maximum value (mg/kg)**	**Mean value ± Standard deviation (mg/kg)**
1	Red bell pepper	6 / 6	0.004 / 0.019	0.011 ± 0.005
2	Garlic	6 / 6	0.010 / 0.084	0.035 ± 0.035
3	Onion	7 / 7	0.004 / 0.071	0.018 ± 0.024
4	Apples	7 / 4	0.003 / 0.015	0.009 ± 0.004
5	Grapefruit	4 / 4	0.003 / 0.013	0.010 ± 0.004
6	Beetroot	4 / 4	0.013 / 0.059	0.040 ± 0.021
7	Broccoli	7 / 5	0.012 / 0.025	0.019 ± 0.005
8	Cabbage	8 / 8	0.004 / 0.008	0.006 ± 0.001
9	Kiwi	4 / 4	0.006 / 0.090	0.028 ± 0.040
10	Lemons	4 / 4	0.007 / 0.018	0.010 ± 0.005
11	Canned corn	3 / 3	0.004 / 0.059	0.022 ± 0.031
12	Beans	4 / 4	0.006 / 0.062	0.020 ± 0.027
13	Rice	7 / 7	0.013 / 0.066	0.039 ± 0.023
14	Mushrooms (*Agaricus bisporus*)	9 / 8	0.007 / 0.078	0.040 ± 0.032
15	White bread	4 / 4	0.006 / 0.030	0.017 ± 0.009
16	Hard cheese	2 / 0	< LOD*	< LOD*
17	Pork pâté	5 / 5	0.019 / 0.070	0.054 ± 0.020
18	Offal sausage	8 / 8	0.021/ 0.085	0.049 ± 0.027
19	Beef	8 / 6	0.008 / 0.090	0.024 ± 0.032
20	Caviar salad	7 / 5	0.004 / 0.029	0.014 ± 0.009
21	Cocoa cake	7 / 7	0.007 / 0.018	0.013 / 0.003
22	Sesame and caramel stick	3 / 3	0.026 / 0.034	0.029 ± 0.004
23	Candies - cherry	3 / 1	0.009 / 0.009	0.009 ± 0.000
24	Crackers	5 / 5	0.008 / 0.032	0.020 ± 0.010
25	Crackers + cacao cream	3 / 3	0.018 / 0.037	0.026 ± 0.009

* Limit of detection

Table 2. Lead concentrations in some foodstuffs from the romanian market

N°.	**Foodstuff**	**N°. of analised samples / n°. of positive samples**	**Minimum/ maximum value (mg/kg)**	**Mean value ± Standard deviation (mg/kg)**
1	Red bell pepper	6 / 4	0.018 / 0.036	0.027 ± 0.007
2	Garlic	6 / 4	0.018 / 0.092	0.048 ± 0.036
3	Onion	7 / 6	0.018 / 0.085	0.043 ± 0.031
4	Apples	7 / 6	0.017 / 0.040	0.025 ± 0.008
5	Grapefruit	4 / 4	0.017 / 0.039	0.027 ± 0.010
6	Beetroot	4 / 3	0.039 / 0.093	0.061 ± 0.028
7	Broccoli	7 / 6	0.019 / 0.091	0.041 ± 0.030
8	Cabbage	8 / 7	0.018 / 0.058	0.041 ± 0.013
9	Kiwi	4 / 3	0.028 / 0.060	0.047 ± 0.016
10	Lemons	4 / 3	0.019 / 0.068	0.036 ± 0.027
11	Canned corn	3 / 3	0.025 / 0.084	0.055 ± 0.029
12	Beans	4 / 4	0.018 / 0.077	0.043 ± 0.030
13	Rice	7 / 7	0.050 / 0.107	0.082 ± 0.021
14	Mushrooms (*Agaricus bisporus*)	9 / 9	0.020 / 0.109	0.070 ± 0.026
15	White bread	4 / 3	0.018 / 0.037	0.029 ± 0.009
16	Hard cheese	2 / 0	< LOD*	< LOD*
17	Pork pâté	5 / 5	0.066 / 0.139	0.094 ± 0.030
18	Offal sausage	8 / 8	0.064 / 0.157	0.116 ± 0.032
19	Beef	8 / 8	0.013 / 0.106	0.077 ± 0.029
20	Caviar salad	7 / 6	0.035 / 0.081	0.059 ± 0.018
21	Cocoa cake	7 / 7	0.022 / 0.037	0.031 / 0.004
22	Sesame and caramel stick	3 / 3	0.044 / 0.100	0.064 ± 0.031
23	Candies - cherry	3 / 3	0.037 / 0.073	0.050 ± 0.019
24	Crackers	5 / 5	0.059 / 0.109	0.086 ± 0.021
25	Crackers + cacao cream	3 / 3	0.072 / 0.090	0.083 ± 0.010

* Limit of detection

In all the samples analysed, the highest concentrations of cadmium were found in animal origin foodstuffs (pork pâté and offal sausage) compared to sweets samples and plant origin samples. The offal sausages are made of organ parts such as kidneys, spleen and liver. These organs contain high amounts of cadmium because this metal is accumulated especially at renal and hepatic level (Satarug et al., 2000). Cadmium levels found in vegetable and fruit samples ranged between 0.006 mg/kg in cabbage, 0.009 mg/kg in apples and 0.039 mg/kg in rice, 0.040 mg/kg in mushrooms and beetroot.

The mean lead leveles in the analysed food samples varied from nondetectable concentrations in hard cheese and higher concentrations which were found in offal sausages (0.116 mg/kg) and pork pâté (0.094 mg/kg). With respect to the level of lead found in fruits, vegetables and cereals, we found lower concentrations in apples (0.025 mg/kg), red bell pepper, grapefruit (0.027 mg/kg) and higher concentrations in rice (0.082 mg/kg), mushrooms (0.070 mg/kg) and beetroot (0.060 mg/kg).

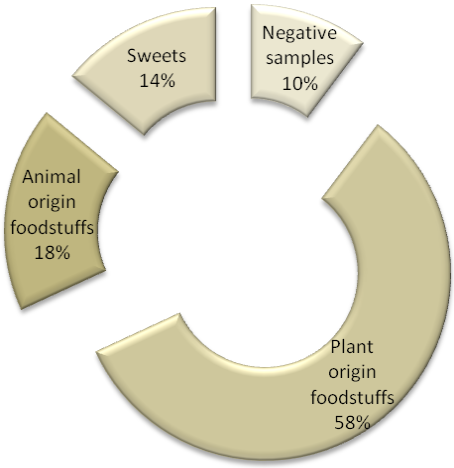


Fig. 1 Cadmium distribution in analyzed samples

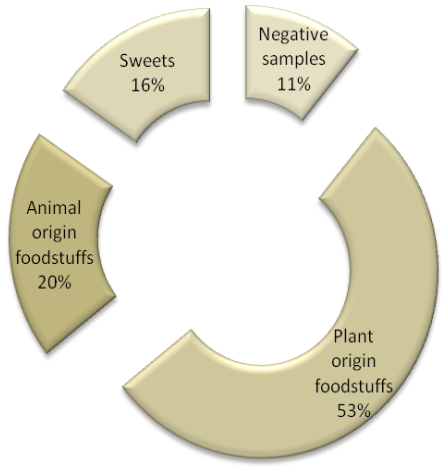


Fig. 2 Lead distribution in analyzed samples

Leblanc et al., in 2005, carried out a study called The First French total diet study. The cadmium values found for rice (0.003 mg/kg), crackers (0.001 mg/kg), bread (0.004 mg/kg), vegetables (0.0108 mg/kg) and fruits (0.0018 mg/kg) were lower compared to the result from our study. The level of cadmium found in offal sausages from our study is similar to the value found in the french study (0.051 mg/kg). Regarding the concentrations of lead found by Leblanc et al. (2005), the levels from the french study are smaller for rice (0.005 mg/kg), crackers (0.019 mg/kg), offals (0.055 mg/kg), vegetables (0.015 mg/kg) and fruits (0.010 mg/kg). The levels of lead in bread found in our study are comparable with those found by Leblanc et al. (0.026 mg/kg).

Another study made by Duran et al. (2009) in Turkey presents the levels of cadmium and lead in candies. They found very high concentrations of metals in candies: cocoa based candies (1.34 mg/kg for lead and 0.681 mg/kg for cadmium), sugar based candies (1.04 mg/kg for lead and 0.207 mg/kg for cadmium) and fruit bases candies (0.473 mg/kg for lead and 0.172 mg/kg for cadmium).

Compared to the turkish study, we obtained very low concentrations in candies (0.050 mg/kg for lead and 0.009 mg/kg for cadmium).

This study reveals that, for the general population from northeastern Romania, the observed levels of the determined metalic contaminants are satisfactory, so the probability of exposure or health risks from such elements is small.

The highest cadmium and lead concentrations found in this study are below the maximum permissible limits set by the Europeean Commission (EC, 2006), therefore the consumer's health probably will not be affected.

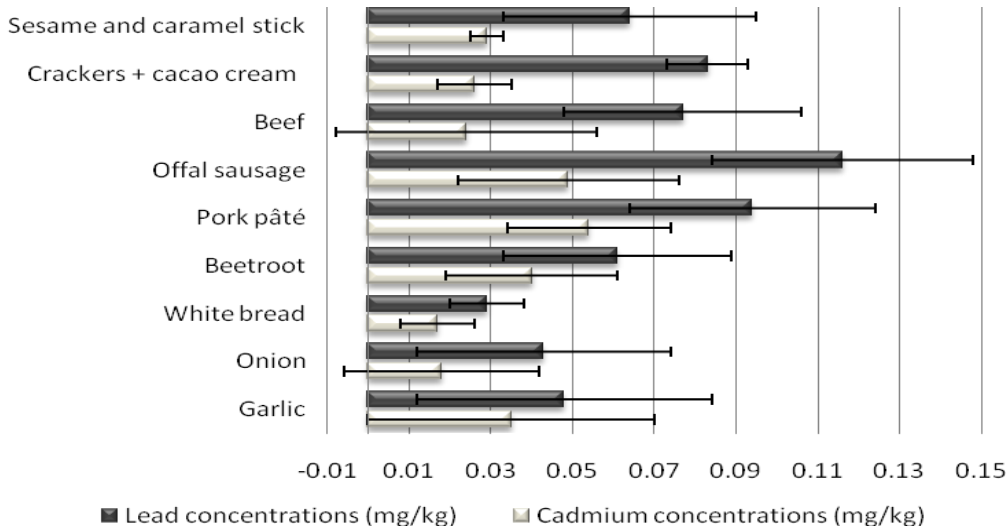


Fig. 3 Comparative results regarding lead and cadmium levels in some analysed foodstuffs

Conclusions

1. This study presents a small insight into the concentrations of two toxic metals (cadmium and lead) in foodstuffs comercialized or produced in the northeastern Romania.
2. The results obtained in this study highlight the fact that the levels of cadmium and lead in analysed foodstuffs were in compliance with the current europeean legislation.
3. The study also underlines that there is no concern about the intake of lead and cadmium through various foodstuffs regarding the general population.
4. The method used for the determination of cadmium and lead (flame atomic absorption spectrometry) is fast, accurate and not too expensive.

Acknowledgements

This work was developed within the European Social Fund project POSDRU 2007-2013 - "Burse doctorale pentru cresterea competitivităţii in domeniul medical si farmaceutic", project id POSDRU/88/1.5/S/58965.

References

1. *** Agency for Toxic Substances and Disease Registry (ATSDR) (2008). Toxicological profile for cadmium. Atlanta: Agency for Toxic Substances and Disease Registry.
2. *** Agency for Toxic Substances and Disease Registry (ATSDR) (2007). Toxicological profile for lead. Atlanta: Agency for Toxic Substances and Disease Registry.
3. *** World Health Organization (WHO) (2010). Seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain food additives and contaminants. *World Health Organization* 2010, 149-177.
4. Bellinger DC (2008). Very low lead exposures and children's neurodevelopment. *Curr Opin Pediatr* ; 20(2): 172-177.

5. Bocio A, Nadal M, Domingo JL (2005).Human exposure to metals through the diet in Tarragona, Spain: temporal trend. *Biol Trace Elem Res* 2005;104:193 – 201.
6. Duran A, Tuzen M, Soylak M (2009). Trace metal contents in chewing gums and candies marketed in Turkey. *Environ Monit Assess*; 149(1-4): 283-289.
7. EC, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union L364, 0005–0024.
8. *** European Food Safety Authority (2009). Scientific Opinion of the Panel on Contaminants in the Food Chain. Cadmium in food. The EFSA Journal, 980, 1-139.
9. *** European Food Safety Authority (2010). Scientific Opinion of the Panel on Contaminants in the Food Chain. Lead in food. The EFSA Journal, 8(4), 1-147.
10. Goyer AR (1997). Toxic metals and essential metal interactions. *Annu Rev Nutr*; 17: 37–50.
11. Jarup L, Akesson A (2009). Current status of cadmium as an environmental health problem. *Toxicol Appl Pharm* ; 238: 201–208.
12. Jarup L, Berglund M, Elinder CG et al (1998). Health effects of cadmium exposure - a review of the literature and a risk estimate. *Scand J Work Environ Health* ; 24: 1-52.
13. Leblanc JC, Guerin T, Noel L (2005). Dietary exposure estimates of 18 elements from the 1st French Total Diet Study. *Food Addit Contam* ; 22: 624-641.
14. Luo C, Shen Z, Lou L et al (2006). EDDS and EDTA-enhanced phytoextraction of metals from artificially contaminated soil and residual effects of chelant compounds. *Environ Pollut* ; 144: 862–871.
15. Munoz O, Bastias JM, Araya M et al (2005). Estimation of the dietary intake of cadmium, lead, mercury, and arsenic by the population of Santiago (Chile)using a Total Diet Study. *Food Chem Toxicol* ; 43: 1647 – 1655.
16. Nicolescu R, Petcu C, Cordeanu A et al (2010). Environmental exposure to lead, but not other neurotoxic metals, relates to core elements of ADHD in Romanian children: performance and questionnaire data. *Environ Res*; 110(5): 476-483.
17. Nordberg GF, Nogawa K, Nordberg M et al (2007). Cadmium. In: Nordberg GF, Fowler BA, Nordberg M, Friberg LT (eds.). *Handbook on the Toxicology of Metals – 3rd edition*. New York: Academic Press, 445-486.
18. Radwan MA, Salama AK (2006). Market basket survey for some heavy metals in Egyptian fruits and vegetables. *Food Chem Toxicol* ; 44: 1273–1278.
19. Rajaganapathy V, Xavier F, Sreekumar D et al (2011). Heavy metal contamination in soil, water and fodder and their presence in livestock and products: a review. *J Environ Sci Technol* 2011; 4(3): 234-249.
20. Skerfving S, Bergdahl IA. Lead ()2007. In: Nordberg G.F., Fowler B.A., Nordberg M., Friberg L.T (eds.). *Handbook on the Toxicology of Metals – 3rd edition*. New York: Academic Press, 599-635.
21. Wasserman MAM (1998). Soil-to-plant transfer of ^{137}Cs related to its geochemical partitioning in oxisols of tropical areas. In: Wasserman JC, Silva-Filho EV, Villas-Boas R (eds.). *Environmental geochemistry in the tropics*. Berlin: Springer, 17-28.