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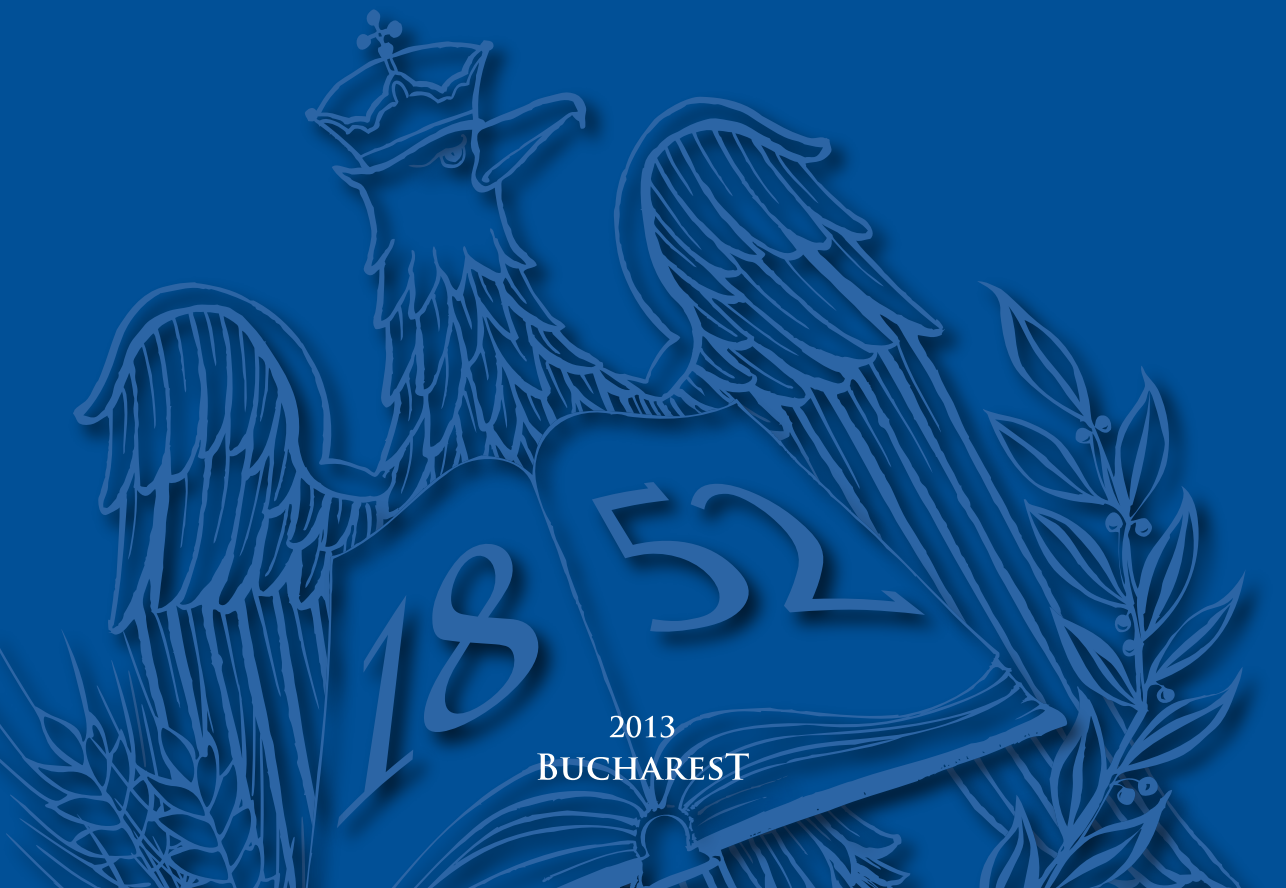

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SUMMARY

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COMPARATIVE ANTIGEN TESTING FOR AN ELISA DIAGNOSTIC METHOD IN OVINE PARATUBERCULOSIS

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Abstract

In this paper it was conducted a study on four types of antigens prepared from Mycobacterium avium subspecies paratuberculosis cultures in order to build an indirect ELISA method with diagnostic sensitivity as high as possible. The optimization of the method's parameters method was developed together with the interpretation's identifications of the cut-off limit. It was found that the antigen Ag 2, is most appropriate for detecting the level of antibodies in sheep serums, is followed that performant by the antigen Ag 3. In testing the serums of 60 sheeps the antigen Ag 2, obtained by molecular filtration, resulted in a diagnostic accuracy of 78.83% compared with the method of commercial kits.

Key words: *antigens, ELISA, paratuberculosis, serums, sheep.*

INTRODUCTION

Paratuberculosis (John's disease) is caused by *Mycobacterium avium subspecies paratuberculosis*, and it remains an important issue for animal and human health. Paratuberculosis diagnosis is performed in two stages: clinical diagnosis and detection of subclinical infections (Samarineanu M. et al., 1996). Determination of subclinical infections are the most important in the prevention and control of paratuberculosis in livestock, both nationally and internationally.

Currently, ELISA (enzyme-linked immunosorbent assay) is considered the most sensitive and specific method for detection of serum antibodies anti-*Mycobacterium avium subspecies paratuberculosis* in ruminants. Sensitivity is higher than the CFR test (complement fixation reaction) allowing identification of infected subclinical carriers (when there are small amounts of antibodies) and their removal from the livestock. The speed of performing this method allows also, a testing of a large number of serums in a short period of time.

In this paper is presented the testing of several antigens prepared in order to choose a suitable antigen for the ELISA technique that can be applied to the testing of ruminant serum.

MATERIALS AND METHODS

It was prepared four types of antigens in view of their application in ELISA.

Antigen Ag 1 – the antigen was obtained by preparing a fraction rich in protein and carbohydrates from the bacterial corpus. From a culture of *Mycobacterium paratuberculosis*, TEPS strain, on the Reid medium, 13 weeks aged, inactivated by autoclaving for 1 hour at a temperature of 100^{o}C and ultrasonicata, it was retained the supernatant which was precipitated with ammonium sulfate up to 25% saturation. The precipitate was slowly dissolved in a small volume of PBS pH 7.2 and was dialysed against the buffer, the obtained antigen is noted Ag 1.

Antigen Ag 2-This type of antigen was prepared from a culture of *Mycobacterium paratuberculosis*, the 8578 strain, 10 weeks aged. The obtaining technique was adapted from the described method by Jark et al., 1997, as follows: it was sampled the bacterial mass, it was inactivated for 15 minutes at 100^{o}C, it was ultrasonicated for 30 minutes, and the supernatant was treated with proteinase K in a Tris-EDTA buffer and subsequently passed through a filter with an exclusion limit of 30,000 daltons.

Antigen Ag 3 – The antigen was prepared according to the method of Milner et al., 1990, as follows: the culture of *Mycobacterium paratuberculosis*, the 8578 strain, on the Reid medium, 12 weeks aged, washed with PBS, was

inactivated by autoclaving. After ultrasonication and centrifugation, the bacterial extract was ultracentrifugated at 10000xg, for 30 minutes. The supernatant was retained as antigen Ag 3.
Antigen Ag 4-This antigen was prepared according to the method described by Molina et al., 1991, with modifications: the culture of *Mycobacterium paratuberculosis*, the 8578 strain, on the Reid medium, 12 weeks aged, washed with PBS, was inactivated by tyndallization 7 hours at 56^{o}C. After centrifugation the supernatant was retained as antigen Ag 4.
The immunoenzymatic assay (ELISA) applied was an indirect type, composed by the following steps:
- The antigen microplate lining: the obtained Mycobacterium paratuberculosis antigens is fixed on the wells of the microplates in carbonate buffer pH 9.6.
- The reaction with sheep serums: the serums to be (tested/examined) were adequately diluted in 0.01 M PBS with 1% bovine serum albumin and 0.05% Tween-20. For optimization were used control serums: the positive serum (P) is a cumulative serum from vaccinated sheeps with a commercial vaccine and the negative serum (N) a serum from an animal unvaccinated and from a herd free of paratuberculosis.
- The reaction with anti-IgG sheep conjugate: the conjugate used was a commercial one, coupled with peroxidase.
- The reaction with chromogen and substrate mixture: the chromogen was 2.2 Azino-di (3-ethyl benztiazolin-6-sulfonic acid) (ABTS) in 2.3 g% citrate buffer, pH 4.
- The stopping of the reaction: it was performed with 1.5% sodium fluoride.

RESULTS AND DISCUSSIONS

The total protein composition determination of the prepared antigens showed that Ag 2 has the highest concentration, the obtained values were: 1.66 mg/ml for antigen Ag 1, 3.80 mg/ml for antigen Ag 2, 1.11 mg/ml for antigen Ag 3 and 0.30 mg/ml for Ag 4.
The immunochemical testing, performed by ELISA, aimed the behavior of the reacting antigens with the control serums and afterwards the diagnosis of some serums from livestocks.
In Ag 1 titration it was observed that at the concentration of 10 mg/ml the concurrent conditions of the biggest ratio and the biggest difference between the optical density values (O.D.) of the control serums. The results are presented in table 1.

Table 1. Values OD x 1000 in Ag 1 antigen titration with sheep serum

	Antigen concentration (µg/ml)									
	160	**80**	**40**	**20**	**10**	**5**	**2.5**	**1.25**	**0.63**	**0.32**
Serum positive (P)	2098	1817	1646	1602	1919	1661	1141	1006	916	583
Serum negative (N)	200	210	253	206	181	161	135	99	62	41
Serum difference P-N	1898	1607	1393	1396	1738	1500	1006	907	854	542
Serum ratio P:N	10.49	8.65	6.50	7.77	10.60	10.31	8.45	10.16	14.77	14.21

Using this concentration of antigen it was performed the testing of 132 sheep serums with a 1/200 dilution. The serums came from 4 farms and were previously tested by the CFR technique. The diagnosis was achieved by imposing an interpretation limit value (cut-off limit) equal to 2.1 x OD negative control serum. In the table 2 are presented the comparative diagnoses by the two techniques and the diagnostic accuracy.
It is found a highly variable diagnostic accuracy between 12.90% and 100% the higher consistents are for negative serums. This aspect may improve if it is chosen a cut-off value which takes into account the possible weaker recognitions between serum antibodies and the antigen.

Table 2. Comparative table (ELISA with Ag 1 and CFR) of the diagnosis for 132 sheep serums

	Number samples	ELISA		CFR		diagnostic accuracy
		Positive	Negative	Positive	Negative	
Ferm 1	28	8	20	13	15	60.71%
Ferm 2	31	4	27	31	0	12.90%
Ferm 3	40	0	40	20	20	52.50%
Ferm 4	33	0	33	0	33	100%

For a greater precision is indicated also to be introduced a category of so-called dubious serums. At the same time, also the using of a purified antigen would help to reduce the nonspecific reactions or to distinguish the small quantities of antibodies in some serums, all this causing a safer diagnosis.

The antigen Ag 2 was titrated using citrate buffer saline pH 6 for fixation, the results are presented in table 3. It is noted that at the concentration of 2.5 μg/ml it is registered the optimal ratio and difference between the control serums.

With this antigen, applied at the above concentration, were tested 12 sheep serums previously diagnosed also by ELISA with the IDEXX kit for ruminants. It was obtained a diagnostic accuracy of 83.33% applying a cut-off limit = 0.2 x OD positive control serum. The more elaborate purification of the antigen and changing the lining conditions led to a variant of the method with high sensitivity and specificity.

The antigens Ag 3 and Ag 4 were simultaneously tested at a concentration of 10 μg/ml, using the sheep control serums with 8 dilutions. The results are presented in table 4.

It was observed that antigen Ag 3, at the 1/200 serum dilution, has the ratio and the difference between the most representative OD values. The antigen Ag 4 showed no appropriate reaction to any serum dilution. The antigen Ag 3 was titrated in 8 decreasing concentrations (from 160 to 1.25 μg/ml) in order to find the optimal concentration for microplate fixation, and the appropriate serum dilution. The data presented in table 5 have revealed the optimal concentration of 10 μg/ml antigen and the optimal dilution of 1/200 for serum.

Table 3. Values DO x 1000 in antigen Ag 2 titration with sheep serums

	Antigen concentration (μg/ml)							
	20	**10**	**5**	**2,5**	**1,25**	**0,62**	**0,31**	**0,15**
Serum positive (P) 1/200	138	417	589	886	835	518	486	522
Serum negative (N) 1/200	63	78	111	140	114	102	133	201
Serum difference P-N	75	339	478	746	721	416	356	321
Serum ratio P:N	2.19	5.34	5.30	6.32	7.32	5.07	3.65	2.59

Table 4. OD values (x1000) in sheep serums titration against antigens Ag 3 and Ag 4

		Dilution sheep serum							
		1/25	**1/50**	**1/100**	**1/200**	**1/400**	**1/800**	**1/1600**	**1/3200**
Antigen Ag 3	Serum positive (P)	3029	2632	2712	2074	1547	899	376	206
	Serum negative (N)	918	591	329	174	109	61	34	18
	Serum difference P-N	2111	2041	2383	1900	1465	838	342	188
	Serum ratio P:N	3.29	4.45	8.24	11.91	14.44	14.73	11.05	11.44
Antigen Ag 4	Serum positive (P)	896	554	418	329	194	101	57	28
	Serum negative (N)	411	233	153	76	43	21	15	3
	Serum difference P-N	485	321	265	253	151	80	42	25
	Serum ratio P:N	2.18	2.37	2.73	4.32	4.51	4.80	3.80	9.33

Table 5. OD values (x 1000) in antigen Ag 3 with sheep serum in 3 dilutions

	Antigen concentration (μg/ml)							
	160	**80**	**40**	**20**	**10**	**5**	**2.5**	**1.25**
Serum positive (P) dilution 1/100	2968	2935	2933	2983	2995	3008	2948	2767
Serum negative (N) dilution 1/100	559	554	627	702	680	524	492	402
Serum difference P-N	2409	2381	2306	2281	2315	2484	2456	2365
Serum ratio P:N	5.30	5.29	4.67	4.24	4.40	5,74	5,99	6.88
Serum positive (P) dilution 1/200	1766	1762	1862	1999	1953	1779	1498	1083
Serum negative (N) dilution 1/200	111	117	125	134	142	275	124	105
Serum difference P-N	1655	1645	1737	1865	1811	1504	1374	978
Serum ratio P:N	15.90	15.05	14.89	14.91	13.75	6.46	12.08	10.31
Serum positive (P) dilution 1/400	568	625	632	794	913	792	575	390
Serum negative (N) dilution 1/400	17	18	24	34	33	38	28	23
Serum difference P-N	551	607	608	760	880	754	547	367
Serum ratio P:N	33.41	34.72	26.33	23.35	27.66	20.84	20.53	16.95

Using Ag 2 and Ag 3 and applying a cut-off limit = 0.2 x OD positive control serum 60 sheep serums were tested previously diagnosed with IDEXX kit. The diagnostic accuracy was 68.33% with Ag 2 and 63.33% with Ag 3.

The 60 sheep serums were compared again regarding the diagnosis applying also a different calculation method for the cut-off limit. Thus, it was calculated a ratio for each sample S (sample) in relation to positive serum, noted S / P% and the interpretation meant the declaration of the serums as: Negative = S / P < 25%, Doubtful = 25% < S / P < 30%, Positives = S / P> 30%. In this way it was found that a better accuracy was obtained for antigen Ag 2, ie 78.83%, where only 13 out of 60 serums had the same diagnosis at IDEXX kit. The accuracy of the method with Ag 3 was 71.66%. Using the MedCalc software the AUC areas were calculated (area under ROC curve) in each testing with the corresponding antigen. ROC curve (receiver operator characteristic curve) is a means of expressing the relationship between sensitivity and specificity of a diagnostic test. Thus, the method with antigen Ag 2 had a value of 0.912, and an estimated cut-off limit of 29.9% (figure 1) and the Ag 3 method had a smaller AUC value of 0.850, and the estimated cut-off limit of 31.5% (figure 2). So the ELISA technique with Ag 2, with a cut-off limit S / P of 30% is more adequate for comparative diagnosis than antigen Ag 3 method.

Milner et al., 1990, who used a type 3 antigen in a ELISA assay on 327 cow serums sampled from a herd infected, obtained a specificity of 98.9%, but the sensitivity varied depending on the stage of the disease in the sense that animals in an early stage could not be detected by the testing. And Molina et al., 1991, who performed an ELISA assay with a type 4 antigen, showed that the clinical signs of the disease appear before the revealing of the serum antibodies.

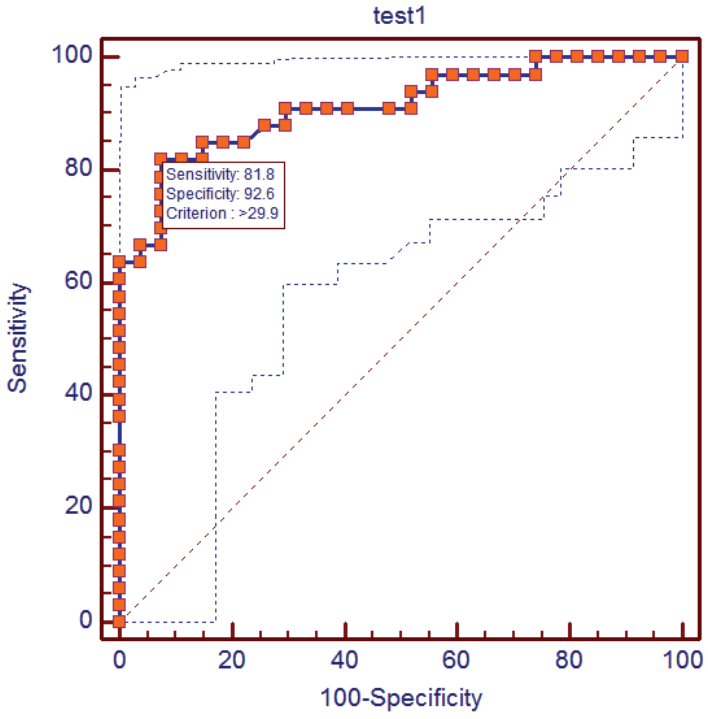

Figure 1. Graph ROC for serum antigen Ag 2 testing (MedCalc software)

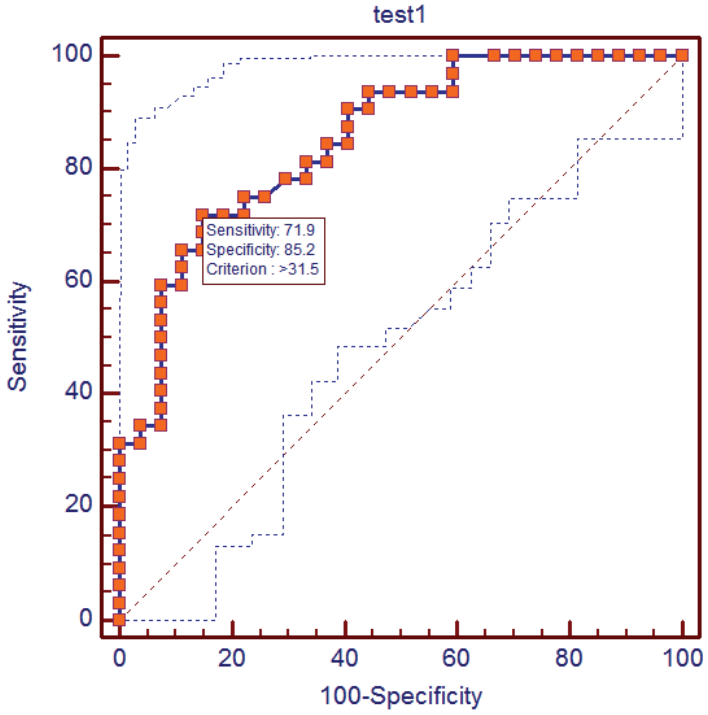

Figure 2. Graph ROC for serum antigen Ag 3 testing (MedCalc software)

CONCLUSIONS

Four types of antigens obtained from cultures of *Mycobacterium paratuberculosis* were prepared and tested by indirect ELISA technique. It was found that the antigen Ag 2, obtained by advanced purification is the most appropriate for detecting the level of antibodies in sheep serums, followed by the Ag 3 antigen performance.

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RESEARCH OF ANTIMICROBIAL EFFECTS OF PROPOLIS FROM PROVINCE OF ORDU

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Abstract

Propolis is a resinous product collected by bees used in their hives to have a safe place. Bees provide it from various plants. It is rich in terms of phenolic compounds so it is very important for the role in contributing to human health. In this study, antimicrobial effects of ethanol extract of propolis were determined against Escherichia coli, Streptococcus mutans, Pseudomonas aeruginosa, Listeria monocytogenes, Candida albicans and Aspergillus niger using disc-diffusion and agar dilution method. According to the results, propolis showed antimicrobial activity against Pseudomonas aeruginosa, Streptococcus mutans, Listeria monocytogenes and Candida albicans. The most sensitive microorganism was Pseudomonas aeruginosa to propolis. This study offers that propolis may provide an alternative to chemical preservatives against several diseases.

Key words: *Antimicrobial, propolis, Ordu.*

INTRODUCTION

Propolis is a strongly adhesive, resinous substance collected, transformed and used by bees to seal holes in their honeycombs. It smooths out the internal walls and protects the entrance against intruders. Honeybees (*Apis mellifera* L.) collect the resin from the cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes added and the partially digested material is mixed with beeswax and used in the hive (Ghisalberti, 1979; Marcucci et al., 1996; Burdock, 1998).

Propolis is extensively used in folk medicine and a number of investigations have shown that propolis have antimicrobial and antiviral properties (Mirzoeva et al., 1997; Park et al., 1998; Kujumgiev et al., 1999; Hegazi and El Hady, 2001; Ota et al., 2001; Kartal et al., 2003; Güler et al., 2003; Prytzyk et al., 2003). The resin contains most of the compounds found in alcohol extracts consumed by people from many countries as food complements or alternative medicine (Gabrys et al., 1986; Marcucci et al., 1996).

It has been shown that there were variations in the antimicrobial activity according to the propolis origin (Hegazi and El Hady, 2001; Stepanovic, 2003). The constituents of propolis vary widely due to climate, season, location and year. Its chemical formula is not stable (Ghisalberti, 1979; Cheng and Wong, 1996). The most important pharmacologically active constituents in propolis are? avonoids (? avones,? avonols,? avononeS), phenolics, and aromatics. Flavonoids are thought to account for much of the biologic activity in propolis (Uzel et al., 2005).

A great enthusiasm characterizes present-day propolis research, driven by positive results in pharmacological tests, dealing not only with antimicrobial activity, the first (Lavie, 1960) and as yet the most investigated effect in propolis research, but also with a wide diversity of effects, including immune activation and cytotoxicity (Banskota et al., 2001).

Turkey, which is the fourth largest honey producing country in the world, has a rare mix of suitable conditions for beekeeping. Turkey has both European and Asian flora characteristics, enriching the bee products, such as honey, pollen and propolis (Aliyazicioglu et al., 2013).

The present study was designed to determine the antimicrobial activities of propolis gathered from Ordu province of Turkey.

MATERIALS AND METHODS

Propolis Sample and Preparation of Extract

Propolis sample in the form of hard lumps were collected from Ordu province of Turkey during

October and November 2012. The crude sample was stored in air-tight glass container in dark at-20°C until used. Propolis extract was prepared by stirring 30 g samples in 150 ml of 95% ethanol at room temperature and the extract was kept at 4°C for a week. The extract was filtered through 45 µm membrane filter and then the solution was dried with an evaporator. The crude extract was stored at-20°C until used.

Test Strains and Culture Media

Strains of bacteria and fungi were obtained from ATCC (American Type Culture Collection, Rockville, USA). Antimicrobial activities of propolis extract sample was assayed against *Escherichia coli* ATCC 25922, *Streptococcus mutans* ATCC 25175, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7677, *Candida albicans* ATCC 25922 and *Aspergillus niger* ATCC 9642. The species of bacteria were grown in Mueller Hinton Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and Mueller Hinton Broth (Merck Co., Darmstadt, Germany). The species of fungi were grown in Sabouraud Dextrose Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and Sabouraud Dextrose Broth (Difco Laboratories, Detroit, MI, USA). The concentrations of bacterial suspensions were adjusted to 10^8 cells/ml, while those of fungal suspensions to 10^7 cells/ml.

Antibacterial and Antifungal Assay

Antibacterial and antifungal activity were measured using methods of diffusion disc plates on agar (Ronald, 1990). In order to test antibacterial and antifungal activity, the fractions of propolis sample was dissolved in ethanol. Mueller Hinton Agar medium (20 ml) for bacteria and Sabouraud Dextrose Agar (20ml) for fungus were poured into a 15 cm petri dish. All bacterial strains were grown in Mueller Hinton Broth medium for 24 h, at 37°C and the fungal strains were grown in Sabouraud Dextrose Broth at 27°C for 48 h. Growth was adjusted to 600 nm of 0.1 by dilution with Mueller Hinton Broth medium for bacteria and Sabouraud Dextrose Broth for fungi. Suspension (100 µl) with approximately 10^8 microorganisms per milliliter was placed in petri dishes. Then, sterile paper discs (6 mm in diameter) were placed on the agar to load 15 µl of the sample (20 mg/ml). One hundred units of nystatin for fungus, ampicillin and cephazolin for bacteria, all obtained from a local pharmacy, were used as a positive control and ethanol as a negative control. Inhibition zones were determined after incubation at 37°C for 24 h for bacterial tests and 27°C for 48 h for fungal tests. All tests were made in triplicates (Aliyazicioglu et al., 2013).

Minimum Inhibition Concentration

The agar dilution method was used for the antimicrobial screening with slight modifications (Vanden Berghe and Vlietinck, 1991). Instead of 96 well microtiter plates 24 well tissue culture (Corning Costar Co., Corning, NY, USA) plates were used. The crude propolis extract was dissolved in ethanol and physiological tris buffer (1:4) and mixed with an equal amount of 3% agar solution at 45°C to a final concentration of 10, 5, 2.5 and 1.25 mg of extract/ml. An amount of 400 µl from the solution was transferred into each well of the tissue culture (Corning) plates. After solidification, each well was inoculated with 10 µl of freshly prepared bacterial suspension of 10^8 bacterial/ml and incubated at 37°C for 24 h. Ampicillin and cephazolin for bacteria and nystatin for fungi, were used at (1.25-10 mg/ml) as positive controls. The microbial growth was assessed by a stereo microscope after the incubation period. All tests were made in triplicates (Aliyazicioglu et al., 2013).

RESULTS AND DISCUSSIONS

In the present study, the antimicrobial activity of ethanol propolis extract from Ordu province, was investigated. The antimicrobial activity of propolis extract was initially evaluated by the disc diffusion method using two gram-positive (*S. mutans*, *L. monocytogenes*), two gram-negative bacteria (*P. aeruginosa*, *E. coli*) and two fungi (*C. albicans*, *A. niger*). The results obtained in the disc diffusion assay regarding the growth inhibition zones of the tested microorganisms are shown in Table 1. Propolis showed the highest antibacterial activity against *P. aeruginosa* (25 mm). Some researches reported that ethanolic propolis extracts inhibited *P. aeruginosa* (Uzel et al., 2005; Aliyazicioglu et al., 2013). The antifungal activity was highly showed against *C. albicans*

(18 mm). Also, propolis showed strong inhibitory action against *S. mutans* (19 mm) and *L. monocytogenes* (17 mm), which correlates well with the literature data (Lepekhin and Leonova, 1970; Gebara et al., 1996; Koo et al, 2000; Ophori et al., 2010; Aliyazicioglu et al., 2013). Propolis extract did not show antimicrobial activity against *E. coli* and *A. niger*. However, in the studies of Aliyazicioglu et al. (2013) and Rahman et al. (2010), propolis showed antimicrobial activity against *E. coli*. It is well known that the type of propolis sample may vary highly according to regional and environmental vegetation.

Evaluation of minimum inhibitory concentration of extract by means of agar dilution experiment method is reported in Table 2. The extract of propolis sample required minimum inhibitory concentration of =1.25 mg/ml for *P. aeruginosa* and > 1.25 mg/ml for *S. mutans*.

The most sensitive microorganism to propolis was *P. aeruginosa* in the gram negative group and *S. mutans* in the gram positive group. According to the results, it may not be concluded that, in general which gram group is more susceptible to propolis sample about antimicrobial action. In the study, the least sensitive microorganism was *A. niger*. A control test run with standard antibiotics revealed that propolis sample have a similar or greater inhibitory effect on *C. albicans* and *P. aeruginosa.*

Table 1. Results of antimicrobial screening of the ethanolic propolis extract determined by the disc diffusion method (inhibition zone in mm).

Samples	Microorganisms					
	E.c.	*S.m.*	*P.a.*	*L.m.*	*C.a.*	*A.n.*
Propolis	10	19	25	17	18	8
AMP	15	25	28	23	NT	NT
CEP	15	30	24	33	NT	NT
NYS	NT	NT	NT	NT	15	15
Ethanol	-	-	-	-	-	-

-: no inhibition, NT: Not tested, *E.c: E. coli*, *S.m: S. mutans*, *P.a: P. aeruginosa*, *L.m: L. monocytogenes*, *C.a: C. albicans*, *A.n: A. niger,* Control: AMP: Ampicillin 10 µg. CEP: Cephazolin 30 µg. NYS: Nystatin 100 Units.

Table 2. Results of antimicrobial screening of the ethanolic propolis extract determined by the agar dilution method (minimum inhibitory concentration, in mg/mL).

Sample	Microorganisms					
	E.c.	*S.m.*	*P.a.*	*L.m.*	*C.a.*	*A.n.*
Propolis	> 10	> 1.25	=1.25	> 2.5	> 2.5	NT

NT: Not tested, *E.c: E. coli*, *S.m: S. mutans*, *P.a: P. aeruginosa*, *L.m: L. monocytogenes*, *C.a: C. albicans*, *A.n: A. niger.*

CONCLUSIONS

Antibacterial activity of propolis depends on chemical composition and concentration of the active components and compounds. It has distinctive features that may be beneficial to our health as an antimicrobial since it has important chemical contents such as flavonoids, phenolics and aromatics. The studies show that propolis which is the polyphenolic-rich natural product may provide an alternative to chemical preservatives and it may be used as a source of natural antimicrobial.

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THE FUNCTIONAL STATUS, RESISTANCE AND ADAPTIVE CAPACITIES OF THE CALVES BEING AFFECTED BY COMBINED STRESSORS DURING THEIR EARLY POSTNATALONTHOGENESIS

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Abstract

The experimental studies revealed and analyzed the dynamics, protein and saline metabolism, resistance to stress factors and adaptive capacities (total proteins and protein fractions, urea, macro elements Ca, P, Na, K, content of glucose, bactericide activity, cortisol concentration) of calves in their early postnatal ontogenesis under the combined influence of stresogen thermal maintenance factor, noise and parasitic factors. There were established that the fluctuations of the researched physiological parameters have the phasic character. The tested indices, in major cases were quantitatively lower in the group of animals affected by the combined stressor factors. Two ascends in changing of the indices have been observed: at the 7-8-th and at the 25-30-th days at birth that coincided with critical development periods: immunodeficiency, depression of the stresogen reaction, depression of dominance and retardation. The positive effect has been registered also upon stress resistance and adaptation capacities of the organism.

Key words: *cattle, resistance and adaptive capacities, parasitar factors.*

INTRODUCTION

The environmental factors (optimal, stressors, extremal) have an effect on organism in multiple situations of combination of such and the impact of their application depends on its nature and intensity (Фурдуй Ф. И. and others, 1982, 1985, 1992).

According to data reflected in scientific literature, the complex of factors applied simultaneously in most cases cause aggravation of the deviations of the functional status of the organism, compared to their separated application, especially in the critical periods of early ontogenesis (Доброволъский Л. А., 1982; Фурдуй Ф. И., and others, 1985; Erhan D. C. etc., 2007; Pavaliuc P. P. etc., 2012).

At the same time, the influence of the simultaneous combined factors can be favorable and the effects of the applied combined environmental factors may be of those three types: additive, synergetic and antagonistic. This classification reflects the essence of the effects that could be observed while factors of various nature affect the organism (Антипов В.В. and others, 1980; Фурдуй Ф.И. and others, 1985; Erhan D., Pavaliuc P., Rusu Ş., 2007; Rusu Ş., 2012).

The specialized scientific literature lack data on effects of the combined action of the maintenance stressor factors on the organism of the calves during their early postnatal ontogenesis. The presented study has been targeting on establishment of the functional status, resistance and adaptive capacities of calves in their early postnatal ontogenesis while affected with stressor thermal maintenance factor being combined with other stressors such as excessive noise and parasitic factors.

MATERIALS AND METHODS

The research has been undertaken on the calves of Black-and-White Holland race during their early postnatal ontogenesis and under controllable conditions. On the leash, similarly to the conditions of households where intensive technologies usually applied, the temperature, noise and parasitic factors are the most predominant one that provoke stress in animals. The thermal factor of the stressful intensity combined with a noise of 70-80 db and parasitic agents (*Eimeria spp., Strongyloides*

papillosus) have been applied to calves following the research purpose.

The animals have been placed in the climacteric camera allowing them to adapt to the new conditions during 40-60 minutes, and afterwards the temperature have been decreased up to 5°C. The stressing temperature combined with noise has been applied on 3-rd, 8-th, 15-th, 20-th, 25-th and 30-th days at birth.

The blood has been collected from the adapted animals just before and after applying combined stimuli. The traditional methods of data processing have been used for the analysis of the collected material. The following blood indices have been researched: the content of total proteins, protein particles, glucose, alkaline reserve, Ca, P, Na, K-levels, cortisol, bactericide activity as well as the growth rate values. The values of the physiological indices studied before applying combined stimuli have been serving as the control set (control group).

RESULTS AND DISCUSSIONS

The previous scientific studies revealed the specifics of influence of the stressing thermal maintenance factor on the functional status, resistance and adaptive capacities of calves during their early postnatal ontogenesis (Pavaliuc P., Erhan D., Rusu Ş. et al., 2012).

The special interest represents the results of the research when stressing temperature is applied along with another such widespread in households' factor as noise. The carried out experiments allowed studying the functional status, resistance and adaptive capacities of the organism of calves during their early postnatal ontogenesis while affected by combined stressor maintenance factors such as temperature and noise. The obtained results are presented in Table 1.

The analysis of obtained data shows that the complex effect of thermal and noise factors at 3-rd and 8-th days at birth, the concentration of total proteins have not been affected considerably compared to its indices before the influence of stressor factors, so that only at the 15-th day this have been increased by 6,5%, and at the 20-rth - by 8,5%, at the 25-th day- by 9,9%, and at 30-th day at birth - by 19%, as compared to the control group. There should be mentioned the relatively stable character of the concentration of blood proteins and its non-essential increase after applying combined stressors under maintenance condition as compared to the control group.

The analysis of the concentration of the protein particles revealed that the concentration of blood albumins has been decreased by 14,0% and by 10,4% correspondingly after applying combined stressors at 8-th and 15-th days at birth as compared to the control group. Just after the 20-th day the increase (by 38,4%) has been observed that reached the maximum level at the 25-th day as compared to the control group. While applying the stressor factors at the 8th day of birth the decrease of the concentration of α-globulins by 2,9 times, and at 25-th day - by 2,3 times has been noted. In this way, the dynamics of changing's in protein particles in calves during their early postnatal ontogenesis is characterized by the decrease of concentration of α–globulins during its first period. Further on, after 15-th day one can observe an increase, especially at the 30-th day. An increase in concentration of β- and γ–globulins at 8-th day, especially of β-globulins (by 1,9 times) have been observed, less marked for γ-globulins (by 1,2 times). Under the influence of combined maintenance stressor factors, the level of β–globulins in blood at the 25-th day at birth has decreased by 1,3 times as compared to its values in control group, and the level of γ–globulins remained approximately at the same level as it was before applying the stressor factors.

Table 1. The dynamics of the protein metabolism indices researched during early postnatal ontogenesis of calves affected in maintenance conditions by combined impulsive thermal and noise factors (n=10 animals)

Age (days)	Total proteins, g/1	Protein particles, %				Urea, mmol/1
		Albumins	globulins			
			α-	β-	γ-	
Before the influence of stressor factors						
3	59.2± 2.49	55.3± 2.64	10.5± 0.31	14.5± 0.39	19.7± 0.56	1.72± 0.08
8	58.3± 2.50	62.2± 3.08	7.2± 0.30	14.3± 0.42	16.3± 0.47	2.15± 0.11
15	51.8± 2.41	72.8± 3.01	6.3± 0.29	11.5± 0.38	9.4± 0.26	2.00± 0.11
20	52.1± 2.48	61.3± 2.74	15.8± 0.43	14.9± 0.43	8.0± 0.21	2.04± 0.11
35	52.5± 2.51	50.0± 2.06	26.7± 0.97	18.3± 0.45	5.0± 0.19	2.01± 0.09
30	51.9± 2.43	65.8± 2.53	15.9± 0.54	9.1± 0.27	9.2± 0.24	3.16± 0.12

After the influence of stressor factors						
3	60.1± 2.63	52.1± 2.41	3.7± 0.12	25.2± 0.41	19.0± 0.29	1.83± 0.09
8	58.3± 2.51	53.5± 2.47	2.5± 0.11	25.4± 0.44	18.6± 0.29	2.30± 0.11
15	55.4± 2.48	65.2± 2.63	6.5± 0.18	20.7± 0.42	7.6± 0.28	1.87± 0.09
20	57.0± 2.50	66.9± 2.71	8.7± 0.21	18.1± 0.38	6.3± 0.21	2.43± 0.11
25	58.3± 2.51	69.2± 2.84	11.5± 0.25	13.6± 0.36	5.7± 0.19	3.00± 0.15
30	54.1± 2.61	66.3± 2.68	13.0± 0.39	11.9± 0.32	8.8± 0.24	3.80± 0.21

$P \leq 0.05$

While applying the combined stimuli the concentration of urea have been increased prominently only at 25-th and 30-th days (by 49,3 and 20,3% correspondingly). The similar increase has been observed also before applying these. This increase could be explained by the introducing after 20-th day in the animal ration of the complex food (hay, haylage).

This being said, one can observe the tendency of the increase of the level of the total proteins starting with 15-th day and the level of urea – starting with 25-th day. The limits of evaluation of these indices have been relatively stable. The level of changing's in the protein particles to a great extent has been correlated with the level of changing's in the total proteins.

Another important physiological indices that characterize the functional status of the organism is the level of saline metabolism that could be explained by determining the concentration of macro elements (Ca, P, Na, K) in the blood, and their correlations (Ca:P; Na:K). The dynamics of the changing's in the concentration of these elements in the blood of calves during their early postnatal ontogenesis before and after applying combined stressor factors – temperature and noise under maintenance conditions-is presented in Table 2.

The data from Table 2 shows that the concentration of Ca during all period of applying the combined stimuli have been higher as compared to the control group and this tendency, starting with 8-th day have been approximately corresponding to 0,2 mmol/l. At the same time, there should be mentioned the decrease in dynamics of levels of Ca, and an increase of its level at 30-th day in both groups. Similarly to situation of concentration of Ca upon applying stressor factors, the concentration of P observed an insignificant tendency of changing′s that still falls into the norm limits (1,45-l,90 mmol/l). While applying the stressor factors, the correlations of these two elements have been slightly increased as compared before applying these, yet again falling into the norm limits.

Table 2. The dynamics of the saline metabolism indices in calves during their early postnatal period at influence of impulsive thermal and noise stressors (n=10 animals)

Age (days)	Ca. mmol/l	P. mmol/l	Ca: P	Na. mmol/l	K. mmol/l	Na: K
Before the influence of stressor factors						
3	2.80± 0.12	1.87± 0.10	1.49± 0.85	155.41± 5.01	6.62± 0.33	23.55± 1.2
8	2.60± 0.11	2.07± 1.11	1.26± 0.78	158.64± 4.63	6.83± 0.29	23.32± 1.2
15	2.55± 0.11	1.62± 0.14	1.57± 0.91	152.40± 5.03	5.80± 0.21	26.28± 1.2
20	2.47± 0.90	1.58± 0.08	1.56± 0.72	153.3± 4.94	6.44± 0.27	23.95± 1.2
25	2.40± 0.80	1.52± 0.06	1.58± 0.84	154.61± 5.00	7.03± 0.31	22.09± 1.2
30	2.60± 0.11	1.62± 0.11	1.60± 0.86	150.0± 5.01	6.41± 0.38	23.47± 1.2
After the influence of stressor factors						
3	2.83± 0.12	1.90± 0.12	1.49± 0.90	132.73± 5.20	6.33± 0.28	21.06± 1.1
8	2.80± 0.12	1.94± 0.11	1.44± 0.75	139.22± 5.00	6.42± 0.31	21.75± 1.2
15	2.75± 0.11	1.74± 0.09	1.58± 0.83	150.61± 5.11	5.71± 0.17	26.42± 1.3
20	2.68± 0.11	1.70± 0.10	1.58± 0.91	152.10± 4.98	6.30± 0.23	22.56± 1.2
25	2.60± 0.90	1.64± 0.08	1.59± 0.87	153.40± 4.57	6.84± 0.30	22.56± 1.2
30	2.85± 0.12	1.68± 0.09	1.70± 0.89	147.41± 4.89	6.21± 0.25	23.77± 1.2

$P \leq 0.05$

The data from Table 2 reveal that the level of Na in blood of the calves affected by combined stressing thermal and noise factors have been increasing from the 3-rd to 25-th days, and its decrease have been observed in the ulterior periods of research. The content of K in blood in the same circumstances remains almost unchanged. Thus, the obtained data show the stable character of Na′ and especially of K′ metabolism that could be observed from its values′ dynamics.

The conducted experimental studies allowed also observing the dynamics of the level of glucose and alkaline reserve in calves′ blood during their early postnatal ontogenesis while

applying combined stressing thermal and noise factors. The obtained data are presented in Figure 1 and 2.

The data from Figure 1 indicate the concentration of glucose in the blood of animals at influence of impulsive thermal and noise stressors during their early postnatal ontogenesis that varied and its dynamics could be characterized by the phasic aspects. Its maximum level has been observed at 8-th and 25-th days (6,66±0,24 and 5,55±0,18 mmol/l).

Under the influence of stressing factors the concentration of glucose have been observed as increasing one during all ontogenesis periods (especially at 15-th day – nearly 2 times) excepting the 25-th day at birth when its sharp decline (by 39,6%) has been observed. These two quite pronounced peaks of changing's have been noted at the 15-th and 25-th days as compared to other periods.

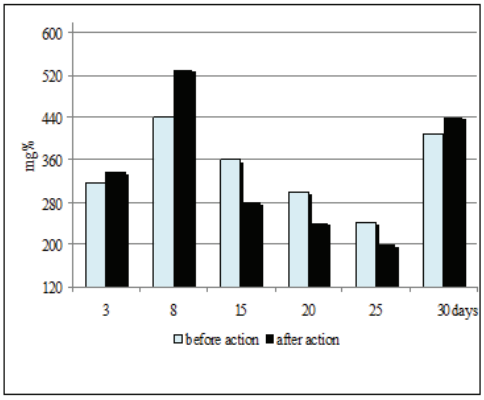

Figure 1. The concentration of glucose in blood plasma of calves during their early postnatal ontogenesis at influence of impulsive thermal and noise stressors

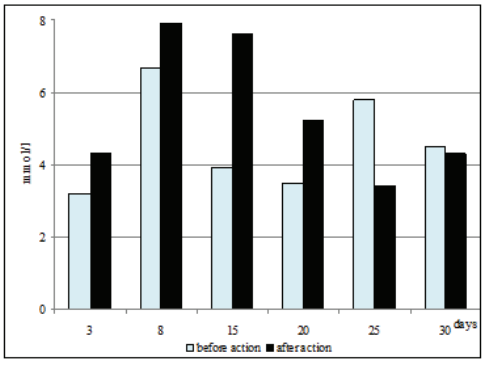

Figure 2. The concentration of alkaline reserve of calves during their early postnatal ontogenesis at influence of impulsive thermal and noise stressors

Similarly, the changings in the dynamics of the alkaline reserve (Figure 2) had the phasic character and its increased level (before and after applying stressing factors) up to 8-th day followed by a decrease of its concentration up to the 25-th day, and a phase of increasing values (on 30-th day). There is a reciprocal correlation of the level of alkaline reserve and level of glucose in the blood. While applying the combined stressing factors at 8-th day at birth the alkaline reserve has been increasing as compared to the control group by 9,1%, and at 15-th day it declined by 22,2%, at 25-th day – by 16,6%. Finally, at 25-th day it declined 2,6 times, if compared to the levels of the 8-th day.

In this way, the decrease in the level of glucose and alkaline reserve demonstrates the mobilezation of the energetic resources in the organism as a response to the developed reaction to stressors. The muscular system became maturated, this period being a dominant one.

Some physiological indices that characterize the resistance of the organism to the stressing factors such as bactericide activity and cortisone concentration in blood have been also studied (Figures 3, 4).

The data from Figure 3 indicate that concentration of cortisone had a tendency of slight increase excepting the 15-th day when it decreesed to the level of the control group. This could be explained by the fact that the major part of maternal immunoglobulins have already disintegrated but the own system not yet matured (period of immunodeficiency). That is why the release of hormone is intensified and its quantity is increasing gradually corresponding to the process of maturation of suprarenal capsules determining the organism to adapt to the changed environmental conditions.

The index that characterizes the stress, resistance and adaptive capacities of the organism to the action of the stressing factors also served the bactericide activity (Figure 4). The obtained data prove that the applying the combined stressing factors cause the increase of this index as compared to the control group, and this have been constant during all period of research constituting 23,9% in average. The biggest increase (by 36,7%) of bactericide activity, compared to the control group, have been registered at 25-th day.

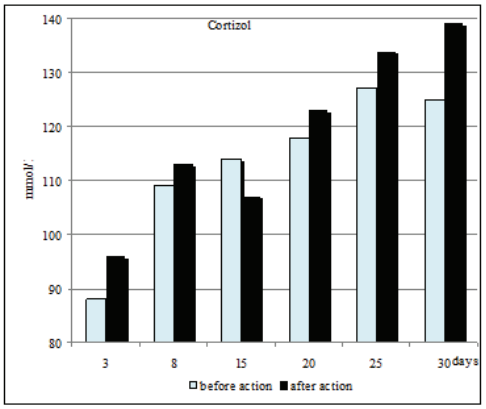

Figure 3. The dynamics of cortisone concentration in blood of calves during their early postnatal ontogenesis at influence of combined thermal and noise stressing factors

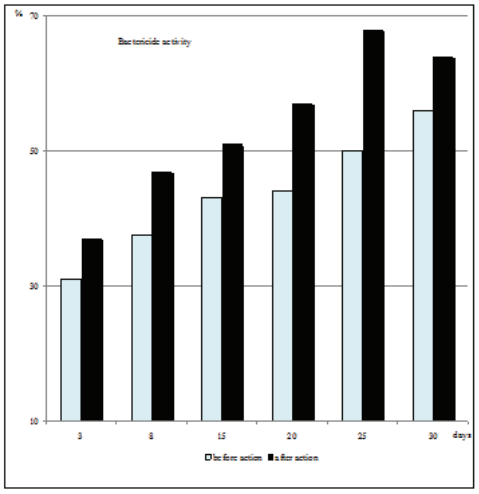

Figure 4. The dynamics of bactericide activity in the blood of calves during their early postnatal ontogenesis at influence of combined thermal and noise stressing factors

The tempo of calves′ growth during critical periods of early postnatal ontogenesis at influence of combined thermal and noise stressing factors have been slightly increased as compared to the control group that could be explained by the induced mobilization of plasticity and energy compensation reactions. This tempo is decreasing in the experimental group compared to the control one just after the critical periods of growth.

During previous studies the influence of stressing abiotic factors on the particularities of the functional status, resistance and adaptive capacities of the calves during their early postnatal ontogenesis have been researched as a separate issue (Pavaliuc P., Erhan D., Rusu Ş. et al., 2012). Later on, 2 series of experiments have been carried out while testing in a separate ways the stressing factors in two groups of calves: 1) applying the combined abiotic stimuli (stressing temperature + excessive noise), the results of which are presented above; 2) control group, applying the biotic parasitic factor, by applying the combined abiotic and biotic stimuli (stressing temperature + excessive noise + parasitic factor). The obtained results are presented below in Table 3.

Table 3. The indices of protein metabolism in calves during their early postnatal ontogenesis at influence of mono- and multifactorial stimuli (n=10 animals)

Age (days)	Total proteins, g/1	Protein particles, %			
		albumins	globulins		
			α-	β-	γ-
Control group					
15	59.27± 2.47	62.37± 2.91	11.90± 0.30	14.17± 0.40	13.35± 0.38
After applying the parasitic factor					
15	83.33± 1.41	37.65± 1.70	8.55± 0.40	13.70± 0.8	31.50± 1.25
After applying the combined stressing temperature, excessive noise and parasitic factors					
15	68.21± 2.40	50.18± 1.89	9.58± 0.19	16.98± 0.33	10.89± 0.26

$P \leq 0.05$

The data presented in Table 3 indicate that the studied indices have been considerably changed in calves under the influence of the biotic parasitic as well as of the combination of abiotic and biotic stressors, as compared to the control group. The concentration of total proteins and protein particles′ spectrum have been changing as follows: while applying the separate parasitic stressor – the content of albumins, α- and β -globulins have been decreased by 39,6; 28,1 and 3,3% correspondingly while the content of total proteins and level of γ-globulins have been increased by 40,6% and by 2,4 times correspondingly. The spectrum of protein particles while applying the combined abiotic and biotic stressors has been changing in the following way: the content of albumins, α- and γ-globulins have decreased by 19,5; 19,50 and 18,4% while the total proteins and

the level of β-globulins have been increased by 15,1% and 19,8%. The analysis of the obtained results reveals that the concentration of total proteins and γ-globulins in calves from the group affected with combined thermal along with excessive noise and parasitic stressors, registered a decrease of 18,1% and by 2,9 times, and the concentration of albumins, α- and β-globulins has increased by 33,3; 12,1 and 23,9% as compared to the group of animals affected with parasitic stressor only. This could be explained by the impact and way of stimuli effect on the organism of calves during their early postnatal ontogenesis.

In this way, two ascents of pronounced changes of the tested physiologic indices at 8-15-th and 25-30-th days at birth have revealed the coincidence with some critical periods of early postnatal ontogenesis–immunodeficiency, depression of the stressogen reaction, depression of dominance and retardation. As a conclusion, the registered changes had a phasic character.

CONCLUSIONS

1. There were observed that the changes in the studied physiological indices (total proteins, protein particles, glucose, alkaline reserve, macro elements Ca, P, Na, K, cortisol, bactericide activity) while applying the combined temperature and noise stressing factors have a phasic character and have been influencing – by increasing or decreasing its values during early postnatal ontogenesis. The most evident changes have been noted at the 8-15-th days and 25-30 days at birth. These changes have corresponding with critical periods of ontogenesis: immunodeficiency, depression of the stressogen reaction, depression of dominance and retardation.

2. A tendency of increased bactericide activity and the level of cortisol in blood (with exception at the 15-th day) have been observed while applying the combined stressor factors, that proves the fact of some increased resistance level and adaptive capacity of the animal organisms.

3. There were observed that the impact of the combined abiotic and biotic factors (stressing temperature + excessive noise + parasitic agents) essential aggravates the metabolic processes in the organism of the calves as compared to these while applying mono factorial stressors.

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COMPARATIVE FEATURES OF THE CALCIUM AND PHOSPHORUS HOMEOSTASIS IN HENS DURING THE LAYING CYCLE

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Abstract

It was determined the evolution of the levels of calcium and phosphorus from blood plasma in White Plymouth Rock hens (a hen breed with high egg production) and White Cornish hens (a hen breed with a low egg production) during the laying cycle, beginning from 22 weeks age up to 40 weeks of age. Parallel, it has been monitored the plasma evolution of the parathyroid hormone (PTH) and vitamin D levels, and the evolution of the following blood parameters: total protein, albumin, and uric acid. The results relive significant differences, according to the breed, concerning the parallel raising of the plasmatic calcium levels and the laying egg percent. Thus, in Plymouth Rock (PLR) hens, the level of calcium (in mg/dL) raised from 10.5 at the beginning of the laying cycle to 33.3 in the peak of the laying, decreasing then, to 30.9 toward the end of the laying cycle. On the other hand, in Cornish (CRN) hens, at the same moments, the values of the plasmatic calcium were: 8.8, 22.5 and 20.4, respectively. The calcium/phosphorus ratio presented an ascendant evolution in both, PLR and CRN breeds, indicating an increasing of the free calcium content of the blood plasma. Plasma albumins ranged between 17.2 and 22.2 mg / mL in the PLR hens and between 19.8 and 22.8 mg / mL in the CRN hens, with significant differences between groups. Uric acid plasma levels have evolved relatively parallel to the laying percentage, showing an intensified protein catabolism, according to laying percentage, in PLR hens. Analysis of the hormone evolution relieves a peak of the PTH level in PRL hens, around 32 weeks of age (amounted to 353 pg/mL). This peak of PTH is behind the laying peak and it is significantly higher in PLR hens than in CRN hens (185 pg/mL at the age of 36 weeks). Regarding vitamin D, its plasma level presented a relatively constant evolution in both hen breeds, seeming to be not influenced by breed or high metabolic requirements that characterize a lay peak. It can be concluded that the high demands of calcium and phosphorus export during the laying cycle in hens with high egg production are supported by high levels of PTH, the main hormone involved in regulating the homeostasis of these two minerals.

Key words: *laying hens, calcium, phosphorus, parathormone, vitamin D.*

INTRODUCTION

Ultra specialized egg laying hen breeds have become true metabolic bombs in which specific processes have experienced maximum acceleration. Metabolic processes are coordinated by hormonal mechanisms which also stress the endocrine system of the animal. Given a maximal metabolism of an animal, the relationship between plasma levels of some elements and the activity levels of the hormonal mechanisms which regulate their plasma levels becomes more complex and more sensitive (Dojana, 2009; Larbier and Leclercq, 1992; Gardinier, 1973). The present paper analyses the interrelation between metabolic demands related to two of the organism minerals (calcium and phosphorus) and the ability of hormone regulating mechanisms to maintain their homeostasis in hens during a period of high metabolic demand (egg laying).

MATERIALS AND METHODS

To achieve the goal of this research, hens from two different breeds have been selected: a hen breed with high egg production and a hen breed with a low egg production. Thus, research has been conducted on a group of 50 Plymouth Rock hens (PLR) aged 22 weeks and a group of Cornish (CRN) hens, aged 24 weeks. The hens were exploited in a special industrial poultry, on a deep litter raising system, in halls having an area of 1200 m^2, achieving a density of 7.2 capita/m^2 for PLR hens and 4.4 capita/m^2 for CRN hens. The light was common for both groups, starting from 11 hours per day and gradually increasing to 16 hours at the age of 27 weeks, being constantly kept up until the

hens' reformation. The hens were fed with age-specific and breed-specific compound feed, in a quantity of 130 g/day, ensuring a quantity of 380 kcal EM/capita/day. The feeds contained 15.4% protein, 4.4 g% calcium and 0.66% total phosphorus, as shown in the manufacturing receipt.

The groups of hens have been monitored in terms of egg production and blood plasma concentrations of the following parameters: calcium, phosphorus, total protein, albumin and uric acid. The plasma evolution of the intact parathyroid hormone (iPTH) (biologically active parathormone) and the vitamin D level have also been monitored. Blood samples were collected every two weeks until the age of 40 weeks, on anticoagulant vacutainers, by *axillary vein* puncture. After collection, the samples were centrifuged at 2,500 rpm in order to fix the blood plasma which was then frozen at-12ºC until processing. The concentrations of calcium, phosphorus, protein and uric acid were determined according the methods described by Manta *et al*. (1976). The hormonal determinations were made by means of an Immulite 1000 analyzer. The results have been statistically processed by determining the mean and standard error of mean. The differences between the groups have been statistically analyzed based on the Students′t test, according to Tacu (1978). The differences between groups were considered to be significant when the probability of the null hypothesis was less than 5% (P=0.05).

RESULTS AND DISCUSSIONS

Figure 1 shows the evolution of the egg production from the two monitored hen groups, starting with the age of 22 weeks and up to the age of 40 weeks.

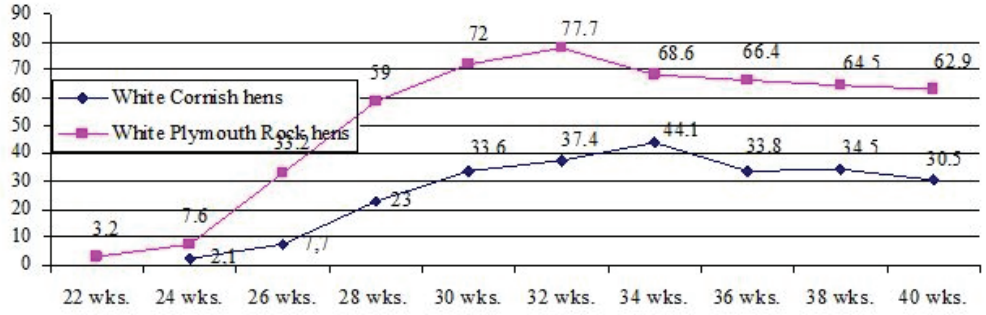

Figure 1. The evolution of the laying percentage in White Plymouth Rock and White Cornish hens during a period form 22 to 40 weeks of age

The analysis of the data presented in figure 1, shows that, in PLR hens, the production peek was achieved at the age of 32 weeks, rate of lay amounting to a value of 77.7%. Comparatively, the CRN hens presented an egg production peek at the age of 34 weeks which amounted to 44.1% rate of lay. The statistical analysis of the differences between the two groups reveals no significant differences between the two groups during the first 4 weeks of monitoring (P>0.05). Starting with week 26 of age, the differences between groups became significant (P<0.05) and starting from week 28, the differences related to the egg production became very significant (P<0.001) and remain significant until the end of the monitoring period.

Table 1 shows the evolution of the main blood biochemical parameters. We found that the level of the plasma proteins was relatively constant, fluctuating around an average value of 43.17 mg/mL in the PLR hens and 46.66 mg/mL in the CRN hens. A similar evolution was also found on the level of serum albumin which fluctuated between 17.2 and 22.2 mg/mL of serum in the PLR hens and between 19.8 and 22.8 mg/mL in the CRN hens, with significant differences between the two groups (P<0.05). Determination of the albumin percentage provides information on the percentage fraction of bound serum calcium: an elevated albumin fraction represents a higher percentage of bound calcium.

Table 1. The evolution of some blood biochemical parameters in Plymouth Rock and Cornish hens during the laying cycle, from 22 to 40 weeks of age

No	Item	References values	Group of hens	Age (in weeks)							
				22	26	28	30	32	34	36	40
2	Total proteins (mg/mL)	35-40*	Plymouth Rock	44.0± 4.9	44.5±	46.5±	46.0±	45.0±	38.0±	40.6±	40.8±
			Cornish	48.0± 8.6	48.5±	46.0±	46.5±	48.5±	44.4±	46.5±	44.9±
	Albumins (mg/mL)		Plymouth Rock	19.1± 4.0	22.2±	21.6±	18.9±	20.5±	17.2±	19.0±	18.8±
			Cornish	22.2±4.4	20.3±	22.5±	21.0±	23.5±	19.9±	21.4±	22.8±
5	Uric acid (mg/dL)	1-7**	Plymouth Rock	4.9± 1.5	4.5±	4.5±	5.8±	6.5±	5.3±	4.7±	4.2±
			Cornish	4.0± 1.3	4.4±	4.8±	5.0±	5.4±	5.8±	4.4±	3.5±
6	Total calcium (mg/dL)	4.5-6[a] 20-98[b]**	Plymouth Rock	10.5± 3.3	18.5±	24.3±	28.4±	33.3±	30.5±	31.6±	30.9±
			Cornish	8.8± 3.1	8.9±	11.7±	14.5±	22.5±	22.5±	20.3±	20.4±
7	Phosphorus (mg/dL)	3-6	Plymouth Rock	4.2± 1.1	4.4± 0.9	5.4± 1.0	5.9± 0.8	6.3± 1.2	5.0± 1.5	5.6± 0.5	5.0± 1.4
			Cornish	2.9± 0.8	3.5± 0.5	3.3± 0.6	4.6± 0.6	4.4± 0.9	4.0± 1.1	3.9± 0.7	3.9± 1.0
8	Ca/P ratio	3.5/1	Plymouth Rock	2.5	4.2	4.5	4.8	5.2	6.1	5.6	6.2
			Cornish	3.0	2.5	3.5	3.1	5.1	5.6	5.2	5.2

So, for every 1-g/dL drop in serum albumin below 4 g/dL, measured serum calcium decreases by 0.8 mg/dL. Therefore, to correct for an albumin level of less than 4 g/dL, one should add 0.8 to the measured value of calcium for each 1-g/dL decrease in albumin. Without this correction, an abnormally high serum calcium level may appear to be normal. For example, an animal with a serum calcium level of 10.3 mg/dL but an albumin level of 3 g/dL appears to have a normal serum calcium level. However, when corrected for the low albumin, the real serum calcium value is 11.1 mg/dL (Agraharkar, 2008).

Analysis of the evolution of the plasma level in the uric acid, as a product of protein catabolism, shows an ascending evolution (from 4.9 to 6.5 mg/mL) parallel with the increase of the egg laying percentage on the PLR hens, marking a peak around the age of 32 weeks (which coincides with the egg laying peek), followed by a descendent trend, decreasing down to 4.2 mg/mL, which was again parallel with the descendent curve of the egg laying process. The parallelism between the evolution curves of the egg laying percentage and the level of uric acid on the PLR hen shows an enhancement of the protein catabolism which is related to the enhancement of the egg production.

Analysis of the evolution of plasma concentration of total calcium shows and ascending curve on both monitored hen groups. The peak was located at a level of 33.3 mg/dL at the age of 32 weeks for PLR hens and at a level of 22.5 mg/dL at the age of 32 and 34 weeks for CRN hens. The statistical analysis of the differences between the two groups in this peak moment shows significant differences ($P<0.01$) between the two groups. Concerning the Ca/P ratio in PLR hens, it was significantly higher than in CRN hens during the entire monitoring period. The higher values of the blood calcium levels in PLR hens are in agreement with a higher production of eggs (a higher percentage of egg laying than in CRN hens). It appears that a higher production of eggs induces an increase in the plasma concentration of calcium, showing a more elevated turnover of the calcium in deposits. On the other hand, the increase of the Ca/P ratio (from 2.5 to 6.1 in PLR hens) shows an increased level of free calcium in hens with high egg production in comparison with hens with low egg production. Chen and Chen (1989) reported breed differences between

ducks and Leghorn hens in terms of serum calcium levels and calcium deposits on bones. Luck and Scanes identified daily evolutions of blood calcium level in hens which were probably related to the evolution of the level of *gonadotropin* releasing *hormones*: ionized calcium showed a sigmoidal pattern over the ovulation cycle reaching a peak within 3–6 hours of oviposition and falling, as shell calcification proceeded, to a minimum 3–6 hours before the next oviposition (Luck and Scanes, 2009).

The plasma level of the intact parathyroid hormone (iPTH) o PLR hens had an initial value of 126.46 ± 44.45 pg/mL (Figure 2).

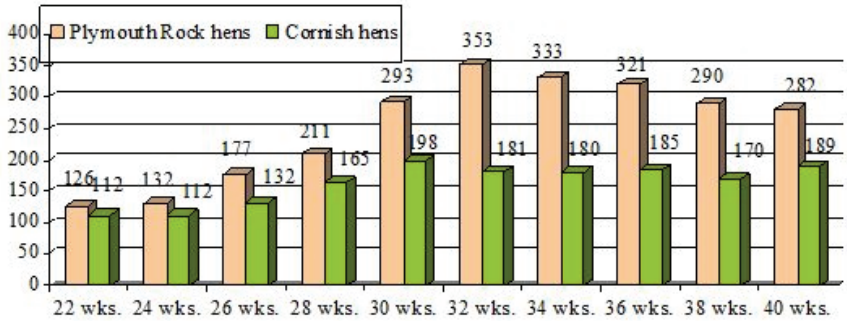

Figure 2. The evolution of the plasma levels of parathyroid hormone (in pg/mL) in White Plymouth Rock and White Cornish hens during the laying eggs cycle

The values remained relatively constant during the following determinations subsequently increasing, reaching a peak at the age of 32 weeks, when the egg production peak was also marked. This peak amounted to a value of 353.16 ± 86.85 pg/mL. Subsequently, the plasma level of the intact parathyroid hormone (PTH) on PLR hens slowly decreased towards the values registered at the beginning of the monitoring period (reaching a value of 282 pg/mL at the end of the monitoring period). On CRN hens, the plasma level of the iPTH had notve an ascending trend, butan evolution which was rather unspecific to the respective physiological period. However, this correlates with a much more reduced egg laying percent. The plasma level of the iPTH on this hens group oscillated between a minimum value of 112 pg/mL at the age of 22 weeks and 198 pg/mL at the age of 30 weeks. Clinically speaking, when the calcium level is high the PTH level needs to be low. An elevated level under this conditions shows and intense activity in the thyroid gland (the producing PTH C-cells). In the case of our experiment, the increase of the PTH concentration in parallel with the plasma calcium level might be explained by an eventual positive feedback mechanism. Rahman *et al*. (2005) found that increased iPTH level occurs even early in the course of CRF and progressive hypocalcemia and hyperphosphatemia are the initiating factors for the development of hyperparathyroidism. This is explained by the occurrence of a push pull mechanism, well known in the specialized literature (Dojana, 2009).

Vitamin D dosage was made taking into account its involvement in the calcium metabolism along with the PTH (Figure 3). Its normal plasma level is amounted to 35 – 40 ng/mL (Larbier and Leclercq, 1992; Mundy and Guise, 1999). Because of its long half-time and a higher concentration, vitamin D is commonly measured to assess and monitor vitamin D status in hens. The plasma level of vitamin D had an unspecific evolution which was not connected to the evolution of the egg laying process for both hen groups (with high egg production and with low egg production) and seemed to not have been influenced by breed or by the intense metabolic stress which characterize an egg laying process peak. Therefore, in PLR hens, the levels of this vitamin fluctuated between 54 and 143 pg/mL and in CRN hens, these levels fluctuated between 54 and 154 pg/mL. This aspect differentiates hens from other species of

animals on which it was found significant correlations between the level of vitamin D and the level of blood calcium (Tsao *et al*., 1985).

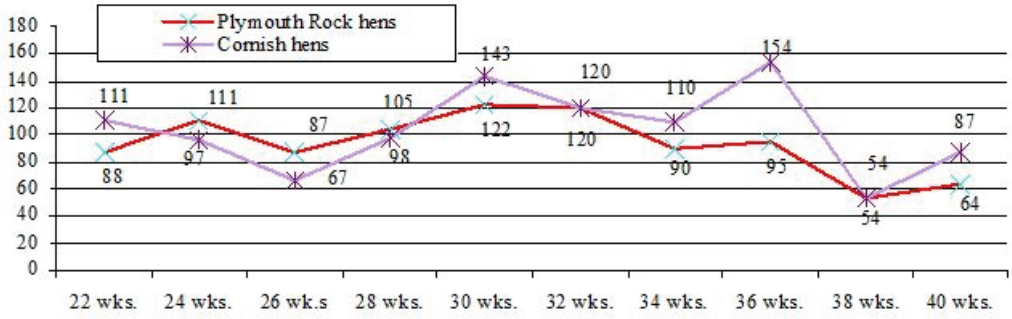

Figure 3. The evolution of the plasma levels of vitamin D (in pg/mL) in White Plymouth Rock and White Cornish hens during the laying egg cycle

CONCLUSIONS

Maximum metabolic requirements of the calcium and phosphorus metabolism during the laying egg peak are supported by an increased parathormone secretion and by the corresponding growth of some blood plasma parameters (calcium and albumins).

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ACTION OF *VARROA DESTRUCTOR* ECTOPARASITE ON EXTERNAL MORPHOLOGY OF THE BEE

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Abstract

The work aims to investigate the histological changes produced by the parasite Varroa destructor in bee species Apis mellifera, using as a liquid fixative Dubosq-Brasil and Masson modified coloration.

Key words: *Varroa destructor, malformations, bees.*

INTRODUCTION

Depopulation of bee colonies affected by *Varroa destructor* is a complex phenomenon that draws important economical loss (De Jong, 1997). Parasitation of colonies with *Varroa* have as direct consequence the nymphe′s death or the loss of viability of eclosionating bees (Kelly et al., 2010). As well, when the parasite affects over 30% of adult bees and drones population, inside the colony, individuals with malformation of wings, members, abdomen and thorax start to appear and also young individuals with unfolding wings after hatching (Rosenkranz, and Engels, 1994, Rosenkranz et al, 2010, Guzman-Novoa et al., 1998).

MATERIALS AND METHODS

Initially, the aim was to realise the histopathological examination on alive bees probes in order to observe tissue modifications as consequence of viral pathogens agents possible transferred by *Varroa destructor*.

In order to determinate the existent structural modifications of intestinal epithelium consecutive hemolymph depletion produced by parasitism with *Varroa destructor*, there has been created a probe stamp with live bees gathered from study apiary, respective experimental apiary of IDAH and have been created two samples, group I that consists in bees clinically healthy, where parasitological, bacteriological and mycological examinations, didn′t evidenced the evolution of specifically disease and group II which consisted in bees from colonies with *Varroa* disease.

The samples have been obtained at the end of active season, in October 2012. Every sample, for each Apiary consists in 25 g alive adult bees parasitized by *Varroa destructor.* For sample creating have been selected bees which has inserted 1-2 Varroa parasites between abdominal tergites, and one of them presents wings malformations or small abdomen.

All bees in the Group II were from honeybee colonies that had isolated brood cells presenting irreversible changes in physical characteristics, of the nymphs and the hatching of bees. Some bees found at hatching and expressed nervous syndrome by shaking the wings.

Digestive system samples collected from bees belonging to consignment, have been fixed in the effectiveness of the jumpers: formalin, Dubosq-Brasil, Carnoy and Lille and at the optimal time interval, i.e. at least 72 hours.

All histologic preparations were examined from permanent optical microscope Zeiss, with magnification power of 150 X to X 1250, transmitted light with AxioVision system for capturing microscopic and computational processing them with IDAH: license 'Zeiss Axioplan 2 Image'.

Stage of dehydration and pre including the paraffin were done automatically with a Pathcentra device, and inclusion in paraffin

wax of anatomical parts was also automatically done with Kunz W – 4.

In order to obtain t has been used he microtomical sections Finesse microtome , for laying them, thermostatically controlled bath Kunz set at 40 ° C and a hob with electronic adjustment.

Fixed anatomical pieces were processed and analyzed in the IDAH Laboratory of Pathology and Health of Useful Insects.

Dubosq– Brasil – mixture consists of picric acid: 0.5 g, 75 ml of 80°C.

Formalin - fixed tissues as a result of the completion of the metilenisation processes and dehydration. It is known as the most widespread and efficient fixative for all types of animal tissues, of the species of mammals, birds, reptiles and fish.

Formalin is used in aqueous solutions of 10%.

Carnoy-mixture consists of: 120 ml 60 ml absolute alcohol, chloroform, 20 ml glacial acetic acid.

Lillie mixture – is made up of: 85 ml absolute ethyl alcohol:10 ml formaldehyde 40%, 5 ml glacial acetic acid.

Taking into account the particularities of the structure of the digestive system from bees, as well as the character of sporozoa and oxyphil of viral image inclusions intended to be outlined in the fasteners operation follow the following conditions:

Fixing of parts of the digestive system's of honey bees (gut, gut the environment together with the insertion of Malpighi tubes and posterior intestine) has been carried out immediately after the harvesting a span of 15 minutes;

The ratio of the liquid Fixer and anatomical parts was at least 1/20 and 1/40.

RESULTS AND DISCUSSIONS

Histological preparations, in a first stage were examined at different grosisments X 100; X 200; X and 1000 X 1200. He showed up as a result of normal small bowel structure clamps at healthy bees from I.

Subsequently, we analyzed the changes produced in the digestive tract and renal Malpighi, depletion of hemolymph caused parasitism with Varroa destructor.

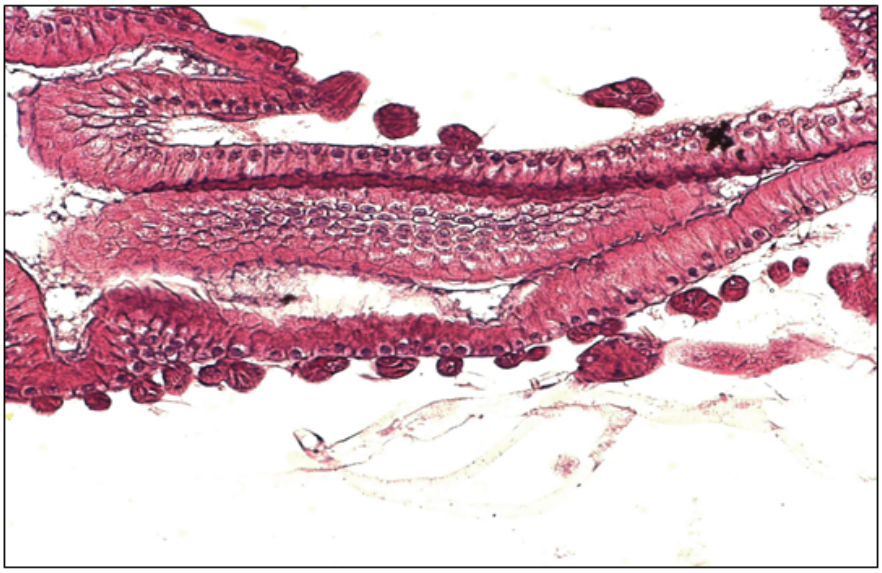

Figure 1. Medium intestine (detail), the adult bee.
Masson, with modified method in liquid Dubosq-Brasil x 200 (original)

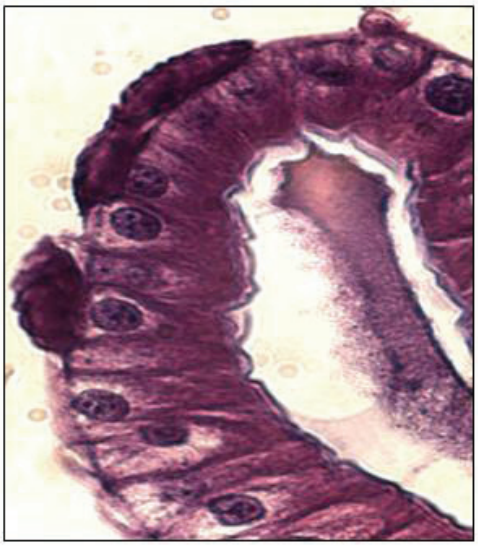

Figure 2. Intestinal ance, adult bee (detail). Masson, with modified method in liquid Dubosq-Brasil, x 1200, NG (original)

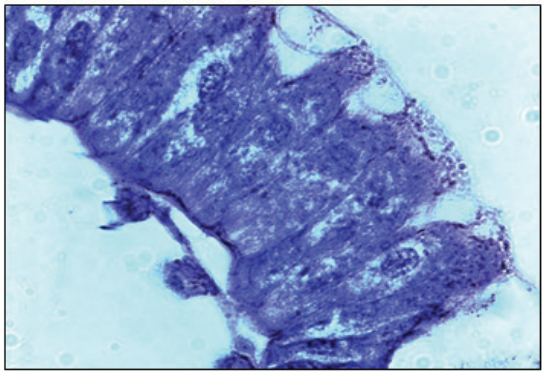

Figure 3. Microvacuolisations, lysis apical cytoplasm in the epithelial cells of the small intestine. Masson, modified method with acid, formalin fixation in X 1000 (original)

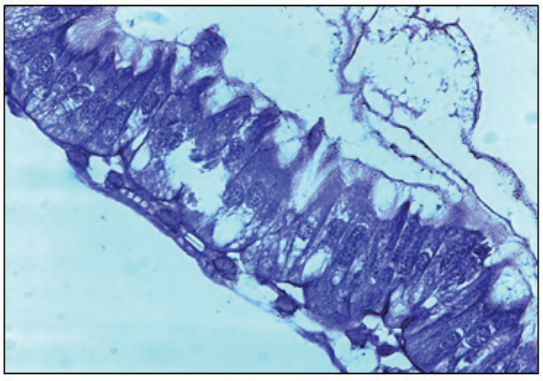

Figure 4. Vacuolizations and intracytoplasmic parabasale and apical destructions of epithelial cells, small intestine-adult bee. Masson, with modified method of fixation in formalin, acid X 1000. (original)

CONCLUSIONS

It has been noticed that hemolymph depletion and virus inoculation develop external morphologic modifications of nymphs and hathced bees.

These modifications appear in abdominal and wings level, which make newly hatched individuals not to be viable. The colony depopulation is occurring step by step following the decrease of newly hatched viable bees.

ACKNOWLEDGEMENTS

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CYTOMORPHOLOGICAL MODIFICATIONS OF INTESTINAL EPITHELIUM OF BEE (*APIS MELLIFERA*) PARASITED BY *NOSEMA* SPP.

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Abstract

The work aims to investigate the histological changes produced by the parasite Nosema spp. in bee species Apis mellifera, using as a liquid fixative Dubosq-Brasil and Masson modified coloration.

Key words: *histological techniques, intestinal epithelium, bees, Nosema spp..*

INTRODUCTION

Apis mellifera is a pollinating insect constantly exposed of different pathogens especially parasites (Webster et al., 2008, Bailey and Ball, 1991). Among these parasites are microsporidians from *Nosema* genre. This represents a numerous group of endoparasites with intracellular localisation, who presents a unicellular structure and have the capacity to make spores (Forsgren and Fries 2010). The election place of all species from *Nosema* genre is represented by the cytoplasm of epithelial cells of digestive mucous tube of adult bee (Webster et al., 2004).

MATERIALS AND METHODS

Initially, we aimed to realise the histopathological examination on living bees samples with scope to observe tissue modifications as consequence of viral pathogens agents possible transferred by *Nosema spp,* by kind of Morison corps.

In order to determinate existent modifications at intestinal epithelium level, consecutive hemolymph depletion produced by parasitism with *Nosema*, it was created a probe stamp with living bees gathered from study apiary, respective experimental apiary of Institute for Diagnosis and Animal Health (IDAH) and have been created two samples, sample I which consisted in bees with no clinical signs of any diseases, where parasitological, bacteriological and mycological examinations, didn′t evidenced the evolution of specifically disease and sample II, which consisted in bees from colonies with nosemosis .

The samples have been obtained at the end of active season, in October 2012. Every sample, for each Apiary consisted in 25 g living adult bees parasitized by *Nosema.* For sample creating there have been selected bees which has inserted 1-2 Nosema parasites between abdominal tergites, and one of them presented wings malformations or small abdomen.

All bees in the Group II were from honeybee colonies that had isolated brood cells presenting irreversible changes in physical characteristics, of the nymphs and the hatching of bees. Digestive system samples collected from bees belonging to consignment have been fixed in the effectiveness of the jumpers: formalin, Dubosq-Brasil, Carnoy and Lille and at the optimal time interval, i.e. at least 72 hours.

All permanent histological preparations were examined from optical microscope Zeiss, with magnification power of 150 X to X 1250, transmitted light with AxioVision system for capturing microscopic and computational processing them with IDAH: license 'Zeiss Axioplan 2 Image'.

Stage of dehydration and pre including the paraffin was done automatically by means of a Pathcentra device, and inclusion in paraffin wax anatomical parts was also automatically with the help of Kunz W – 4.

In order to obtain the microtomical sections has been used Finesse microtome, for laying them, thermostatically controlled bath Kunz set at 40 ° C and a hob with electronic adjustment.

Fixed anatomical pieces were processed and analyzed in the Laboratory of pathology and health of IDAH useful Insects.

The method used was represented by the specific histopathological techniques.

Dubosq– Brasil mixture, formalin in aqueous solutions of 10%, Carnoy-mixture and Lillie mixture were used for histological preparations obtaining.

Fixing of parts of the digestive system's Anatomy of honey bees (gut, the gut environment together with the insertion of Malpighi tubes and posterior intestine) has been carried out immediately after the harvesting a span of 15 minutes;

The ratio of the liquid Fixer and anatomical parts was at least 1/20 and 1/40.

RESULTS AND DISCUSSIONS

Histologically, viral inclusions are highlighted in the Morison epithelial cells of the small intestine, intracytoplasmic. They must be in the form of corpuscles, rounded 1-2 micrometers in diameter, opaque, more numerous in the area of insertion of Malpighi tubes. Also, the spores of *Nosema spp*, causing destruction of the intestinal epithelium, and do not turn entirely through Mason changed colour.

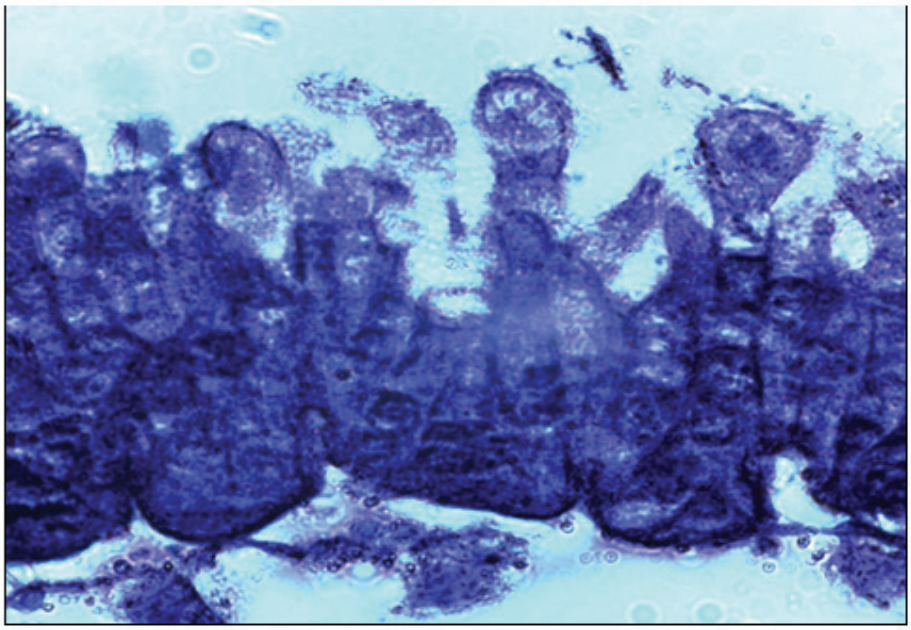

Figure 1. Intranuclear microvacuolisations and intracytoplasmic, with the apical epithelial cell lysis. Masson staining method, Carnoy fixation , X 1000. (original)

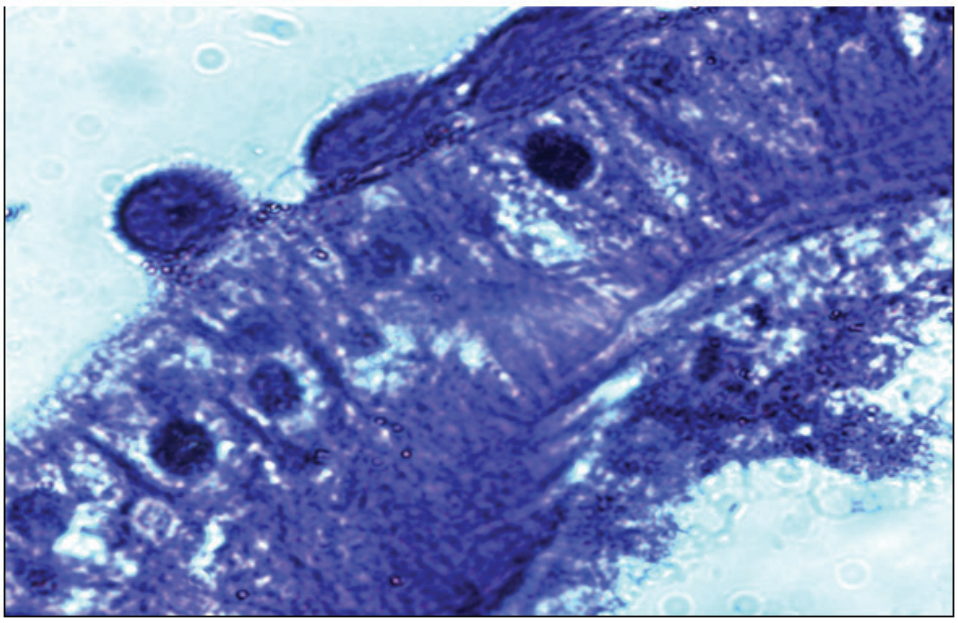

Figure 2. Granular and vacuolar alteration-confluante in the cytoplasm of epithelial cells. Thin bowel adult bee. Modified Masson staining method, in liquid Lilie fixation X l000 (original).

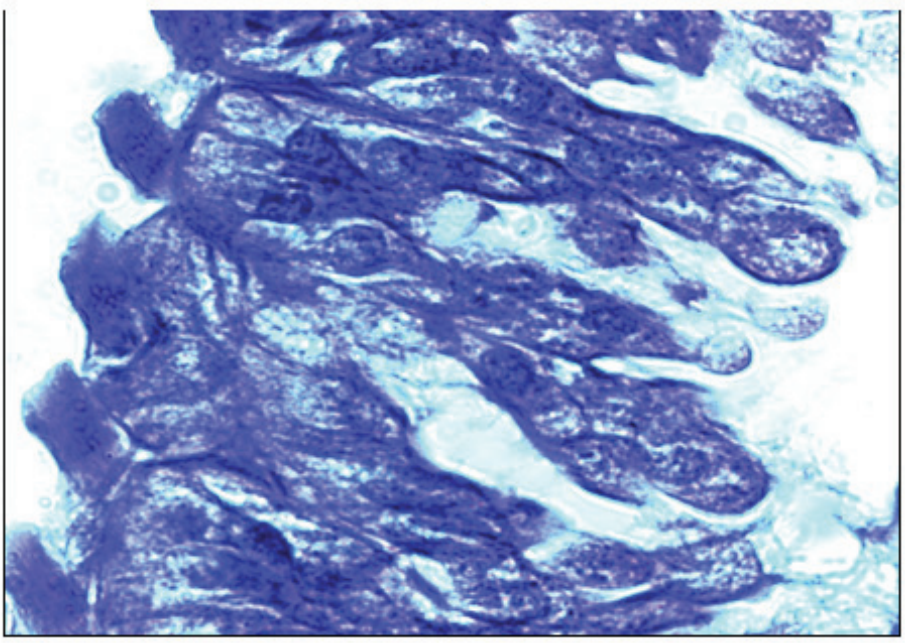

Figure 3. Medium intestine, the adult bee. Modified Masson staining method in liquid Dubosq-Brasil, x 1000, without filter NG (original)

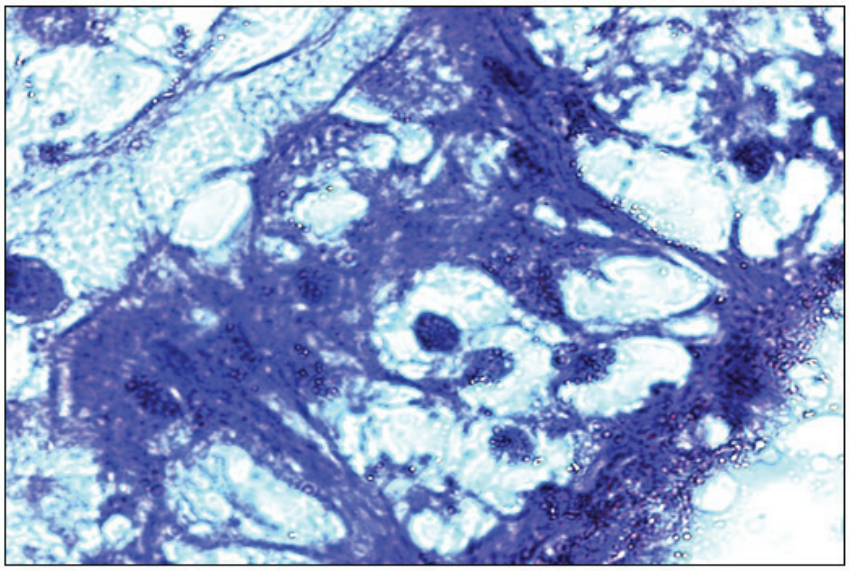

Figure 4. Vacuolisation and cytoplasmic aggregation, cariolisis, with dilaceration of the basal membrane. Infection with spores of Nosema spp., adult bee

Liquid fixative Dubosq-Brasil and modified Masson method had shown the structure and and cellularity of medium intestine and also the structure and characteristic of Malpighi tubes.

The histopathological examination done has allowed level of infection establishment with Nosema spp, which produced irreversible modifications of intestinal epithelium structure, as a result of digestive function alteration in bees and finally the bee′s death.

In the process of examining histological preparations obtained from samples of bees belonging to the lot on intestine and fastened with solutions, they noticed a lot of changes (microvacuolisations, lysis, epithelial cells, etc.) in the structure of small intestine epithelial cells and thin.

CONCLUSIONS

Fixing with the utmost efficiency and colouring Dubosq-Brasil Masson changed have highlighted in the control group, another type of histological changes that can be translated through a proliferative process observed only in the small intestine epithelium, the environment where intense metabolic reactions take place

ACKNOWLEDGEMENTS

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STUDY OF THE MORPHOLOGICAL BASIS IMPLICATED IN INHALATION ANAESTHESIA AT DOGS: A PERSONAL RESEARCH

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Abstract

The study presents one of anesthesia techniques, the inhalation anesthesia, frequently used in veterinary medicine. The tested substance is a general anesthetics whose role is to act selective or at global mode, above one kind of structures form central nervous system, producing specific effects for his team.
There have been observed the anesthetic effects of Izofluran dosed through mask induction and maintained with tracheal sonde at 5 dogs from different species and weights on a private clinic.
There have been recorded the hematological and biochemical values of every patient, and also evaluated the vital constants: cardiac frequency, respiratory frequency, temperature, induction time, wake up time, metabolisation time and seconds products eliminating. There have been monitored the effects on cardiovascular, respiratory, neuromuscular, renal systems and live. In order to find the best inhalator there have been made comparative tests between Izofluran and Sevofluran.
After inhalation administration of Izofluran it was observed a nesemnificative clinical grow of enzymatic activity for aminotransferase (GOT/AST), alaninaminotranferase (GPT/ALT), gama glutamil transferase (GGT), total amylase, glycaemia, creatinine, urea and compared with initial moment. There were not recorded major differences between initial moment and after anesthesia. The steps of anesthesia have characterized through induction of anesthetic in one moderate time (10-15 minutes) with respiratory complications (apnea, larynx spasm and cough), passing from anesthesia was done in 15-25 minutes.
Secondary reactions (vomit, convulsions or events) were not present at anesthetized cases with Izofluran, and the temperature, cardiac frequency, respiratory frequency and oxygen saturation of peripheral tissues were on normal parameters. The phases of anesthesia have been characterized with a longer time of induction and waking up against Sevofluran with some respiratory complications.

Key words: *izofluran, anesthesia, intubation, dog, enzymes.*

INTRODUCTION

The anesthesia represents a medical procedure which decreases or suppresses, completely or partially, the organism′s sensitivity, by chemical substances called anaestethics. The anesthesia is used throughout surgical procedures to allow animals to bear the surgical intervention, with minimal painful effects. The anesthesia is taking place when one or more types of sensitivity disappear and when sensitivity is reversibly abolished, by using anaesthetics.
The general anesthesia consists in the reversible loss of consciousness. The general anesthesia is realized through three types of actions: narcosis (which represents the loss of consciousness), by reason of the administration of an inhalation anaesthetic (sevoflurane, isoflurane, halotane) or intravenous; analgesia (the disparity of pain), obtained through the administration of analgesics; curarisation (paralyzing substances), that allow the muscles to relax, in order to have a good consecution of the surgical intervention.
The morphologic base implicated in the inhalation anesthetics is represented by the respiratory system, with intrapulmonary and extrapulmonary tracts, and their integrity is contributing in a great way in the good consecution of the phases of narcosis and in the wakening process (Ang et al, 1998).

MATERIALS AND METHODS

Previous published results suggested the procedure of anaesthetic administration (Tacke et al., 1998, Topal et al., 2003, Yuan et al., 2012). We have followed the anesthetic effects of Isoflurane, administered through mask induction and maintained through endotracheal

intubation with a flexible plastic tube, in five dogs of different size and breed (in the age interval of 2-12 years, three males and two females, two of European breed, two poodles and one bichon), in a private clinic. The surgical interventions they underwent did not have a great ampleness and their duration did not exceed 70 minutes. Each patient received the same premedication.

On each case were recorded the hematological and biochemical values, and we evaluated the vital constants: the cardiac frequency, the oxygen saturation of the peripheral tissues, the induction time, the wakening time, the metabolisation and secondary waste products elimination. The effects on the cardiovascular system, respiratory, neuromuscular and renal systems and the liver were also monitored.

The anesthesia circuit that we used for the experiment is a closed circuit, which is made from a system of tubes that ensure the oxygen input and the elimination of carbon dioxide, that is absorbed by a conventional soda lime, thus creating the artificial ventilation. In the enclosed circuit, the gaseous blend is reinhalated, thus ensuring an adequate oxygen level. This method has the advantage that it needs a small amount of volatile anesthetic substances, it realizes a deep narcosis that has a controllable duration and has the possibility of controlling the pulmonary ventilation. The endotracheal intubation was realized as we'll see next:

- we opened the mouth;
- we have visualized the epiglottis in the laryngeal opening and we have exteriorized the tongue and applied the laryngoscope at the base of the tongue;
- the head of the animal was fixed in an orthopnea position, to reduce as much as possible the orotracheal angle, and then the flexible plastic tube was inserted up to the anterior third of the trachea. The protrusion of the tube up to the trachea was visualized also in the radiography.
- the flexible tube has a plastic bubble that, when air is inserted, remains fixed in the windpipe; the air is inserted into the plastic bubble with the use of a syringe and the exterior end of the tube was closed (it needs to be closed if the plastic tube doesn't have an automatic valve);
- to check that the plastic tube is correctly positioned into the trachea (there is a risk that, if the intubation is made under difficult conditions, the plastic tube may be accidentally inserted into the esophagus), we proceeded to the auscultation of the lungs with the stethoscope after an insufflation with the balloon. The vesicular murmur and the thoracic distension once the air was insufflated confirmed that the plastic tube was correctly inserted;
- the plastic flexible tube was connected on to the anesthetic circuit and narcosis was induced;
- the tube was fixed to the animal's jaw, to prevent the intratracheal movement of the tube and to avoid tracheal lesions.

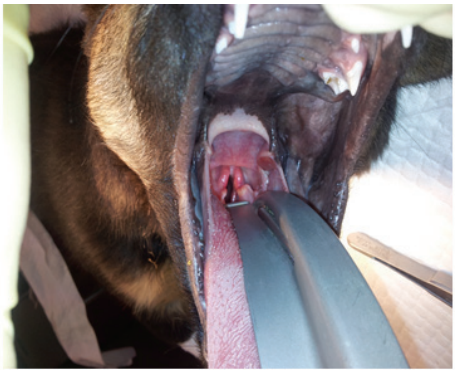

Figure 1. Visualization of the tracheal opening

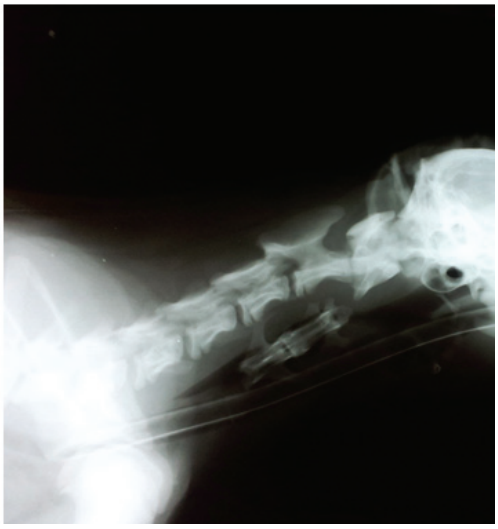

Figure 2. Radiological visualization of the endotracheal plastic tube

Figure 3. Monitoring the patient's vital signs

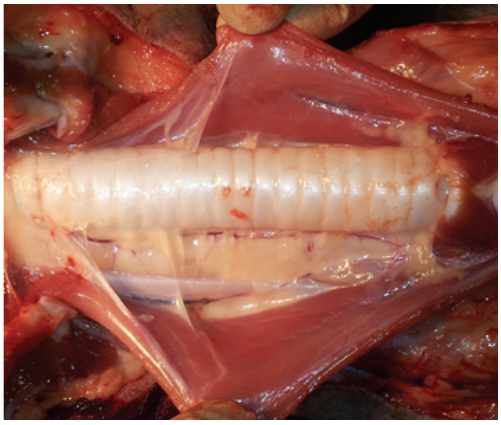

Figure 4. The dissection of the ventral region of the neck

RESULTS AND DISCUSSIONS

After the inhalation administration of the Isoflurane, we noticed: an insignificant growth of the enzymatic activity of the aspartate amino-transferase (GOT/AST), alanine amino-transferase (GPT/AST), gamma glutamil-transferase (GGT), the total amylase, glycaemia, creatinine and urea above the initial determination. Comparing Isoflurane with Sevoflurane, in the case of Isoflurane, the enzymatic activity is higher.

The phases of anesthesia were characterized by induction of the anesthesia in a moderate time (15-20 minutes), with some noticeable respiratory complications (apnea, laryngeal spasm, coughing), the wakening took 15-25 minutes. The minimal alveolar concentration (MAC) of the Isoflurane is about 1,5 %, according to the animal's weight and body structure, lower than Sevoflurane; it is lower in older patients in which a larger amount of anaesthetic administered for a longer period of time produces a respiratory depression faster than in a younger organism.

The accentuation of the depth of the anesthesia allows endotracheal intubation without using muscle relaxants. The smell, that is irritating and stinging, allowed mask induction in a higher rate of the respiratory complications (apnea, abolition of the respiration, laryngeal spasm and coughing) than Sevoflurane. Thus, the smell slowly affects induction.

The rapid growth of the alveolar inspiratory concentration with Isoflurane is translated by a fast anesthetic induction. The frequency of salivation, stopping of the breathing, laryngeal spasm, are easily increased than in Sevoflurane and are controllable. The anesthesia induction was realized by increasing the inspirited concentration of Isoflurane, the induction time was shorter without complications (cardiac and respiratory), using a high concentration, the technique of the prepared circuit (primed circuit), comparing with an conventional method of progressive induction.

By using practical tests, we have demonstrated the fact that the substance rapidly enters the system, after an inhalation administration, and after the administration is stopped, the blood concentration of the anesthetic has quickly decreased.

We have attempted to demonstrate that there is a certain nephrotoxicity and a liver toxicity in the case of using Isoflurane. According to the physical and chemical data, the organism biotransformation of the Isoflurane is reduced, but larger than Sevoflurane. Approximately 0.25% of the administered quantity is found as metabolites, most of them are excreted by the kidneys as trifluoroacetic acid and fluorine. These metabolites, when are found in a great plasmatic concentration, may become nephrotoxic, by changing the renal perfusion.

From point of view of nephrotoxicity and liver toxicity, the pre- and post-surgery biochemical tests have demonstrated that the renal and liver function where unaffected in canines.

Like any other inhalation anaesthetic, a large blood concentration of Isoflurane determines a respiratory depression and a an increase in the partial arterial pressure of CO_2.

CONCLUSIONS

In the dogs that were anesthetized using Isoflurane, there were no side effects (vomiting, seizures, agitation) and the temperature, cardiac frequency, respiratory frequency and the oxygen saturation of the peripheral tissue were normal.

The inhalation anesthesia did not modify the postop blood parameters.

The respiratory elimination of the anaesthetic allows a better control of the anesthesia and a rapid wakening in case of any complication.

The pleasant, yet acrid and irritating smell allows mask induction and intubation without the use of muscle relaxants, but with weaker effects than Sevoflurane.

We have noticed that the side effects on organs and systems are minimal.

The side effect metabolites fluorine based that have a great plasmatic concentration may influence the renal function in a negative way.

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THE ANESTHESIA THROUGH INHALATION IN ONE SURGICAL EMERGENCY OF DIGESTIVE TRACT OF A CAT

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Abstract

This paper is based on the clinical experience in emergency surgery in pets, consisting of foreign bodies that remain stranded in certain parts of the digestive tract. We present the clinical case of a cat, 8 years old, European breed, that was brought by its owners at a veterinary hospital with the following symptoms: dyspnea, polypnea, dysphagia, apathy, sialorrhea, a normal vesicular murmur, normothermia. We made an abdominal ultrasound, took blood for analysis and afterwards, a radiographic exam, a lateral-cervico-thoracic and a ventro-dorsal radiography of the same region. From the data obtained from the owners, the animal never swallowed anything in the past. Until the examination day, there were no signs of illness or another pathology. The biochemical exams showed no alteration of the main organs (the pancreatic, liver, and renal function) and the hematologic parameters were also normal. After the radiographic examination of the cervical region, a foreign body was discovered. It had almost 6 centimeters, was stuck in the anterior third of the esophagus, had a needle-like shape, with one sharp end ventrally oriented, and the other blunt one, dorsally positioned. An emergency surgery was made, with endotracheal intubation and the removal of the foreign body, which was represented by a sewing needle, which had perforated the esophagus, positioning itself transversally through the latero-cervical muscles, and having a ventrally end positioned into the esophagus and the other end, subcutaneous in the dorsal cervical area. Among the emergency surgeries in pets, foreign body pathology has an important role, because of the need to establish a quick diagnose and to treat the animal as fast as possible. The clinical and radiological exam has tracked down a foreign body in the esophagus. We used endotracheal intubation for the anesthesia. Through specific and adequate surgical maneuvers, we managed to extract the foreign body. Knowing the animal's habits has an important role, thus making the diagnose and treatment more accurate and not to threaten the animal's life.

Key words: *emergency, anesthesia, digestive tract.*

INTRODUCTION

This study approaches one of the currents of veterinary medicine, emergency medicine in fact. Most of the times, the fast actions of the vet may mean the difference between life or death. This work is based on the clinical experiences in emergency medicine of pets, of a private clinic, consisting of foreign bodies that remain stranded in various portions of the digestive tract. In such a situation, the first thing we have to do is to ensure the safety of the respiratory pathways, breathing, circulation and the assessment of the potential external hemorrhages.

MATERIALS AND METHODS

We present a clinical case of an apart complexity. We are talking about a cat, 8 years old, European breed, that was brought at a veterinary hospital with the following symptoms: dyspnea, polypnea, dysphagia, apathy, sialorrhea, a normal vesicular murmur, normothermia, normal cardiac output, normal blood pressure.

RESULTS AND DISCUSSIONS

From the data obtained from the cat′s owner, we noticed that it had never eaten any other foreign body. Until the examination day, there were no signs of illness and no other pathologies.

Abdominal ultrasound results: the liver had easily increased diameters, the gallbladder had a slightly transonic content; the kidneys had a normal shape, no dilations, both kidneys had hyperecogenous images, probably microscopic stones, and the urinary bladder had a large amount of hyperecogenous deposit.

Biochemical test results: GOT-34.5, GPT-47.1, GGT-6.4, FA-57.8 , BIL-0.5 , PR-7.8 ., ALB-3.2 , UREE-35.4 , CRE-1.2 , ALFA AMIL.-1548 , GLI-65. The biochemical results showed no alteration of the main organs (normal liver, pancreatic and renal function).

The hematological results: WBC-15, LYM-2.1, GRA-10.3 , RBC-8.5, HGB-17 , HTC-42 ,MCV-70, PLT- 218. Neither these parameters were not altered.

Figure 1. The biochemical and hematological laboratory

After the talk with the owners, the biochemical, hematological and ultrasound investigations, we started the radiological investigation. When the cranial-cervico-thoracic region, in a lateral and ventro-dorsal position was examined, a foreign body was discovered. It had almost 6 centimeters, was stuck in the anterior third of the esophagus, had a needle-like shape, with one sharp end ventrally oriented, and the other blunt one, dorsally positioned.

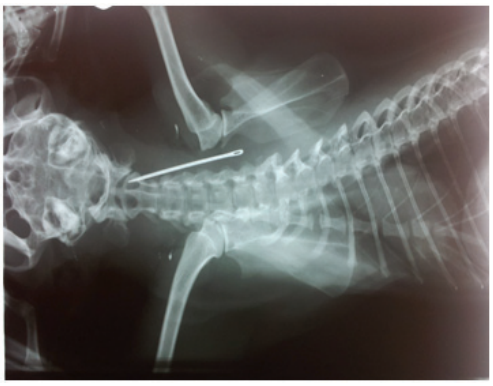

Figure 2. The cranial-cervico-thoracic region, ventro-dorsally examined

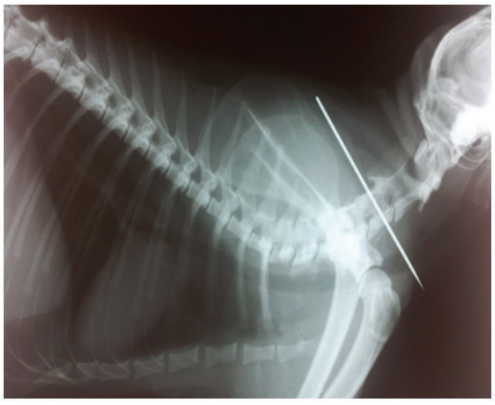

Figure 3. The cranial-cervico-thoracic region, laterally examined

We had to proceed to an emergency surgery, with endotracheal intubation. The intubation procedure was a very difficult one, because of the foreign body′s positioning in the trachea. The surgery was a catchy one, because after the intubation, the animal was apneic, and the pulse was weak and it had a low oxygen saturation.

In the first step, we dissected the cervical region, to bear away the anatomic structures and not to threaten the animal′s life when the foreign body was removed. We managed to extract the foreign body, in fact a sewing needle that had attached to itself about 40 centimeters of string. It had perforated the esophagus, positioning itself transversally, with one end situated laterally from the tracheal rings and the other one, dorsally subcutaneous, after it had perforated the cervical muscles. The string that was attached to the needle got into

the cat′s stomach, and it was removed at the same time as the needle.

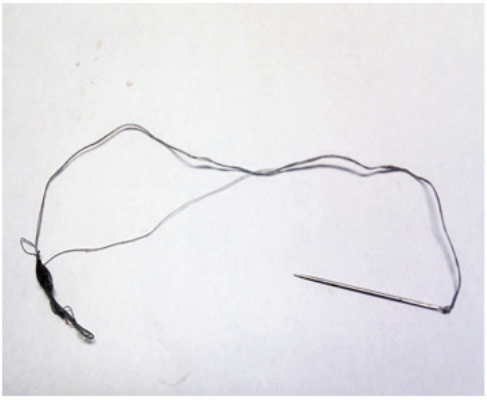

Figure 4. The sewing needle and the string in the animal′s esophagus

CONCLUSIONS

The clinical and radiological examination tracked down the esophageal foreign body.

The surgery was made with endotracheal anesthesia.

Through specific and adequate surgical maneuvers, the foreign body was successfully extracted.

Knowing the animal′s habits allows a proper and fast diagnose and an adequate treatment.

ACKNOWLEDGEMENTS

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ULTRASONOGRAPHIC ASPECTS IN URETEROHYDRONEPHROSIS IN DOGS

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Abstract

Ureterohydronephrosis is characterised by progressive dilatation and urine accumulation inside the renal basinet and/or ureters. A number of 40 dogs were evaluated, from different breeds with different ages by physical, imagistic and laboratory (hematologic and biochemical) examination. The aim of this study was the evaluation of ultrasonographic aspects and blood changes in ureterohydronephrosis in dogs. Physical examination revealed abdominal distension (23/40), pain sensibility (31/40) and uremic syndrome signs (21/40), Laboratory investigations revealed hemoconcentration (18/40), hypochrome anemia (13/40) and leucocytosis (12/40). Ultrasonographic, different degrees of basinet and ureters dilatation were noticed accompanied by renal structural changes.

Key words: *dogs, ultrasonography, ureterohydronephrosis.*

INTRODUCTION

Ureterohydronephrosis is characterized by a nephropathy with reduced incidence in dogs, caused by progressive dilatation by urine accumulation in renal basinet level and/or ureters. This aspect is the result of urinary ways obstruction and is accompanied by atrophic changes of compressive type in renal parenchyma level. (Codreanu M.D. 2010)
Ureterohydronephrosis presents clinical aspects of different intensities produced by varied causes and some metabolic correlations depending on renal lesion.
Usually, hydronephrosis appears unilateral and remains without a clinical response because of function overtaking by the other kidney (M. Codreanu, 2008). Bilateral hydronephrosis appears rare, usually secondary to lesion in trigon, prostate or urethral level (Christie, B.A. Bjorling, 1993).

MATERIALS AND METHODS

This study was carried out inside the clinic of Faculty of Veterinary Medicine Bucharest on a number of 40 dogs with ages between 6 to 15 years suspected for ureterohydronephrosis, which were examined clinically, ultrasonographic, hematologic and biochemical, suspicioned of ureterohydronephrosis.
Clinical examined was carried out through general methods according to instruction from specialty materials (Vlăgioiu, 2007). In hematologic examination, red cellular series were investigated (direct erythrocyte constants, derived erythrocyte constants) as well as leucocyte series by quantity (number) and quality (percentage). In biochemical examination, the following parameters were examined: urea, creatinine, albumin, globulin and total protein.
Imagistic, ultrasonographic investigations were carried out, appreciating abdominal distension degree as well as renal lesions as a result of ureterohydronephrosis.

RESULTS AND DISCUSSIONS

Clinical examination revealed abdominal distension of different degrees, uremic syndrome (inapetence, vomiting, hypothermia and neurodepresive syndrome) and pain sensibility in profound palpation in renal level. Abdominal distension was present in 23 of 40 dogs (57.5%), uremic syndrome in 21 of 40 dogs (52.5%) and pain sensibility in 31 of 40 dogs (77.5%)
Hematologic examination revealed leuocytosis aspects (increase in white cells number) in 12 out of 40 dogs (30%), hemoconcentration and hemoglobin level increase in 18 out of 40 dogs

(45%) and hypochrome anemia (decrease of corpuscular hemoglobin concentration) in 13 out of 40 dogs (32.5%).

In biochemical examination, increased values of serum urea were noted as follows: between 45 and 95 mg/dl in 22 out of 40 dogs (55%), 95-160 mg/dl in 12 out of 40 dogs (30%) and over 160 mg/gl in 6 out of 40 dogs (15%). Increase in serum creatinine level was noted in 17 out of 40 dogs (42.5%) with values between 2 and 3 mg/dl, 16 out of 40 dogs (40%) with values between 3 and 6 mg/dl and values over 6 mg/dl in 7 out of 40 dogs (17.5%).

Decrease in serum albumin was severe in 15 out of 40 dogs (37.5%) and moderate in 21 out of 40 dogs (52.5%).

Abdominal echography has revealed ureterohydronephrosis aspects which affected one kidney (unilateral) in 35 out of 40 dogs (87.5%) or both kidneys (bilateral) in 5 out of 40 dogs (12.5%)

From renal lesion degrees point of view, the following were determined:

- ureterohydronephrosis of first degree (Fig. 1), in 5 out of 40 dogs (12.5%), by dilatation of basinet, ureter and calices. Caliceal structure was appreciated as normal.

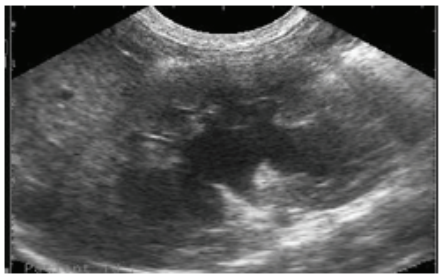

Figure 3. ureterohydronephrosis third degree

- ureterohydronephrosis of second degree (Fig. 2), in 12 out of 40 dogs (30%) characterized by basinet, ureter and renal calices dilatation.
- ureterohydronephrosis of third degree (Fig. 3) in 11 out of 40 dogs (27.5%), with basinet, ureter dilatation and caliceal cup reverse (excentric convexity)
- ureterohydronephrosis of fourth degree (Fig. 4) in 10 out of 40 dogs (25%) with obvious basinet, ureter and calices dilatation with reduction of parenchyma index.
- ureterohydronephrosis of fifth degree (Fig. 5) in 2 out of 40 dogs (5%), with extreme dilatation of urinary ways and lack of renal parenchyma.

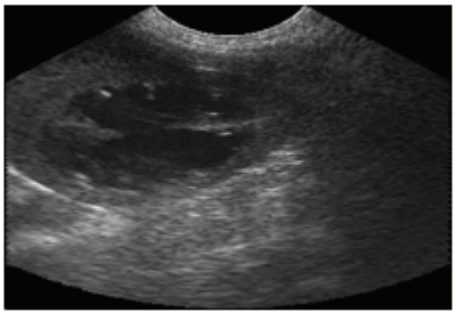

Figure 1. ureterohydronephrosis first degree

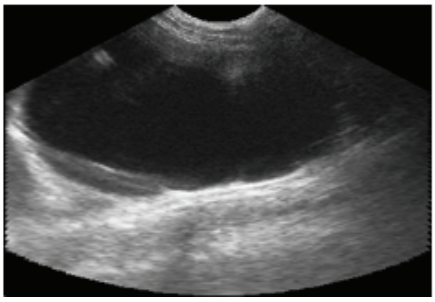

Figure 4. ureterohydronephrosis fourth degree

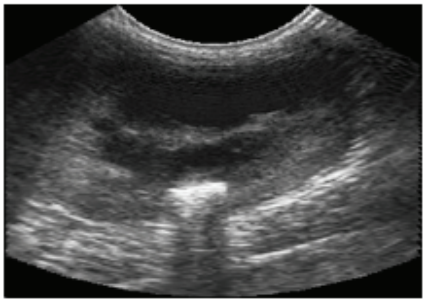

Figure 2. ureterohydronephrosis second degree

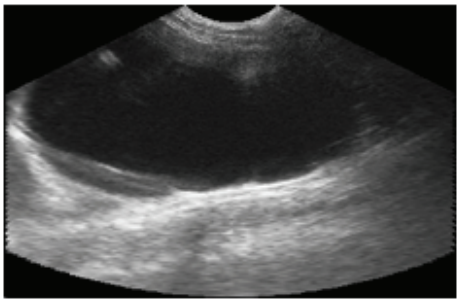

Figure 5. ureterohydronephrosis fifth degree

Analysis of obtained data evidences abdominal pain presence, of different intensities, in ureterohydronephrosis, being the main sign in this lesion type, fact related in previous studies (Ettinger, 1989; Stone, 1990; Osborne et al, 1995; Abd-El-Roofy, 2005).Hematologic changes registered in this study, represented by hemoconcentration and hypochrome anemia are similar with results obtained by Rousset et al. (2011).Abd-El-Roofy (2005) didn't meet red cellular series changes in his study.Regarding biochemical examination, our results showed increase in serum urea and creatinine levels, changes noted by other autors as well (Abd-El-Roofy, 2005; Mehmet et al., 2005). Serum albumin has registered low values, our results being similar to those recorded by other authors (Nelson and Cottfo, 1992; Dan woo and Chang, 2001; Abd-El-Roofy, 2005).

Causes that lead to ureterohydronephrosis, in dogs evaluated in this study, were connected to urethral obstructions in most cases, fact known in specialty literature (Osborne and Finco, 1995; Mehmet et al., 2005). Other than these causes, in ureterohydronephrosis pathogenesis, adherences after ovariohysterectomy can be included (Dragu I., 2012).

Age of animals included in this study varied from 6 to 15 years with an average of 10.4 years. Still, a large number of cases were registered in 7 and 13 years old dogs. Our results demonstrate that this affection was encountered in adult and respectively old animals. Unlike these results, previous studies have identified ureterohydronephrosis in much younger animals, 6 months (Mehmet et al., 2005), respectively 9 months (Rousset et al., 2011), being considered as consequences of embrio development disorders.

CONCLUSIONS

1. From clinical examination of 40 dogs with ureterohydronephrosis, abdominal distension of different degrees, pain sensibility in profound palpation in renal level as well as specific signs for uremic syndrome (inapetence, comiting, hypothermia and neurodepresive syndrome).

2. Hematologic, changes in red cellular series were encountered, translated by hemoconcentration, hypochrome anemia, and in white cellular series, leucocytosis.

3. Biochemical analysis registered increased values in serum urea and creatinine levels and decrease of serum albumin, specific in renal functional failure.

4. Ultrasonographic, basinet, ureters and calices dilatation of different degrees ending with renal parenchyma disappearance.

5. Anamnestic information correlated with clinical and paraclinical (ultrasonography, hematology and biochemistry) in a number of 40 dogs suspicioned with ureterohydronephrosis, represent an optimum protocol in diagnosis of urinary system pathology.

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MEDULLARY SYNDROME OCCURRED DUE TO A FIBROSARCOMA OF BONE OF THE VERTEBRAL BODY

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Abstract

In this report, a 7 years old male Pekingese dog case is described.
The dog presented for a medical investigation with a complex medullar syndrome (motility and sensitivity of the hind limb abolished). From history, we deduced that this paralysis of the hind limb was installed gradually.
Clinical examination was followed by X-ray examination, which showed significant osteolysis of the L6 vertebral body. This lesion has a direct connection with the symptoms being the cause of the hind limb paralysis. Sampling of biological material by excisional biopsy and later of the histological examination of it, confirmed the presence of a malignant process, namely a fibrosarcoma of bone.
It is important to point out that malignant neoplastic process of the spine have significant compressive effects on the nerve substance (spinal cord) causing clinical expression with a progressive character, from motility and sensitive mild dysfunction to its abolition.

Key words: *medullary syndrome, excisional biopsy, fibrosarcoma of bone.*

INTRODUCTION

Fibrosarcoma of bone is a mixed malignant tumor that affects the bone tissue being usually localised in the apendicular skeleton, and rarely in the afferent axial skeleton of bone tissue (Moore et al., 2000).
As all chronic progressive diseases common to tissues localised near to the spinal cord, also fibrosarcoma of bone causes some motor and sensory progressive dysfunction. It is very important that these spinal disorders be diagnosed earlier to determine the prognosis and therapeutic protocol as soon as possible (Dickerson et al., 2001).
The diagnosis protocol is very complex, consisting of clinical, radiological examination, RMN, (Aembrust et al., 2004 and Pooya et al. 2004) but for a certain diagnosis it is used the histological examination, preceded by excisional biopsy.

MATERIALS AND METHODS

The case is represented by a 7 years old male Pekingese dog.
The subject presented for a medical investigation, clinically expressing paraplegia (parlaysis of the hindlimb), with the motive and sensory function of the hindlimb abolished (Fig. 1).
After the clinical examination, a spinal compression syndrome was suspected. In order to establish a diagnosis of certainty, the animal was then successively subjected to a radiological examination, biopsy excision and histological examination.

Figure 1. Motive and sensitive disfunction of the hindlimbs

RESULTS AND DISCUSSIONS

After the clinical examination, the animal was subjected to the radiological examination, using a ventro-dorsal incidence, with a maximum extension of the pelvic limbs.
After the radiological examination (Fig. 2) the osteolysis of L6 vertebral body was emphasized with the lack of the left transverse process.

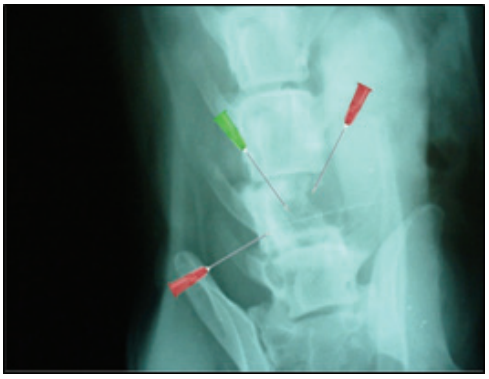

Figure 2. Osteolysis of L6 vertebral body.

Considering that the bone structure affected by severe osteolysis (lumbar vertebrae) has an intimate contact with the nerve substance (spinal cord), it is clear that the damage on the nerve substance is irreversible, for which the prognosis is grave. There were subsequently taken modified tissue samples for histological examination, using radio guidance (Fig. 2). It was seen on the smear the presence of an unstructured necrosis (necrotic cell rests with nude nuclei), gaps of non-uniform compact bone, rich fibroblastic cellularity which delimitates the necrotic area and penetrates the bone gaps, disorganizing the compact structure. The morphological appearance is specific to a fibrosarcoma of bone (Fig. 3). Fibrosarcoma of bone like other chronic lesions with peridural localization can cause some nervous syndromes expressed by sensitivity and motility disturbances of variable intensity.

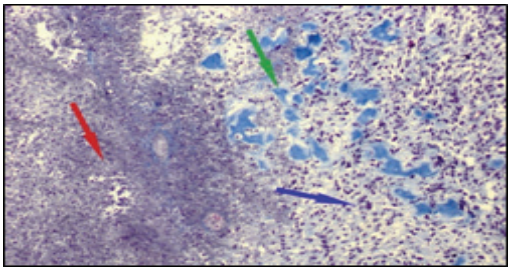

Figure 3. Necrotic cell rests (red arrow), lacunae in compact bone tissue (green arrow) and disorganized compact structure (blue arrow). Fibrosarcoma. (H& E stain, 10x.)

CONCLUSIONS

Medullary syndrome is a complex etiopathogenesis as etiological factors are involved: inflammatory, degenerative processes, neoplastic processes, met nearby anatomical structures in the spinal cord.

Neoplastic processes present in spinal bone structures cause motive and sensory dysfunction, being directly correlated with the intensity of the lesion.

Radiological excisional biopsy has significant importance for establishing confirmatory diagnosis in suspect neoplastic diseases of the spine.

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TICK FAUNA OF GOAT AND SHEEP IN BELGRADE AREA

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Abstract

The study about tick fauna and season distribution of tick of small ruminant at spread Belgrade area was started in March and finished in November 2011. During study we examined a total of 91 flocks of goats and sheep from 6 Belgrade districts. In total we examined 281 sheep and 122 goats. Ticks infestation we occurred at 169 (60.14%) sheep and 42 (34.42%) goats. Most abundant were Ixodes ricinus, followed by Dermacentor marginatus, Rhipicephalus sanguineus, R.bursa, Haemaphysalis punctata, D.recticulatus.

Key words: *ticks, goats, sheep, Belgrade area.*

INTRODUCTION

Breeding of sheep and goats were increased during last decade on Belgrade area. Today, small flocks of sheep and goats play an important role in providing animal protein for diet, especially for those people who live in village at mountains part of Belgrade area. They are usually kept under extensive conditions and graze or brows on any land that is not being cultivated (Pavlovic et al. 2009).
Way of breeding had prerequisite to a lot of infections including parasites. Examination of goats and sheep parasitizes were only sporadically performed at last 20 years in Serbia and we had only a few paper about it. Most of the research related to helminthes infection (Pavlovic et al. 2011a,b). At the same time, survey of the fauna and ectoparasites are almost done and we are only sporadic presence of certain species of arthropods in sheep and goats (Pavlovic et al.1995, 1997).
In pasture breed condition tick infestation are common especially during late spring and autumn months (Pavlovic et al.1995, Milutinovic et al. 1998) and aim of our examination are to established tick fauna at flocks of goats and sheep in Belgrade area.

MATERIALS AND METHODS

The study about tick fauna and season distribution of tick of small ruminant at spread Belgrade area was started in March and finished in November 2011. During study we examined a total of 91 flocks of goats and sheep from 6 Belgrade districts. In total we examined 281 sheep and 122 goats. Ticks were collected from sheep and goats by means lightly sprung forceps. All specimens were placed into glass specimen bottles which had a piece of hard paper inserted bearing the name of locality name of host and date and hour of collection. The tick species were detected using keys given by Pomerancev (1950) and Kapustin (1955).

RESULTS AND DISCUSSIONS

Ticks infestation we occurred at 169 (60.14%) sheep and 42 (34.42%) goats. Most abundant were *Ixodes ricinus,* followed by *Dermacentor marginatus, Rhipicephalus sanguineus, R.bursa, Haemaphysalis punctata* and *D.recticulatus.* The collected tick specimens, a total of 2768 were adult′s females and males belonged to the *ixodidae* family.
The population dynamics of recorded tick species are known for their two maxima a year- in spring (April-May) and in autumn (September-October). The considerable interchange between spring and autumn tick populations can be attributed mainly to environmental conditions. The population maximum for three species *Dermacentor marginatus, D.recticulatus* as well as *Haemaphysalis punctata* occured in April. May was the month of the population peak for

I.ricinus and it was noted that this species started to decrease in abundance in June. R.*hipicephalus sanguineus* and *R.bursa* reached their maxima decreasing gradually until August, and disappearing completely in September and October.

The autumn population peak in September and in October occurred for the *I.ricinus, Dermacentor marginatus* and *Haemaphysalis punctata.*

Ticks were found on 60.14% of examined sheep. Relative abundance analysis revealed that the species at sheep *I. ricinus* was absolutely dominant 41.91%, followed by *Dermacentor marginatus* (32.91%), *Rhipicephalus bursa* (17.22%), *R.sanguineus* (6.72%), *Haemaphysalis punctata* (2.21%) and *D. recticulatus* (1.17%).

Ticks were found on 34.42% of examined goats**.** Relative abundance analysis revealed that the species at goats *I. ricinus* was absolutely dominant 64.42%, followed by *Rhipicephalus bursa* (17.22%), *R. sanguineus* (6.72%), *Haemaphysalis punctata* (4.22%) and *Dermacentor marginatus* (2.91%).

Similar results we obtained during examination of ticks fauna in west and east part of Serbia where *Ixodes ricinus* and *Dermacentor marginatus* are dominant tick species at sheep (Milutinovic et al.1996, 1998)

At the same time in the investigated areas at the goat *I.ricinus* and *Haemaphysalis punctata* were most abundant species in contrast of Belgrade area where, except *I.ricinus,* second dominant species were *Rhipicephalus bursa.* During examination performed in Belgrade area by Milutinovic et al. (1997), Dimitric (1999) and later by Pavlovic. et al. (1999,2002) most abundant tick species were *I. . R.sanguineus, D.recticulatus* and *D.marginatus*. Those ticks' species were occurred in dog population and at foxes and badgers hunted at spread Belgrade area (Pavlovic et al. 1997b).

Our studies conducted over a dozen years later, it was determined that the situation has no changed in terms of ticks species and its number and confirmed dominate role of *I.ricinus* and *Rhipicephalus* species at Belgrade area.

CONCLUSIONS

During study performed in 2011.we examined a total of 91 flocks of goats and sheep from 6 Belgrade districts.. Ticks infestation we occurred at 169 (60.14%) sheep and 42 (34.42%) goats. Most abundant were *Ixodes ricinus,* followed by *Dermacentor marginatus, Rhipicephalus sanguineus, R.bursa, Haemaphysalis punctata* and *D.recticulatus.* These findings are of great epidemiological importance because these types of ticks transmit a multitude zoonotises like *Borellia burgdefori, Erlicihia spp., Anaplasma spp., Tick-born encephalitis, numerous haemorrhagic fewer* and etc.

ACKNOWLEDGEMENTS

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EFFECT OF BALANCED ANAESTHESIA ON CANINE LYMPHOCYTE APOPTOSIS

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Abstract

It is well established that major surgery, anaesthesia, and sedation compromise a wide range of immune function that may predispose patients to postoperative infections, septic complications, and tumour spread.
The immunosuppressive effects of general anaesthesia are quite different and depend on the used anesthetic agents, dose and combinations. We tested the hypothesis that perioperative lymphocytopenia is due to apoptosis of these cells induced by balanced anesthesia. The relation of lymphocyte apoptosis to the anaesthesiological stress and concentrations of the main pro-and anti-inflammatory cytokines was also investigated.
Based on the results we concluded that balanced anaesthesia used in the present study induced lymphocytopenia by activation of apoptosis of these cells which was due to the combined apoptogenic effects of halothane, fentanyl, and pancuronium, but neither to the anaesthesia-related stress-response nor to changes in the main pro-and anti-inflammatory cytokines TNF-alpha and IL-10. Total lymphocyte count was diminished on the expense of B-lymphocytes without significant changes in CD 5+ and CD 8+ cells.
Clinical implications: balanced anaesthesia disturbs normal humoral immune response by decreasing the count of B-lymphocytes for minimum 24 hours after anesthesia.

Key words: *apoptosis, balanced anaesthesia, dog, lymphocyte.*

INTRODUCTION

It is well established that major surgery, anaesthesia, and sedation compromise a wide range of immune functions that may predispose patients to postoperative infections, septic complications, and respiratory dysfunction (Bolke et al., 2001). Many investigations reported lymphocytopenia induced by anaesthesia and surgery (Oka et al., 1996; Isitmangil et al., 2002; Yokoyama et al., 2005). Very few are studies on the immune effects of anaesthesia alone without surgery. The aim of our work was to explore the effect of balanced anaesthesia on some aspects of immunity with elimination of surgery related influences such as pain, tissue damage, blood loss and transfusion, organ dysfunction, inflammation.
The immunosuppressive effects of general anaesthesia are quite different and depend on the used anesthetic agents, dose and combinations. We investigated the hypothesis that perioperative lymphocytopenia is due to apoptosis of these cells induced by balanced anesthesia. Balanced anesthesia was chosen because it has the advantages that combination of narcotic, opioid and muscle paralyzing agents enables to decrease the total dose of narcotic by 75%. Moreover, this kind of anesthesia is very useful for cesarean section because a fetal respiratory suppression is minimal.
There are some discrepancies about induction (Matsuoka et al., 2001) or not (Ohara et al., 2005) of lymphocyte apoptosis by anaesthesia. Balance between cell survival and death is under strict genetic control. Deviations in any direction can lead to disease process. Decreased apoptosis is connected with the development of cancer (Bogler et al., 1995), autoimmunity disorders, or viral infections (Young et al., 1997). Excessive apoptosis is related to endotoxaemia, sepsis and multiorgan failure (Nielsen et al., 2005).
The relation of lymphocyte apoptosis to the anaesthesiological stress and concentrations of the main pro-and anti-inflammatory cytokines was also investigated.

MATERIALS AND METHODS

Anaesthetic protocol

The study was performed on 8 healthy mixed breed dogs, aged between 3 and 5 years, with mean body weight of 17.9 ± 3.5 kg, equalized in gender. In order to avoid an environmental stress the animals were kept under equal living conditions and fed on a commercial diet for a month. They received also antiparasitic drugs and were routinely vaccinated.

All dogs received one and the same scheme of balanced anesthesia that was achieved by a combination of halothane, pancuronium bromide, and fentanyli cytrate. Atropini sulfas (Sopharma-Bulgaria, 0.02mg/kg.m, S.C.), and acepromazini maleas (CombistressÒ, Kela-Belgium, 0.1 mg/kg.m, I.M.) were used for premedication. Twenty minutes later thiopentalum natrium (Biochemie GmbH-Austria, 10 mg/kg.m, I.V.) applied as a 2.5% solution was injected for induction of anaesthesia and endotracheal tube was inserted. Anaesthesia was maintained as follows: an inhalation agent halothane 0.5% in 100% oxygen was inhaled through a semicircuit respiratory system; a muscular paralyzing agent pancuronium bromide (PavulonÒ, Troyapharm-Bulgaria) was applied intravenously by 0.06 mg/kg.m initially with repetition of a half of the initial dose when a single spontaneous respiratory effort recurs; an opioide agonist fentanyli citras (StobiumÒ, Research Institute of Chemistry and Pharmacology-Bulgaria) was applied every 30 minutes I.V.). in dose 0.01mg/kg.m. Respiration was maintained by artificial ventilation of the lungs by oxygen flow of 20ml/kg and rate of 12 minutes $^{-1}$.

Depth of anaesthesia was maintained in surgical stage by tracing of some important unconditioned reflexes (eye globe in central position, on the average dilated pupils, lack of corneal, palpebral, patellar, anal, and swallowing reflexes). Venous line was created by canulating of a cephalic vein using a venous catether 22G, 25mm (Vygon GmbH & Co., Germany). The infusion rate of saline solution was 10ml/kg.m/h.

Total duration of anaesthesia was 120 minutes. After recurrence of four spontaneous respiratory movements 10 mg galanthamini hydrobromide (NivalinPÒ, Sopharma-Bulgaria) was used I.V. for recovery from neuromuscular blockade.

Extubation was performed after recurrence of the swallowing reflex. The animal was considered brought out of anaesthesia when it was able to raise his head and took sternal recumbence.

Blood sampling and processing

Venous blood samples were obtained immediately before (0 minute), during deep anaesthesia (120 minute), and on the next day (24 hour). The blood was withdrawn from v. jugularis, which was preliminarily canulated. KF + Na_2EDTA 15mmol solution was used as an anticoagulant. Half of the collected blood was used for counting of lymphocyte numbers, measuring the proportion of apoptotic lymphocytes, and determining lymphocyte subpopulations. Plasma was separated from the second half of the collected blood after centrifuging 30 minutes by 1000 rpm and was stored at -25°C. Stored plasma was used later for quantifying of stress hormones and cytokine concentrations.

Total lymphocyte count was calculated from differential leukocyte formula on blood smear. The percentage of B-lymphocyte subset was found out by complement-zimozane indicative complexes that bind to the receptor of C3 component of the complement located on the surface of these cells (ZC-rosette test, Kajdacvy-Balla & Mendes, 1976). Determination of percentage of apoptotic cells as well as T-lymphocyte subsets was performed after isolation of peripheral blood polymorphonuclear cells (PBMCs).

Collection of PBMCs

Isolation of PBMCs was performed under strict sterile conditions with the help of separation medium Histopaque with density 1.083 (Sigma Aldrich, St. Luis, MO, USA). Firstly, 2.5ml whole blood was diluted 1:1 with saline solution. After that 3ml of Histopaque was placed in tube and 4ml of diluted blood was deposited. Centrifugation in 1880 rpm for 40 minutes was accomplished and stratification was attained from the bottom to the surface as follows: red and white blood cells, separation medium, thin ring of lymphocytes, and finally plasma. The lymphocyte ring was fully drown out with pipette, washed three times with

RPMI-1640 medium (Sigma Aldrich, St. Luis, MO, USA), and centrifuged for 10 minutes by 1500 rpm.

Annexin V-FITC-Propidium iodide staining and flow cytometry

Apoptotic lymphocytes were determined by the method of Vermes et al. (1995). This method uses the property of Annexin V to bind to the membrane phospholipid phosphatidilserine in the presence of calcium. Phosphatidilserine is expressed on the cell surface very early in apoptosis, before DNA fragmentation to occur. A percentage of the cells stained with FITC conjugated-Annexin V (Apoptest, Dako Cytomation, Denmark) from total lymphocyte count were determined by flow cytometry analysis (EPICS flowcytometric analyzer Beckman Coulter Inc., USA). The translocation of phosphatidylserine from the inner side of the cell membrane to the outer layer occurs also in necrotic cells. To distinguish cells that had lost membrane integrity, propidium iodide dye was added before analysis. Necrotic and late apoptotic cells are stained with propidium iodide (PI), a DNA-binding dye, because of the loss of the cell membrane integrity. Therefore, AV (+) /PI (-) cells are regarded as early apoptotic, AV (-) /PI (-) as vital, AV (+) /PI (+) as late apoptotic or secondary necrotic cells, and AV (-) /PI (+) as primary necrotic cells.

Lymphocyte subsets were analyzed using FITC-conjugated rat monoclonal antibodies specific anti-canine CD-5 clone and CD8-clone (Bio Source International Inc., USA).

Determination of stress hormones and cytokines

Plasma concentrations of the cytokines tumour necrosis factor-alpha (TNF-alpha) and interleukine − 10 (IL-10) were measured by immunoenzymatic assay ELISA (Sunrise reader, Columbus washing mashine, and Magellan V3.11 software) using mise monoclonal antibodies specific for canine TNF-alpha and IL-10 (R& D Systems, USA).

Plasma adrenaline and cortisol levels were determined by radio immune assay using RIA kits (Amersham Biosciences, UK).

Statistical analysis

Statistical significant differences between three investigated periods were assessed by analysis of variance ANOVA/LSD (Statmost for Windows, DataMost Corp. 1994-1995) and probability lower than 0.05 was considered to be significant.

RESULTS AND DISCUSSIONS

Our results revealed that balanced anaesthesia with halothane, pancuronium, and fentanyl lead to decreased lymphocyte number on the expense of B-lymphocytes without changes in total T-lymphocyte number and T-suppressor cells (table 1).

Table 1. Changes in total lymphocyte count, percentage of apoptotic PBMCs, lymphocyte subpopulations, plasma concentrations of stress hormones and cytokines TNF-alpha, and IL-10 before, during and after balanced anaesthesia in dogs. Data are presented as mean ± SD.

Parameter	0 min	120 min	24 h
Lymphocytes, G/L	4.30± 0.56	1.46± 0.27*	4.42± 0.42
Apoptotic PBMC,% B-lymphocytes,% CD 5+,% CD8+,%	1. ± 1.79 2. ± 2 3. ± 12.4 4. ± 7.4	1. ± 1.52 *** 2. ± 1*** 3. ± 12.6 4. ± 8.6	1. ± 1.70* 2. ± 1*** 3. ± 6.1 4. ± 9.7
TNF-alpha, pg mL^{-1}	0.0± 0.0	0.84± 2.06	0.0± 0.0
IL-10, pg mL^{-1}	36.17± 43.35	36.12± 45.90	36.44± 40.93
Adrenaline, ng mL^{-1} Cortisol, nmol L^{-1}	25.9 ± 4.3 74.5± 40.7	51.7 ± 5.0*** 99.1± 68.1	31.3 ± 4.6* 31.9± 25.3*

*p< 0.05, **p< 0.01, ***p< 0.001 versus initial period;

Balanced anaesthesia induced apoptosis of PBMCs that lasts at least 24 hours (figure 1).

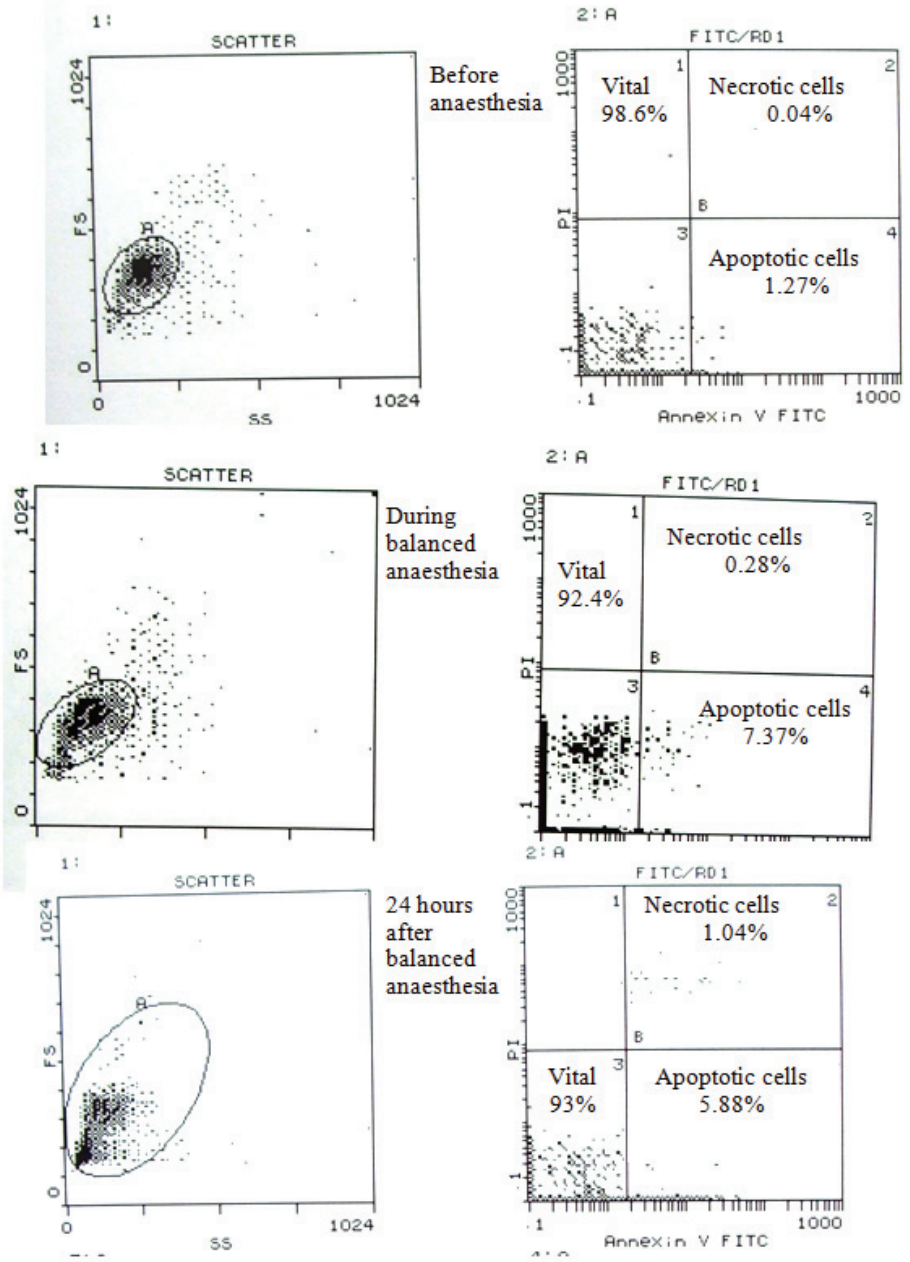

Figure 1. Flow cytometry report of discrimination between vital, early apoptotic, and secondary necrotic lymphocytes using Annexin V FITC/Propidium iodide staining in a dog immediately before, at 120 minute of balanced anaesthesia, and 24 hours after anaesthesia.

Cytokines did not show any alterations during and after anaesthesia. Plasma concentration of adrenaline increased at 120 minute of anaesthesia but with the tendency to decrease on the next day. Cortisol increased insignificantly during anaesthesia, but 24 hours later its concentration dropped bellow the initial level.

Balanced anaesthesia that we performed on dogs was comprised by halothane, fentanyl and

pancuronium after induction with thiopenthal sodium. Barbiturates as anaesthesia-inducing agents have been reported to have a cytocidal action on lymphocytes in vitro (Trowwll, 1958) but at clinical dose this effect appears to be negligible.

Almost all inhalation anaesthetics induced apoptosis of normal peripheral lymphocytes in vitro by time and dose-related manner (Matsuoka et al., 2001; Loop, 2005). These investigations can not be extrapolated in clinical environment because they are accomplished in vitro on human and mice cells but not on canine cells.

Yamada et al. (2001) carried out a similar to our study on dogs anaesthetized with thiopental and halothane or isoflurane without any surgery. They concluded that both anaesthetics induced lymphocytopenia that was due to increased apoptosis of these cells.

There are several evidences that opioids modulated cell survival and death (Wang et al., 2002; Ohara et al., 2005). Immune competent cells express opioid receptors thus under the impact of the opioids they submit to apoptosis (Tegeder & Geisslinger, 2004). Fentanyl showed a strong apoptogenic effect on isolated peripheral lymphocytes in vitro (Delogu et al., 2004). In this research fentanyl initiated time-dependant lymphocyte apoptosis by alterations in their membrane redox metabolism. In another study Delogu et al. (2003) revealed that incubation of lymphocytes with clinically applicable concentrations of pancuronium also induced apoptosis of these cells.

Apoptosis is a genetic controlled process that could be induced by several activators such as stress, reactive oxygen species (ROS), and cytokines by three different main ways: binding of TNF-alpha to the specific receptors, mitochondrial mechanism or by apoptosis-inducing factor (AIF).

One of the most potent inductors of apoptosis is ROS. In our previous study we did not fund an increased plasma malondyaldehyde levels as a parameter of oxidative stress (Simeonova et al., 2004) thus we excluded the possibility of ROS-inducible lymphocyte apoptosis during balanced anaesthesia.

Direction and the strength of the immune response could be affected by cytokines. They work on an apoptosis of the cells by different ways (Matsuda et al., 2001). IL-10 increased the viability of the cells by increasing the expression of protective molecule Bcl-2 and thus suppressing apoptosis (Cohen et al., 1997). Delogu et al. (2001b) found out that increased production of IL-10 during anaesthesia and surgery had been in correlation with the increase apoptosis of T-lymphocytes. According to our results induction of lymphocyte apoptosis by balanced anaesthesia was not in relation to IL-10 because we did not observe any statistical significant changes in its plasma concentrations. Moreover, there were not any alterations in the levels of TNF-alpha which is considered as a direct inductor of apoptosis and these results were contrary to the data of Kotani et al. (1999) who claimed that inhalation anaesthetics halothane, isoflurane, enflurane, and sevoflurane increased gene expression of proinflammatory cytokines including TNF-alpha. Obviously, the rise in pro-inflammatory cytokines has been referred to be due to the stress response to surgery but not to anaesthesia alone. The increase in concentrations of proinflammatory cytokines TNF-alpha, IL-1, and IL-6 was approximately proportional to the severity of the surgery (Kumar et al., 2002).

After examination the mechanism of stress-induced apoptosis Fumarola & Guidotti (2004) concluded that different cell types engage more than one intracellular signaling pathway for induction of apoptosis. Stress-related apoptosis is mediated by the insufficient expression of the protective molecule Bcl-2. Most of the investigations have been performed upon concomitant anesthesiological and surgical stress. We did not perform any surgery in order to avoid the influence of surgery on the immune function. In our research the anesthesiological stress response was connected with the increase plasma levels of adrenalin but not of cortisol. The endocrine parameter that has a central role in modulating cytokine synthesis and lymphocyte migration is cortisol (Wilckens and De Rijk, 1997). Glucocorticoids enhance IL-10 production which is known to inhibit T cell function. Cortisol decrease up to 2 hours in response to single fentanyl application (Hoehe et al., 1988) which explains the lack of cortisol-based stress response in our balanced anaesthesia. In

contrast, both epinephrine and norepinephrine inhibit the production of TNF-alpha while increasing the production of IL-10 (Munford and Pugin, 2001).

According to Papadima et al. (2009) combined general and epidural anesthesia failed to blunt the increase in lymphocyte commitment to apoptosis caused by surgical stress. They also concluded that decrease in total lymphocyte count and increase in percentage of the late apoptotic lymphocytes correlated positively with the increased cortisol levels. Immunosuppressive effects of surgical trauma and anaesthesia reported Yamada et al. (2002). Their results showed an increased lymphocyte apoptosis in dogs induced by general anaesthesia and surgery that correlated with the increased plasma cortisol levels. This data are not in accordance with our results because we did not found any correlation between plasma cortisol concentrations and lymphocyte apoptosis during balanced anaesthesia. Moreover, the latter authors revealed a greater increase in apoptosis of T-lymphocyte population compared with the B-lymphocytes because of a bigger susceptibility of T-cells to corticosteroids than B-lymphocytes. The reason for that difference was probably due to the influence of opioid fentanyl in our study. In vivo animal models have shown that opiate agonist suppress both humoral and cell-mediated immune response (Bayer et al., 1990; Bryant et al., 1992). In clinical conditions, however, fentanyl may suppress mostly humoral immune response.

CONCLUSIONS

In conclusion, balanced anaesthesia used in the present study induced lymphocytopenia by activation of apoptosis of these cells which was due to the combined apoptogenic effects of halothane, fentanyl, and pancuronium, but neither to the anaesthesia-related stress-response nor to changes in the main pro-and anti-inflammatory cytokines TNF-alpha and IL-10. Total lymphocyte count was diminished on the expense of B-lymphocytes without significant changes in CD 5+ and CD 8+ cells.

Clinical implications: balanced anaesthesia disturbs normal humoral immune response by decreasing the count of B-lymphocytes for minimum 24 hours after anesthesia.

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EFFECTS OF LONG LASTING ANAESTHESIA AND EXPERIMENTAL ABDOMINAL SURGERY UPON SOME VITAL PARAMETERS IN HORSES

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Abstract

The aim of the experiment was to study changes in blood gases, electrolytes, acid-base and some coagulation parameters occurring during long lasting anaesthesia and strangulation obstruction of the small intestine in horses.
Five healthy ponies were used for that purpose. They were submitted to 9 hours isoflurane anaesthesia with CRI of dexmedetomidine and ketamine. Strangulation obstruction of the small intestine was performed for 6 hours for histomorphometric investigations and was restored with enteroanastomosis afterward. Arterial and venous blood samples were taken in three periods and investigated parameters were measured.
Instead of periodic IPPV oxygenation progressively decreased judged from lowered PaO_2 and saturation with time. Moreover, a respiratory acidosis developed at 3rd hour and went deep at 9th hour. With regard to coagulation system alterations affected only D-dimer. Increase in D-dimer values corresponded with elevation of blood lactate level.
Long lasting anaesthesia and abdominal surgery does not impaired significantly oxygenation and coagulation in healthy horses but could contribute to further worsening of already compromised functions in colic horses.

Key words: *blood gases, haemocoagulation, horses, isoflurane anaesthesia, strangulation obstruction.*

INTRODUCTION

An anaesthetic-related death has been reported to be 10 times higher in colic horses undergoing emergency abdominal surgery in comparison with healthy horses undergoing elective anaesthetic procedures (Johnston et al., 2002). Each of strangulation obstruction (McClure et al., 1979), general anaesthesia (Day et al., 1995) or abdominal surgery instigates respiratory and coagulation systems of horses. Colic horses enter anaesthesia with already compromised functions that could result in intensification of respiratory depression and coagulation disorders. We tested the combined effects of the intestinal damage, anaesthesia and surgery upon some parameters of respiration and coagulation.

The adaptive mechanisms against hypoxemia such as increases in ventilation, cardiac output, and contraction of the spleen are obtunded during inhalation anaesthesia in contrast to the awake horses (McDonell and Kerr, 2007). Moreover, oxygenation is disturbed in anaesthetized horses due to ventilation-perfusion mismatching within lung parenchyma because of compression and absorption atelectasis (Nyman et al., 1990). Dorsally recumbent horses are more prone to compression atelectasis than other species as they have long slope diaphragm (Nyman and Hedenstierna, 1989). These effects are enhanced in colic horses because of the pressure applied to the diaphragm by exaggerated abdominal organs.

Coagulopathies are common in horses with colic and have been associated with increased morbidity and mortality (Schaer and Epstein, 2009). According to the results of Imaz et al. (2002) all tested parameters of haemostatic profile such as PT, APPT, fibrinogen, AT III, FDP and platelet count changed with the severity of colic horses.

The aim of the experiment was to study changes in blood gases, electrolytes, acid-base and some coagulation parameters occurring during long lasting anaesthesia and strangulation obstruction of small intestine in horses.

MATERIALS AND METHODS

The study was approved by the Institutional Ethical Committee on Animal Experiments in Stara Zagora, Bulgaria. It was part of another research relating to histomorphometrical

changes in the intestine during strangulation obstruction. Five healthy ponies aged between 2 and 7 years, weighing 213.2 ± 71.2kg (mean ± SD) were donated for the experiment. Horses were allowed to adapt for a month and were fed and bred in one and the same conditions. Deworming was performed using mebendazole 7.5mg/kg PO (Telmin® paste, Janssen Animal Health, Belgium).

Animals were submitted to 9 hours anaesthesia using isoflurane and continuous rate infusion (CRI) of dexmedetomidine and ketamine. Meanwhile, 6 hours lasting strangulation obstruction was induced, biopsy tissue samples were taken in two-hour periods, whereupon an enteroectomy and enteroanastomose were performed.

Food but not water was withdrawn 12 hours before anaesthesia. Premedication was performed with xylazine hydrochloride (Alfasan International, Holland) 0.8mg/kg given IV 15 minutes before induction. Both jugular veins were cannulated with 14G venous catheters (Venocan plus®, Kruuse, Denmark). Induction was did with a mixture of diazepam (Sopharma, Bulgaria) 0.05mg/kg and ketamine hydrochloride (Anaket®, Richter Pharma, Austria) 2.2mg/kg IV. After tracheal intubation using silicone tube 20-22 OD (Cook, USA) horses were hoisted and placed on the padded surgical table in dorsal recumbence.

Anaesthesia was maintained with isoflurane (Aerane®, Baxter, Slovenia) in 100% oxygen given by Penlon Sigma Delta vaporizer mounted on large animal anaesthesia machine LDS 3000 equipped with mechanical ventilator DHV 1000 (Surgivet, USA). A continued rate infusion (CRI) of 1.75μg kg^{-1} h^{-1} dexmedetomidine (Dexdomitor®, Orion Pharma, Finland) plus ketamine 1mg kg^{-1} h^{-1} diluted in saline solution was applied using microinfusion pump WZ-50C6, All Pro, China). Ringer solution (Actavis, Bulgaria) was given at rate of 10ml kg^{-1} h^{-1} through the second venous jugular catheter. The required surgical anaesthetic depth was maintained by adjusting the vaporizer setting.

Left or right facial artery was used for arterial access by cannulating it with 22 G catheter (Venocan plus®, Kruuse, Denmark). Arterial blood samples were withdrawn in heparinized syringes immediately after catheter placement, 3 hours and 9 hours later for blood gases, electrolytes and acid-base status and lactate measurements. Repiratory/Blood gases VetStat® cassettes and VetStat® electrolyte and blood gas analyzer (IDEXX Laboratories, Inc., USA) were used for that purpose. Lactate levels were determined by colorimetric method using enzymatic Roche/Hitachi lactate reagent (Roche Diagnostica, Germany). The mean alveolar arterial oxygen gradient (P (A-a) O_2) was calculated for every period using the following equation:

P (A-a) O_2=PAO_2-PaO_2= (FiO_2 x (BP-PH_2O))-$PaCO_2$/RQ, where

BP means barometric pressure in the alveolus at see level and is equal to 760mmHg; PH_2O – water vapor pressure in alveolar air at 37°C is equal to 48mmHg; RQ – respiratory quotient assumed 0.8.

The arterial access was necessary also for invasive measurement of blood pressures.

Intensive monitoring of the animals was made in 5-minute intervals all the time by means of multi-parameter patient monitor PM-9000Vet (Mindray, China). The following main parameters were traced: heart rate (HR), respiratory rate (RR), saturation (Sat), one-waveform electrocardiography (ECG) using three lead wires placed in sternal-withers configuration, invasive systolic (SYS), mean (MEAN), and diastolic (DIA) blood pressures, capnography, inspired and expired concentrations of oxygen (FiO_2, EtO_2 respectively), carbon dioxide ($FiCO_2$, $EtCO_2$ respectively), and isoflurane (FiIso, EtIso respectively), minimal alveolar concentration (MAC) of isoflurane.

Horses were allowed to breathe spontaneously. If the arterial partial pressure of CO_2 ($PaCO_2$) increased above 60mmHg, the arterial partial pressure of O_2 (PaO_2) decreased bellow 100mmHg, or RR was lower than 4 breaths $minute^{-1}$ for more than 3 minutes, an intermittent positive pressure ventilation (IPPV) was applied. Tidal volume of 20ml kg^{-1}, peak inspiratory pressure (PIP) no more 30cm H_2O, RR 8 breaths $minute^{-1}$, and inspiratory time 2.5 seconds were set in assisted-controlled mode of respiration.

Mean arterial blood pressure was maintained above 60mmHg by speeding up the rate of Ringer solution or by infusion of dopamine

hydrochloride (Warsaw Pharmaceutical Works, Polfa SA, Poland) at rate of 0.5-1 μg kg^{-1} min^{-1}. Ventral abdominal wall was aseptically prepared and covered with surgical drapes. A 20 cm long ventral midline skin incision was followed by white line incision starting from the umbilical mark forward. Caudal jejunum was exteriorized and 2 meter long segment was isolated. Double ligatures from Polyglactin 910 (DemeCRYL®, USP1, Demeteck Corporation, USA) were placed on blood vessels going to it and obstruction of both ends of segment was made with the same absorbable but heavier sutures (Sutupak®, USP 2, Ethicon, Germany). Tissue biopsy samples were taken from intestine in 2-hour intervals for histomorphometrical investigations. After 6 hours of strangulation obstruction had passed damaged intestinal segment was removed, the two ends sutured with polydioxanone 2-0 (PDS®II, Ethicon, Germany) by one-layer Lembert continuous pattern and abdominal incision closed routinely using polydioxanone USP 2 (Suturak®, Ethicon, Germany).

Coagulation parameters fibrinogen, prothrombin time (PT), activated partial protrombin time (APPT), thrombin time (TT), and D-dimer were measured in blood samples taken in citrated tubes during three periods − before premedication, at the 3rd hour of anaesthesia and operation, and at the 9th hour. The first four parameters were measured by mean of coagulometer Amelung KC 1A, Germany and tests of Human Diagnostica, Germany. For D-dimer measurement latex-aglutination colorimetric method and test was used (Spinreact, Spain).

After the end of the procedure animals were allowed to recover in padded stall. A standard postoperative intensive therapy was performed on the next days.

Statistical analysis was performed by means of Statistica® 6-0 version computer program (StatSoft Inc. USA). Kolmogorov-Smirnov test was used to test the distribution of data. Mann-Whytney U test was used afterward to calculate the differences of respiratory, acid-base and coagulation parameters between the three investigated time periods. P-value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSIONS

Surgical plane of anaesthesia was keep with comparatively low concentrations of isoflurane (fig. 1). Vaporizer setting, EtIso and MAC were adjusted in narrow ranges of 1.23/0.96/1.8%; 0.98/0.84/1.16%; 0.92/0.78/1.04% respectively (mean/minimal/and maximal values) decreasing with time after the first hour.

The main clinical parameters were in acceptable limits (fig. 2 and 3) and unexpected life threatening events were not observed. All horses were administered dopamine for maintenance of adequate blood pressure. Not anyone of them developed whatever type of arrhythmia throughout anaesthesia and surgery.

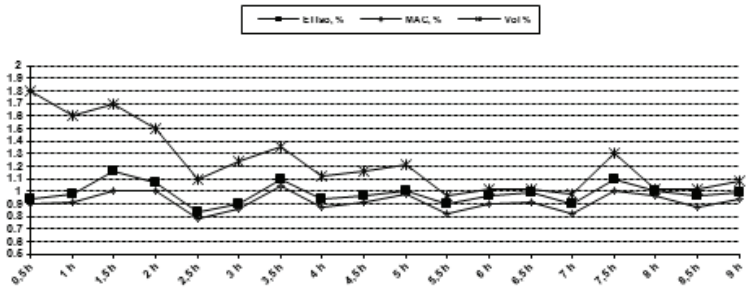

Figure 1. Changes in anaesthesiological parameters during anaesthesia and abdominal surgery in horses. EtIso − end tidal of isoflurane; MAC − minimal alveolar concentration; Vol%-volume percentage

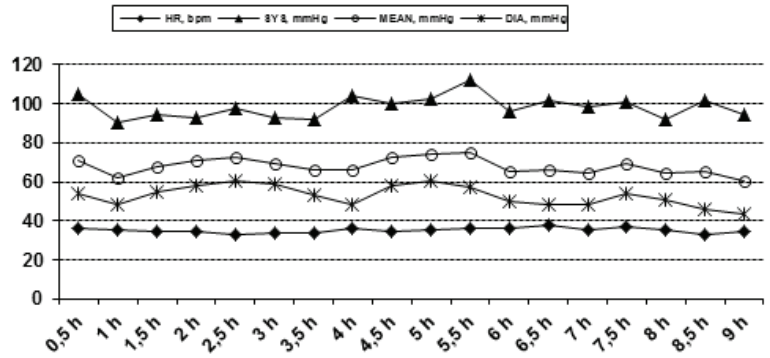

Figure 2. Changes in the main cardiovascular parameters during abdominal surgery under isoflurane anaesthesia. HR – heart rate; bpm – beat per minute; SYS – systolic blood pressure; MEAN – mean blood pressure; DIA – diastolic blood pressure

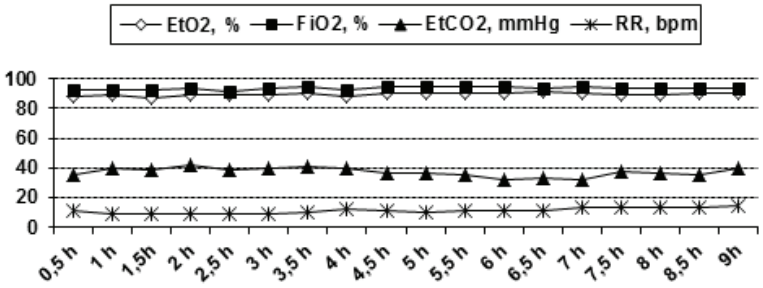

Figure 3. Changes in the main respiratory parameters during abdominal surgery under isoflurane anaesthesia. EtO2 – end tidal of oxygen; FiO2 – fraction of inspired oxygen; EtCO2 – end tidal of carbon dioxide; RR – respiratory rate; bpm-breath per minute;

Most of the time horses breathed spontaneously with IPPV applied periodically. Instead of periodic IPPV oxygenation progressively decreased with time judged from lowered PaO_2 and saturation (table 1).

Table 1. Changes in haemocoagulation parameters and blood lactate during experimental 6 hours lasting strangulation obstruction upon isoflurane anaesthesia in horses. Mann-Whitney nonparametric analysis was used to test the differences in parameters between investigated periods. Data are presented as mean ± standard deviation.

Parameter	n	0 hour	3rd hours	9th hour
pH	5	7.44 ± 0.05	7.33 ± 0.04 *	7.23 ± 0.09 *
$PaCO_2$, mmHg	5	43.2 ± 4.87	56.2 ± 9.52	65.2 ± 4.87 *
HCO_3, mmol/l	5	26.92 ± 1.94	27.46 ± 9.52	28.38 ± 5.04
Anion gap, mmol/l	5	12.0 ± 1.93	12.24 ± 1.3	9.9 ± 8.67
tCO_2, mmol/l	5	28.26 ± 1.99	29.2 ± 2.52	31.0 ± 6.85
BE, mmol/l	5	2.9 ± 2.19	0.5 ± 1.46	-1.2 ± 2.77 *
PaO_2, mmHg	5	195.4 ± 30.96	88.4 ± 27.19 *	90.6 ± 38.2 *
Hb, g/l	5	99.2 ± 29.88	115.0 ± 35.9	102.6 ± 24.71
Sat,%	5	99.2 ± 0.84	93.2 ± 5.36	88.2 ± 5.39 *
P (A-a) O_2, mmHg	5	419.88 ± 15.03	510.63 ± 29.4*	497.18 ± 20.25*
Sodium, mmol/l	5	140.4 ± 3.85	141.6 ± 8.41	141.6 ± 11.44
Potassium, mmol/l	5	4.58 ± 0.82	3.44 ± 0.51	4.0 ± 1.49
Clorides, mmol/l	5	106.2 ± 2.17	105.2 ± 7.46	108.0 ± 5.18

-$p < 0.05$ with regard to the "0 hour" period

* -$p < 0.05$ between "3rd hour" and "9th hour" periods

General anaesthesia using isoflurane was connected with high perioperative morbidity rate due to the high concentration of inhalation anaesthetics (Johnston et al., 1995) as well as to their dose-dependant cardiopulmonary depression (Steffey et al., 1980). Several methods had been tried to reduce the concentrations required for surgery since then. Partial intravenous anaesthesia (PIVA) combines volatile anaesthesia with continuous intravenously administration of analgesic and anaesthetic agents in low doses, with the aim of stabilizing physiological parameters.

Surgical anaestesia usually requires 1.2-1.4 times the MAC of inhalation agent. This means that for isoflurane EtIso commonly should be 1.6%. The addition of lidocaine by CRI to isoflurane anaesthesia in horses decreased the need for maintainance of surgical anaesthesia by 25% (Schunbeck et al., 2012).

When CRI of medetomidine, the forerunner of dexmedetomidine, was given to horses which were also receiving CRI of ketamin and lidocain a reduction the concentration of isoflurane necessary to maintain satisfactory anaesthesia for surgery was achieved, and reduced the dobutamine required to maintain mean arterial pressure (Kempchen et al., 2012). The reported values of EtIso (0.65%) were very similar to our results. We chose the newest alpha-2 agonist in our PIVA in order to decrease isoflurane requirements and to improve cardiopulmonary function, and to counteract to the pain.

The results of Pascoe et al. (2005; 2006) showed that dexmedetomidine infusions decrease the intra-operative requirements of isoflurane in dogs undergoing surgery in a dose dependant manner and low doses (0.5 μg kg^{-1} h^{-1}) appeared to have minimal effect on cardiopulmonary values, whereas the high doses (3 μg kg^{-1} h^{-1}) caused typical changes expected with an alpha-2 agonist.

Hubbell et al. (2011) observed atrial fibrillation in one of five horses submitted to 3 hours isoflurane anaesthesia without surgery but no changes in cardiovascular function was detected with the exception of blood pressure which had to be maintained with dobutamine. We did not find any arrhythmia in our experimental protocol but as in the before mentioned work we had to counteract to hypotension with dopamine infusion. The lack of arrhythmias could be explained with low concentrations of all used anaesthetics.

It is well known that volatile anaesthetics decrease ventilation dose-dependently suppressing directly the medullary and aortic and carotic body chemoreceptors' stimulation. In contrast, alpha-2 agonists are known to have the least effects on respiration in comparison to other sedatives. Intravenous administration of low dose dexmedetomidine did not alter arterial pH, PaO_2 and $PaCO_2$ in horses (Bettschart-Wolfensberger et al., 2005).

The same as our protocol of isoflurane anaesthesia but without CRI and surgery caused respiratory disturbances in both 50% and > 95% inspired oxygen (Hubbell et al., 2011). The reason for that was the increase of shunt fractions and alveolar dead space ventilation during 3 hours of anaesthesia. Our results showed development of hypoxemia and hypercarbia as was reported by many other investigators (Day et al., 1995; Kazuto, 2001). In contrast to previous report, IPPV did not ameliorate significantly oxygenation in our study. This may be due to the longer anaesthesia applied in our experiment, to the combined effects of anaesthesia and surgery, or because of the fact that improvement only occurs when PEEP values that compromise cardiac output are employed (Wettstein et al., 2006). Administration of 100% oxygen will usually improve hypoxemia in patients with ventilation-perfusion mismatch, but not in patients with significant right to left shunt (Muir and Morais, 2007). This might have been happened in our experimental animals as we calculated an elevation of A-a gradients with time provided animals inspired over 90% oxygen during the whole procedure.

Isoflurane anaesthesia without surgery resulted in increased venous lactate concentrations independent on inspired oxygen concentrations (Hubbell et al., 2011). In our study the increase lactate levels could be due not only to increased production during hypoxemia but also to decreased venous drainage causing an accumulation of produced lactate within the muscles during long lasting anaesthesia. Similar increased plasma lactate levels were

reported in isoflurane anaesthetized healthy horses which were much more pronounced during anaesthesia in colic horses (Edner et al., 2007). This finding implies that during anesthesia there is a demand for energy through anaerobic metabolism even in the healthy horse because of inadequate muscle oxygenation and metabolism.

Major surgery is associated with a hypercoagulable and proinflammatory state that persists into the postoperative period. Perioperative inflammatory responses to trauma can trigger hypercoagulability, especially in patients undergoing colic surgery.

Inflamation affects coagulation and fibrinolysis, and horses with inflammatory and ischemic disease are at risk of coagulopathies (Cesarini et al., 2010; Mendez-Angulo et al., 2010). Early stages of these diseases are characterized by hypercoagulability. Conventional coagulation testing supported the presence of hypercoagulation (decreased AT and increased D-Dimer concentrations), thrombelastography and coagulation abnormalities were rarely found in the same horses and the methods were not statistically related (Dunkel et al., 2010). Epstein et al. (2012) found out that the odds ratio of nonsurvival horses with gastrointestinal diseases and SIRS were 23.75 times higher if APTT was greater than 85.6 sec on the second day of admission.

Pablo et al. (1983) induced strangulation obstruction of distal jejunum just like in our experiment and found out that 6 hours, but not 2 or 4 hours, lasting strangulation resulted in severe ischemic damage to the intestines that was associated with significant coagulopathy consistent with disseminated intravascular coagulation (DIC). We did not observe notably prolonged clothing times and changes in fibrinogen levels but found out a significantly increase D-dimer concentrations with time that corresponded to the elevations in lactate levels.

In clinical studied horses with different types of colics, especially those with enteritis or peritonitis, was found significantly higher plasma D-Dimer concentrations and more severe coagulopathies on admission than in horses with other diagnoses (Cesarini et al., 2010). Moreover, a potential cut-off value for nonsurvival was found at approximately 4 ng/mL with odds ratio 8.8 for nonsurvival. D-dimer proved to be an early and more reliable parameter than FDP in predicting DIC development in colic horses (Stokol et al., 2005). Moreover, it turned out to have a predictive value for survival (Sandholm et al., 1995).

In the present study was tested if anaesthesia could contribute to coagulation disorders related to intestinal strangulation and surgery. Generally, anaesthesia alone was not reported to change coagulation parameters in healthy patients. Aydilek et al. (2007) studied the effects of xylazine-diazepam-ketamine, our induction anaesthetics, on the APTT, PT and platelet count in horses and did not find any significant alterations outside normal reference values. Several studies of the effects of inhalation anaesthetic agents on coagulation system reported that halothane (Kohro and Yamakage, 1996) and sevoflurane but not isoflurane had an inhibitory action on platelet function (Elrashidy et al., 2007). Dinev and Andonova found out increased concentrations of thromboxane B_2 between 1 and 2.5 hours of halothane anesthesia and during the corresponding stages of the surgical intervention which suggested that the anesthetic technique and surgery caused similarly a significant increase in thromboxane B_2.

CONCLUSIONS

Therefore, we could conclude that minor changes in coagulation during our experiment probably were due rather to the bowel ischemia and inflammation than to anaesthesia itself.

Finally, with regard to clinical relevance long lasting anaesthesia and abdominal surgery does not impaired significantly oxygenation and coagulation in healthy horses but could contribute to further worsening of already compromised functions in colic horses.

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THE FIRST CASES OF INFESTATION WITH *AELUROSTRONGYLUS ABSTRUSUS* IN CATS FROM TIMIS COUNTY

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Abstract

Study was taken in five cats from Timis County. All cats were under 24 months of age, except one which had seven years. The five cats were clinically, radiographically and coprological examined. The most common symptoms were coughing, wheezing, sneezing, and nasal discharge. Were seen bronchopneumonia at the radiographically examination. At the coprological exam larvae were present in fresh fecal smears.

Key words: *Aelurostrongylus abstrusus, cats, bronchopneumonia.*

INTRODUCTION

Aelurostrongylus abstrusus (*Nematoda, Strongylida*), the most common lungworm of cats, is found in many parts of the world, including the USA, Europe, and Australia. It has an indirect life cycle. It lives in the alveoli, bronchioles, bronchi and trachea of cats (Traversa, 2010). They are small parasites (males 7 mm, females 10 mm), deeply embedded in the lung tissues. The eggs are forced into alveolar ducts and adjacent alveoli where they form small nodules and hatch. First-stage larvae (L1) are coughed up, swallowed, and passed in the feces (Lopéz et al., 2005). The larvae seen in the feces of infected animals are tightly coiled, have an undulating tail with a spine, and are < 400 μm long (Lopéz et al., 2005). Larvae may survive in faeces for about 2 weeks until they penetrate terrestrial gastropod molluscs, in which they continue their development to the third larval stage (L3), which is infective to the final host (Lopéz et al., 2005). When one of these transport hosts is eaten, the larvae migrate from the stomach to the lungs via the peritoneal and thoracic cavities. They reach the lungs within 24 hr and are seen in the feces in ~1 month (Lopéz et al., 2005). Although prevalence can be high, clinical and diagnostic signs are often lacking. Chronic wasting, cough, dyspnea, and pulmonary wheezes may be seen. The lungs usually have solidified, gray, raised nodules 1-10 mm in diameter; generalized alveolar disease has been seen in chronic cases (Traversa et al., 2009). Diagnosis can be made by recovering larvae from faeces, bronchoalveolar lavage or necropsy. Treatment still has to be defined, but ivermectin is the most recommended drug (Traversa et al., 2009). In the past few years, case reports of aelurostrongylosis have been reported from many European countries with prevalence values between 0,7% and 1% in Germany (Epe et al., 1993 and 2004) and 22% in Croatia (Grabarevic et al., 1999). The occurrence of *Aelurostrongylus abstrusus* in cats from central and southern Italy (Capuano et al., 1995; Pennisi et al., 1995; Traversa et al., 2008) as well as in northern Italy (Grandi et al., 2005) suggests that this parasitic infection is not occasional. However, due to the inherent limits of classic diagnostic approaches, it is likely that feline aelurostrongylosis is often underestimated (Payo-Puente et al., 2008; Traversa et al., 2008).

The aim of this study was to report and describe five cases of aelurostrongylosis diagnosed in Timis County.

MATERIALS AND METHODS

Study was conducted during March 2010 to September 2011. Five fecal samples were analyzed from five cats. The cats included in

this study were from Timis County and were examined in the Veterinary Clinics of the Faculty of Veterinary Medicine Timisoara. All cats were privately-owned and were brought to medical examination because of respiratory signs. Age of cats studied was two months, six months, two year and seven year. Breed of cats taken in study was the Burmese breed (one cat) and European one (four cats). Cats were examined clinically, radiographically and coprological. The samples were taken from each cat. Fresh stool specimens were collected in clean plastic containers and stored at $+4^0$C. The examination of the sample was accomplished using flotation method (Willis) and direct examination using Lügol solution (Cosoroaba, 2002). For the flotation procedure the standard technique described by Cosoroabă (2002) was respected. Lugol's staining method is to make a native preparation of stool was added a drop and mix Lugol's solution. It is necessary to remove coarse food particles with a syringe needle and cover with a cover slip (Cosoroaba, 2002). The prepared slides were examined under a microscope with 400× magnification.

RESULTS AND DISCUSSIONS

Clinical signs were respiratory symptoms and the most common were: cough, dispnea, sneezing, and nasal discharge (see Table 1.).

Table 1. The clinical signs, coprological exam and radiographic features of A. abstrusus infection in the five cats studied

Cats	**European breed, 2 month, F**	**European breed, 2 year, M,**	**European breed, 7 year, M,**	**European breed, 6 month, M**	Burmese **breed, 2 year, F**
Clinical signs	dispnea, cough, tachycardia, tachypnea, sneezing, normal temperature	dispnea, cough, sneezing, normal temperature	dispnea, cough, sneezing, diarrheal feces vomit, anorexia, normal temperature	dispnea, cough, sneezing, diarrheal feces, anorexia, normal temperature	dispnea, cough, sneezing, normal temperature
Radiographic signs	severe bronchopneumonia	severe bronchopneumonia	severe bronchopneumonia	severe bronchopneumonia	severe bronchopneumonia
Coprological exam	*Toxocara* spp., *Trichocephalus* spp., *A. abstrusus*	*A. abstrusus, Giardia* spp.,	*A. abstrusus*	*A. abstrusus*	*A. abstrusus*

Symptoms like cough and dispnea associated with radiographic evidence of lung inflammation should alert the veterinarian to include aelurostrongylosis in the differential diagnosis. In the picture below you can see *Aelurostrongylus abstrusus* highlighted by different diagnostic methods (Figure 1.).

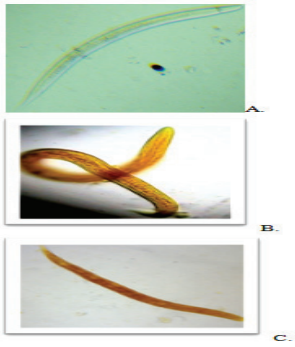

Figure 1. *Aelurostrongylus abstrusus* with Willis method (A) and with Lügol solution (B, C.) (original)

The evaluation of the radiographs showed abnormalities such as bronchial thickening, bronchial opacity, focal or generalized alveolar lung disease and increased vascular and focal parenchyma densities, in infected cats (Figure 2).

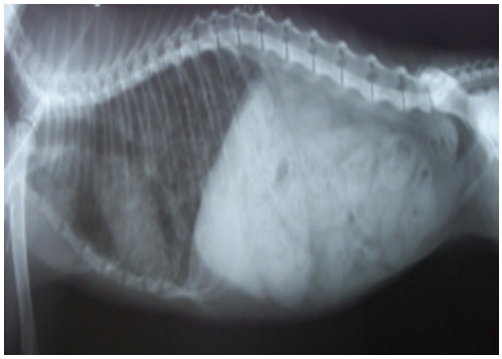

Figure 2. Thoracic radiographs in cat (original)

A. abstrusus infection is relatively rare. Recent surveys report prevalence's that range from 0.7% to 2.6% (Canestri Trotti et al., 1990; Epe et al., 2004; Miro´ et al., 2004; Robben et al., 2004). Feline infections have been described by several authors (Dubey and Crane, 1968; Scott, 1972; Pampiglione et al., 1990; Barrs et al., 1999; Sherding, 2004). In Romania Mircean et al., (2010) estimate at 5.6% the prevalence of infection with *A. abstrusus* in Transilvania.

CONCLUSIONS

The five fecal samples have been identified with *Aelurostrongylus abstrusus.* These results indicate that cat aelurostrongylosis is of clinical importance and, thus, needs to be included in differential diagnosis of feline respiratory diseases. Thoracic radiographs showed a bronchial pattern with thickening of the bronchial walls and infiltrates into the peribronchial regions.

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COMPARISON OF TRAMADOL AND ROBENACOXIB POSTOPERATIVE ANALGESIC EFFICACY IN DOGS

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Abstract

Analgesia is the main concern regarding pacient's postoperative rehabilitation. The main aim of this study was to compare the analgesic effect of Robenacoxib and Tramadol when administrated after surgery. Forty client-owned dogs undergoing genital surgery at the Clinic of Obstetrics and Gynecology (Faculty of Veterinary Medicine of Bucharest) were taken into study. Anaesthetic and supportive care protocols were standardized. Tramadol (2 mg/kg) was administrated postoperatively every 6-8 hours, while Robenacoxib (1 mg/kg) was administrated once a day. Pain scores were estimated according to Glasgow scale of pain for animals. Patients in Tramadol group (n=20) were more quiet (15/20), they ate sooner after surgery (10/20), fewer of them cried and whimpered (10/20). Dogs in Robenacoxib group (n=20) did not lick around the incision line (17/20) and the wound healed faster (15/20). Tramadol alone provides longer-lasting analgesia compared to Robenacoxib, but does not have the same anti inflammatory effect. Robenacoxib has a better effect in wound healing. The combined administration of Tramadol and Robenacoxib should be the subject of a further study.

Key words: *analgesia, Tramadol, Robenacoxib, dog.*

INTRODUCTION

Pain is "an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Watson 1979). Surgical intervention are associated with perioperatory pain and inflammatory response. Surgery suppresses the immune system and this suppression is directly proportionate to the invasiveness of the surgery (Pollock et al. 1991). Pain and the resulting response have marked physiologic effects (lactic acidosis, gastrointestinal ileus, increased protein catabolism, decreased food intake, delayed wound healing) resulting in increased morbidity and mortality (Stafford 2006; Chapman and Gavrin 1999; Hansen et al. 1997) . Therefore, effective analgesia is an essential part of postoperative management. Postoperative pain is expected for at least the first 24 to 72 hours (Hellyer 2002).

Opioids and nonsteroidal antiinflammatory drugs are the usual treatment options for postoperative pain (Lee 2011).

Tramadol is a synthetic opioid of moderate potency. It has a dual mechanism of action: it binds to the μ1 opioid receptor and inhibits the monoaminergic pathway, which is responsible for noradrenaline and serotonin reuptake (KuKanich and Papich 2004) . In the dog it can be administered orally (Giorgi et al. 2009), intravenously (McMillan et al. 2008) , subcutaneously (KuKanich and Papich 2004) , intramuscularly (de Sousa et al. 2008) and epiduraly (Guedes et al. 2005).

Robenacoxib is a new nonsteroidal anti-inflammatory drug approved for the treatment of pain and inflammation in the dog (King et al. 2010; King et al. 2009; Jung et al. 2009) . Robenacoxib has a high degree of selectivity for inhibition of the cyclooxigenase 2 (COX-2) isoform and a short blood half-life combined with longer residence times at sites of inflammation (King et al. 2010; King et al. 2009) . In healthy beagle dogs, robenacoxib has a high safety index, causing no detectable toxicity when administered at daily dosages as high as 40 mg/ kg for 1 month or 10 mg/ kg daily for 6 months without gastrointestinal side effects (King et al. 2011) .

The aim of the study was to evaluate postoperative analgesia of tramadol and robenacoxib administered orally in dogs.

MATERIALS AND METHODS

Forty client-owned dogs (n=40) undergoing genital surgery at the Clinic of Obstetrics and Gynecology (Faculty of Veterinary Medicine of Bucharest) were taken into study. Age of the dogs ranged from 2 years to 7 years with a mean of 4.5 years. The body weight of the dogs ranged from 12 kg to 42 kg (mean=27 kg).
All animals were evaluated (physical examination, complete blood cell count, biochemistry profile) and were classified according to the American Society of Anesthesiologists scale (ASA 1 to 5). Only the ASA 1 to ASA 3 dogs were included in the study.
Dogs were fasted 12 hours and water was withdrawn 4 hours before the surgery. The animals underwent the same anesthetic protocol (table 1).

Table 1. Anaesthetic protocol

Substance	Dose
Medetomidine (Domitor®, Pfizer Animal Health, Romania)	10 µg/kg IM Wait 10 minutes
Butorphanol (Butomidor®, Richter Pharma, Austria)	0,2 mg/kg IM Wait 10 minutes
Propofol (Lipuro®, B Braun Medical, Romania)	6 mg/kg IV bolus
Endotracheal intubation	
Isoflurane (Anesteran®, Rompharm Co., Romania)	1.5 – 2%

Lactated Ringer's solution (B Braun Medical, Romania) was infused IV (10 ml/kg/h) throughout the surgical procedure.
After extubation the dogs were randomly divided in two groups. The first group (n=20) was treated with tramadol (Tramadol®, Ozone Laboratories Group, Romania) 2 mg/kg PO every 8 hours starting 2 hours after the extubation for 3 days. Robenacoxib (Onsior®, Novartis Animal Health, Switzerland) 2 mg/kg was administered once a day, PO to the second group (n=20) starting 2 hours after the extubation for 3 days.
Physiologic indicators of acute pain in animals include increased heart rate, increased heart pressure, peripheral vasoconstriction, cardiac disrhythmias, sweating, hyperventilation and reduced peristaltism (Pearson 2007).
Pain was assessed according to the Glasgow Composite Measure Pain Scale (GCMPS) for dogs, a practical and recognized way of evaluating postoperative pain (Murrell et al. 2008; Reid et al. 2007) . The GCMPS comprises six behavioural categories with associated descriptive expressions: vocalisation, attention to wound, mobility, response to touch, demeanour and posture/activity. Items are placed in increasing order of pain intensity and numbered accordingly. Pain scores were assigned before surgery (as a baseline) every 24 hours and maximum possible score was 24.
Data were recorded as mean±SD. The date were analyzed using an analysis of variance and unpaired t-test (IBM SPSS software, ver. 19 for Windows; IBM, New York, USA). A P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Patients in tramadol group (n=20) were more quiet (15/20), they ate sooner after surgery (10/20), fewer of them cried and whimpered (10/20). Only one dog in tramadol group required additional analgesia in the first 24 hours after surgery. Adverse effects (nausea, salivation) appeared at 3 of the patients of the Tramadol group. 6 dogs of the tramadol group developed an acute inflammation around the incision site. The apparent calmer attitude of the dogs in this group is correlated with the sedative effects of tramadol. Similar observations were made following the administration of tramadol by epidural (Guedes et al. 2005), intravenous (McMillan et al. 2008; Giorgi et al. 2010) , intramuscular (de Sousa et al. 2008; Giorgi et al. 2010) or subcutaneous route (Buhari et al. 2012) . Also the analgesic effect is similar regardless of the route of administration, the oral route is easier to manage by the owners.
Dogs in robenacoxib group (n=20) did not lick around the incision line (17/20) and the wound healed faster (15/20). This is probably due to the anti-inflammatory properties of robenacoxib. There were no side effects (nausea, vomiting) in the robenacoxib group.

Our results confirm the analgesic proprieties of robenacoxib as already proven by previous studies for oral and subcutaneous routes of administration (Gruet et al. 2011; Edamura et al. 2012)

The median pain score did not significantly differ between the tramadol and robenacoxib treatments at any time point (figure 1).

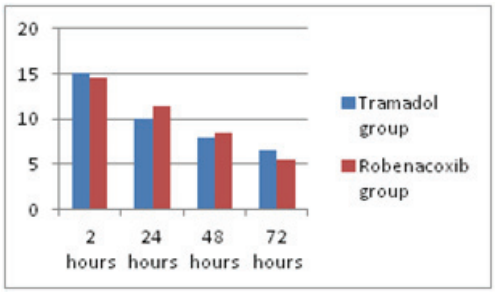

Figure 1. Evolution of pain scores in 72 hours

We can not exclude the influence of anesthetic substances on pain scores, especially during the first 2-4 hours after surgery.

Tramadol alone provides longer-lasting analgesia compared to Robenacoxib, but does not have the same anti inflammatory effect. Robenacoxib has a better effect in wound healing. The tolerability of both treatments was good as assessed from adverse effects and clinical signs. Although our study is the first to compare the postoperative analgesic efficacy of Tramadol and Robenacoxib, the combined administration of these two should be the subject of a further study.

CONCLUSIONS

Pain control method should depend on the type of procedure, severity of pain associated to the surgery, economic reasons and individual factors. Postoperative pain management is improved by including drugs that can be administered orally. Both tramadol and robenacoxib can be used for postsurgical pain management in the dog.

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ACROPODIAL DISEASES IN HORSES DIAGNOSED RADIOGRAPHICALLY: RETROSPECTIVE STUDY OF 7 CASES

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Abstract

The lameness associated with pain localized within acropodial region is common in horses, which enforces the use of radiological equipment in tracking changes in bone level as well as in adjacent soft tissues level. The most common acropodial diseases are represented by traumatic processes, followed by degenerative and infectious, which can evolve acute or chronic. The purpose of this study was describing semiological aspects noticed in radiological examination of acropodial region in horses. Radiographic images from animals with acropodial diseases were selected and examined inside the radiology clinic inside the Faculty of Veterinary Medicine Bucharest. The assessment of radiological images has revealed changes in acropodial level in 7 horses, ages varying between 8 months and 11 years, of which 4 males and 3 females. Acropodial changes were represented by 3 cases with middle phalanx fracture and the rest with: distal interphalangeal luxation, degenerative processes of the proximal interphalangeal joint, hoof wall exongulation, proliferative processes in middle phalanx.

Key words: *acropodial diseases, horses, radiological examination.*

INTRODUCTION

Lameness is a manifestation of an inflammatory process or mechanical defect, including pain which determines abnormal movement characterized by lameness (Ross et Dyson, 2011). Frequent diseases which affect horses toes are represented by fractures – consecutive acute trauma, chronic disorders (desmitis, tendonitis, enthesopathy, degenerative joint disease) – caused by chronic repetitive traumas and infectious processes (Riedesel, 2007). Radiographic investigations in horses are carried out every year in some countries with horse breeding traditions, to discover limb disorders as a pre-purchase trial (Kane et al., 2003; Furniss et al., 2011). Such investigations are not carried out at the moment in our country. The purpose of this study was to identify imagistic aspects of acropodial disorders in 7 horses admitted for radiologic investigations.

MATERIALS AND METHODS

Radiographic examination, from standard and complementary views depending on the case, was carried out and radiographic images were evaluated for 7 mix-breed horses (3 females and 4 males), ages varying between 8 months and 11 years, which presented lameness. Information about data of every animal and medical history was taken from the owners.

RESULTS AND DISCUSSIONS

Radiologic examination carried out in horses taken into study has revealed traumatic disorders presence (4/7), inflammatory (1/7), degenerative (1/7) and proliferative (1/7). Traumatic disorders were represented by fractures (3/4) and luxation (1/4). Fractures were identified in middle phalanx (P2) level, affecting the left posterior limb (2/3) and right anterior limb (1/3).

Case 1 – an 8-month-old mix-breed male – severe lameness in posterior left limb, consecutive to a car accident. Four views were taken (standing dorsolateral-palmaromedial oblique, dorsoproximal-palmarodistal flexed and dorsal 15° lateral-palmaromedial oblique flexed). Radiographic examinations reveal comminuted fracture of P2. From dorsolateral-palmaromedial oblique view the following were identified: fracture in palmar lateral proximal eminence, monoarticular, complete; physeal fracture, oblique incomplete fracture towards medial condyle (Fig. 1). From dorso-palmar view, physeal and lateral proximal eminence fractures were confirmed.

Dorsoproximal-palmarodistal flexed view has revealed dorsal sagittal plain incomplete fracture, monoarticular and ventral sagittal plane fracture, incomplete, monoarticular (Fig. 2). In dorsal 15° lateral-palmar medial oblique flexed view physeal fracture and palmar eminence lateral proximal fracture was noticed.

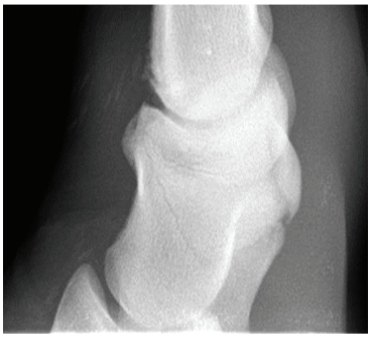

Figure 1. Comminuted fracture in P2

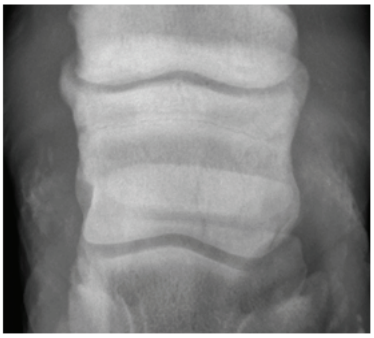

Figure 2. Sagittal plain incomplete fracture in P2

Case 2 – a 5-year-old mix-breed male – severe limping in right anterior limb, consecutive to a work accident. Two views were taken (lateromedial and dorsopalmar). Radiologic image has revealed P2 comminuted fracture. A number of fracture lines were noticed which resulted in multiple fragments varying in size. Also, enlargement of distal interphalangeal auricular space was noticed.

Case 3 – a 6-year-old mix-breed female – severe lameness in left posterior limb. Two views were taken (lateromedial and dorsopalmar) which evidenced P2 comminuted fracture, complete, biarticular (Fig. 3).

Case 4 – a 3-year-old mix-breed male – severe lameness in anterior left limb. Lateral-medial view evidenced the hyperextension (luxation) of distal interphalangeal joint and dorsal movement of the distal phalanx (P3), with highlight of digital common extensor tendon insertion and separation of navicular bone from P2 and P3 (Fig. 4).

Case 5 – an 11-year-old mix-breed female – chronic lameness, neglected, anterior right limb. Two views were taken (latero-medial standing and dorsopalmar flexed). Radiographic images revealed the presence of a joint degenerative process in proximal interphalangeal joint level. Degenerative disorders were represented by periosteal reactivity on the cranial side of distal proximal phalanx extremity (P1), periosteal reactivity on the cranial side of P2 proximal extremity and joint space narrowing. Also, entesophites at the insertions site of superficial digital flexor on P2 and in sesamoidean oblique ligament insertion site on P1, lateral view (Fig. 5). Dorsal palmar view reveals entesophites presence in collateral medial/lateral ligament attachment site of proximal interphalangeal joint (Fig. 6).

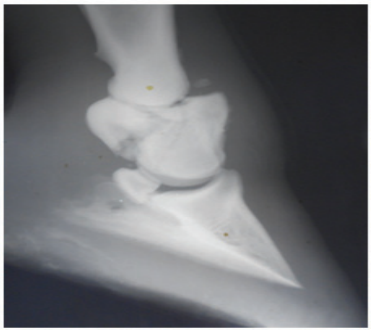

Figure 3. Comminuted fracture, complete, biarticular

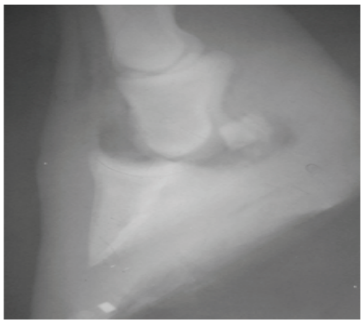

Figure 4. Luxation of distal interphalangean joint

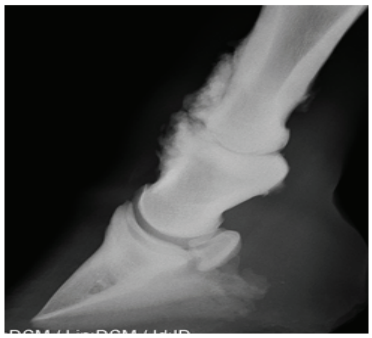

Figure 5. Degenerative disorders in the proximal interphalangian joint

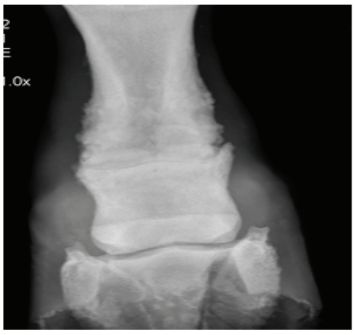

Figure 6. Entesophites in collateral medial/lateral ligament insertion site

Case 6 – an 8-year-old mix-breed female – lameness in posterior left limb consecutive to trauma, which resulted in hoof wall exongulation. Radiographic images (lateromedial flexion and dorso-plantar flexion revealed hoof wall separation from P3 and distal movement of P3. The presence of a radio transparent area was noticed between the hoof wall and soft tissue of the coriom which indicates air presence consecutive to hoof wall exongulation.

Case 7 – a 6-year-old mix-breed male – proliferative formation in pastern level, on the dorso-medial side of the anterior right limb (Fig. 7). Radiographic images taken from caudal 20° palmar-dorsomedial oblique view evidenced, in distal extremity level of P1, evidenced the presence of a bone-like radiopaque formation covered in soft tissue. Hyperostotic process was noticed on the caudal side of P1 (Fig. 8). Samples could not be taken for cytological or histopathological examination in order to identify lesion type, which lead to a general terminology of proliferative lesion.

Figure 7. Prolipherative formation in pastern level

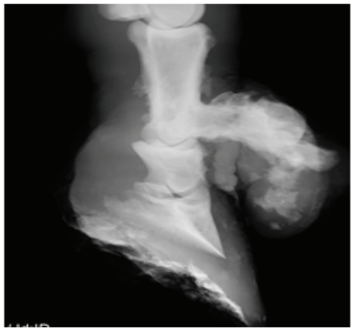

Figure 8. Hyperostotic process in P1

Obtained results highlight the presence of a large array of bone structure changes in acropodial level in horses. Acute trauma, which produces fractures in toes level, are the main causes common in horses (Riedesel, 2007), especially the ones used for work purposes. Under the action of external and internal forces, the bones respond with an elastic deformation, thereby, after the force stops, bones back to the original shape. The moment when force becomes excessive, fractures are produced (Riggs, 2002). Combined action of powerful compression and traction forces on the achropodium, when limbs are fixated on the soil sustaining body weight, determines varied structural modification. Bone fractures can be classified in three categories: traumatic fractures caused by transient overload, pathological fractures and fatigue fractures

caused by repeated stress to the bone (Riggs, 2002). Comminuted fracture was the most frequent lesion found in examined subjects, posterior limbs being the most affected. Owen et al. (2010), in a study carried out in UK noticed that traumatic lesions were frequent in posterior limbs (48%) compared with anterior limbs (17%), and Riedesel (2007) noticed that posterior limbs are twice more affected compared with the anterior limbs. Following analysis results obtained in the study shown here, P2 was considered to be the most affected site. This aspect can be attributed to intense stress on the respective segment during traction in work horses. Dates from literature sustain that phalanx fractures are common in sport horses and target especially the proximal phalanx and less regarding medial phalanx (Tanase, 2003). Ramza et al. (2011) in a study carried out on young race horses, noticed that fractures are most common in tibia level (20.7%) and proximal phalanx (14.5%). Variation can be attributed to the different number of cases taken into study, as well as different destination of animals (working vs. sport).

Hyperextension of interphalangeal distal joint can be congenital (in foals) or acquired (consecutive to trauma or distal phalanx infections) (Riedesel, 2007). Our case presented luxation of distal interphalangeal joint, with dorsal movement of P3 consecutive to avulsion of flexor digital profound tendon insertion and traction of extensor digital common tendon. Consecutive to major trauma, tendon laceration was produced as well as bone ray ratio and ligament structures were modified. Furniss et al. (2011), examining a number of 269 pure breed horses have revealed distal joint hyperextension presence in a reduced proportion (7.56%) in left limb compared with right limbs (9.66%).

Degenerative joint disorders are associated with limps and are chronic modifications characterized by osteophytes (new bone formation) on the periarticular edges of bone segments which form joints (Riedesel, 2007). It is known that the diseases evolve insidious, and bone disorders are produced way before noticing radiological changes (Brommer et al. 2003). In severe situations, new bone production can narrow articular spaces, subcondral sclerosis and osteophytes extension (Butler et al., 2000), in the past the term "high ringbone" was used (Edwards, 1984). Causes are varied, but traumas are frequently associated with periostitis and new bone production (Riedesel, 2007). In our examined case, degenerative disorders were represented by ostephytes, evident from lateromedial view and enthesophytes at ligament insertion sites, more obvious from dorsopalmar view. It appears that repeated trauma represented basis for degenerative disease occurrence in proximal joint level.

Exongulation is a disorder caused by the detachment of the hoof wall from the cheratogen membrane (Tănase, 2003) following the destruction between lamellae coriales and lamellae epidermalis. There are multiple causes (primary or secondary), trauma being the main cause, but infectious or circulatory disorders are not to be neglected. Our examined animal, from what the owner told, suffered an accident which leads to hoof wall avulsion. This phenomenon was probably produced following chronic circulatory processes, previously the animal presenting lameness in posterior limbs (possibly laminitis). Lameness presence, concurrent in a biped (pelvic/thoracic) or in all limbs pleads for laminitis presence (Tănase, 2003). As follows, in our case study, it can be stated that it was a mechanical exongulation of the hoof wall due to a chronic laminitis.

Neoplastic processes are extremely rare in toes level (Riedesel, 2007). Lack of histopathological examination of the formation from distal extremity of proximal phalanx makes enunciating a definitive diagnosis impossible.

CONCLUSIONS

Following obtained analysis results it was found that the middle phalanx represented the most affected segment; radiological examination allowed the establishment of type, localization and extends of lesion processes.

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THE USE OF MEDETOMIDINE AND BUPRENORPHINE FOR PREMEDICATION, KETAMINE FOR INDUCTION AND ISOFLURANE TO MAINTAIN GENERAL ANESTHESIA IN RABBITS. CASE STUDIES

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Abstract

The aim of this study was to assess the level of analgesia and safety of this aesthetic protocol. Many other anaesthetic protocols presented in scientific works are not enough detailed regarding their safety or their analgesic predicted effects. For this study we anesthetized three healthy rabbits. Two of them suffered a standard orchiectomy procedure and one suffered an orthopaedic intervention on humerus. All three received the same anaesthetic protocol. They were premedicated using medetomidine 0.25 mg/kg. After 10 minutes the rabbits were induced using ketamine 15 mg/kg subcutaneous. For analgesia we administered buprenorphine 0.03 mg/kg subcutaneous and meloxicam 0.6 mg/kg. Anaesthesia was maintained with isoflurane administered by facemask 1-1.5% in pure oxygen. During anaesthesia we assessed respiratory and cardiac parameters using a pulse-oximeter. After analysing our results we concluded that this protocol gives a predictable level of analgesia sufficient for most of procedures, even orthopaedic surgeries, and that our subjects were free of any cardio-respiratory secondary effects during anaesthesia.

Key words: *rabbit, medetomidine, buprenorphine, ketamine, isoflurane.*

INTRODUCTION

Injectable anaesthesia is frequently used in rabbits because it is an easy way to obtain general anaesthesia. In the scientific works there are only two other articles that present general anaesthesia using this protocol (Difilippo et al., 2004; murphy et al., 2010). Difilippo et al. assessed this protocol during cardiothoracic surgery while in murphy et al., the rabbit did not suffer any surgery. The lack of research over this protocol convinced us to study it more and especially the level of analgesia during painful surgeries. This research will help the implementation of this protocol in clinical practice for routine and special surgeries. Our results regarding safety, efficiency, and analgesia are confirming the other studies (Difilippo et al., 2004; murphy et al., 2010).

MATERIALS AND METHODS

For this study we used four rabbit subjects that came to Clinique vétérinaire universitaire de Liège for routine, castration by prescrotal technique, and orthopaedic surgery, one radial reduction with intramedullary nailing and one tibia reduction with intramedullary nailing and external fixators. They weighted between 0.37 and 4 kg and they were not starved before anaesthesia. The subjects were risk classified as ASA 2.

Rabbits were premedicated with medetomidine (sedator® 1mg/ml, eurovet animal health) 0.25 mg/kg subcutaneous. For analgesia subjects received buprenorphine (vetergesic ® 0.3 mg/kg, ecuphar) 0.03 mg/kg subcutaneous 10 minutes after premedication and meloxicam (metacam® 5 mg/kg, boehring ingelheim) 0.6 mg/kg subcutaneous at the end of anaesthesia. For anaesthesia induction the rabbits received ketamine (ketamine 1000® 100 mg/ml, CEVA) 15 mg/kg subcutaneous 10 minutes after premedication. From the moment that rabbits lost standing position we started to administer isoflurane (iso-ver® 1000mg/g, eurovet animal health) 1-2% in pure O_2 2 L/minute by face mask. An intravenous catheter was placed in the marginal ear vein and we administered a mixture fluid 10 ml/kg using an injection

pump: colloid (voluven® 6%, fresenius kabi deutschland gmbh) 44%, lactated ringer's (excel®, b. Braun medical inc.) 44% and glucose (glucose 30% lavoisier fl 500ml, chaix et du marais®) 12% of the mixture.

During anaesthesia they were monitored using clinical examination and pulse-oximeter every 5 minutes and data was recorded in an aesthetic report. Using clinical examination we monitored righting reflex, palpebral reflex and pedal reflex. Respiratory rate was measured by visually counting spontaneous breaths. Heart rate and oxygen tissue saturation was monitored using a pulse-oximeter applied over the tongue.

In the recovery period rabbits first received atipamezol (revertor® 5 mg/ml, virbac) 1 mg/kg intramuscular as antidote for medetomidine. They were also inoculated with metoclopramide (vomend® 5mg/ml, dechra veterinary products) 0.5 mg/kg to stimulate appetite and digestion after surgery. Recovery took place in a quiet place were the rabbits had access to fresh food and water. During recovery they also received pure oxygen 4 L/min in the cage.

Surgery technique was not part of this research and then it is not presented it in this case report.

RESULTS AND DISCUSSIONS

Premedication, induction and recovery took place smooth as murphy k.l. et al. (2010) also recorded in his study. After medetomidine premedication rabbits were sedated but they did not lose standing position or any reflexes. Ketamine administration produced smooth induction with losing sternal position. Ocular reflexes and pedal pinch reflex were also abolished after ketamine induction. Buprenorphine did not change anything in the anaesthetic status after administration. In this phase intubation was tried on all rabbits unsuccessfully by the blind technique contradicting the study of murphy k.l. et al. (2010). Difilippo s.m. et al. (2004) also recorded that using this anaesthetic protocol, but using higher dosages, all rabbits could be intubated. Other study used the same protocol for premedication and induction but no buprenorphine and still rabbits could be intubated by blind technique (Grint et al. 2008).all rabbits recovered gently from anaesthesia after stopping isoflurane and atipamezol inoculation.

Venous catheters were applied to all subjects in the lateral ear vein (Tutunaru et al. 2012). This protocol is efficient to desensitize external ear and to apply a venous catheter.

Cardio-respiratory parameters were first depressed just after induction but they returned to normal after 10-15 minutes without any analeptic treatment and it was not clinical important. During anaesthesia heart rate had a mean value of 220 beats/minute (140-300) equal to those recorded by murphy k.l. et al. (2010) and higher to those recorded by Difilippo s.m. et al. (2004). Heart rate variation was not correlated with surgery pain stimulation. Rabbits did not suffer of bradycardia during anaesthesia even if using medetomidine heart rate might fall (figure 1) (Tutunaru et al. 2011; Tutunaru et al. 2010).

Tissue oxygen saturation did not get lower than 92%, with a mean value of 97%. Respiratory rate was relatively constant during surgery with a mean value of 50 breath/minutes (30-100). Apnea was not recorded in any rabbit, not even in the induction phase. Apnea might not be recorded even if higher doses were used (Difilippo et al., 2004).

Buprenorphine reduces ventilation and tissue oxygenation by his depressive action on the respiratory function in conscious rabbits but do not influence blood pressure or heart rate (Shafford and Schadt 2008). This study did not assess end tidal carbon dioxide but still it did not record any fall in oxygen tissue saturation. This may be due to the fact that rabbits received pure oxygen by mask before, during and after anaesthesia.

The protocol achieved a level of analgesia sufficient for orthopaedic surgeries. In an earlier study over orthopaedic surgery were

Figure 1. Aspect during general anaesthesia

anesthetised with a lower dose of medetomidine and ketamine, maintained with isoflurane, and still analgesia was sufficient for the intervention (Zuijlen et al. 2010).

CONCLUSIONS

The article shows that the protocol used provides a safe anaesthesia for rabbit patients ASA II risk class.

The research also proves that the protocol provides sufficient analgesia for routine surgeries but also for orthopaedic intervention.

The study also wanted to demonstrate the applicability of this anaesthetic protocol in small animal clinics. All clinics equipped with an anaesthetic inhalator machine can embrace this protocol. Even if the clinic is not equipped with such machines this protocol might work but duration of anaesthesia is limited.

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PROXIMATE AND MINERAL ANALYSIS OF ATLANTIC SALMON *(SALMO SALAR)* CULTIVATED IN BULGARIA

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Abstract

Problem statement: *Only limited information exists on nutrients in salmonoids meat in Bulgaria, which may to be different and vary to a greater extent than the nutrient composition of other fish items. The present paper is aimed to determine the proximate composition, macro and trace elements of Atlantic salmon`s meat. These data could be helpful in judging the value of nutrient composition data as a base for dietary recommendations.*
Organisms: *12 species of Atlantic salmon (Salmo salar).*
Approach: *The aim of this study was to determine the proximate composition and levels of iron, potassium, sodium, calcium, phosphorus, magnesium, copper, selenium and zinc in Atlantic salmon cultivated for the first time in Bulgaria. The content of protein, fat and ash and concentrations of iron, potassium, sodium, calcium, phosphorus, magnesium, copper, selenium and zinc were determined by automatic systems and electro thermal atomic absorption spectrometry (ETAAS) after microwave digestion. Mean values and their respective coefficients of variation were calculated from the measured concentrations.*
Conclusion: *In order to provide an accurate overview and to be able to calculate reliable dietary intakes, it is important to know the fish composition data.*

Key words: *Atlantic salmon, Proximate composition, Macro elements, Trace elements.*

INTRODUCTION

The first evidence connecting humans to salmon was found in southwestern France and northern Spain in caves that were occupied during the Upper Paleolithic period. Salmon fish traps from around 6000 B.C. have been found in Sweden and salmon fish nets from around 6250 B.C. have been found in Danish bogs. Atlantic salmon, once abundant throughout the North Atlantic, were prized food by Gauls, Romans, and Native Americans alike (Clay, 2004).

Today more than 50% of the global salmon supply was farmed (Johnson, 2001). According to the United Nations Food and Agriculture Organization (2005), salmon is farmed in 24 countries, as the major producers of salmon are Norway and Chile (Bostick et al., 2005).

Among seafood species consumed in the world, salmon is an important contributor of many nutrients. She is preferred fish species for consumption because of its rapid growth and rich and diverse composition of the meat (Exler, 2007; Stancheva et al., 2010). In other side fish tissue is an excellent source of macro and essential trace elements such as iron (Fe), zinc (Zn) and selenium (Se) (Briggs and Schweigert, 1990). The accurate determination of these elements is therefore important in nutrition studies, particularly because meat, as a biological material, exhibits natural variations in the amounts of nutrients contained (Greenfield and Southgate, 2003). Therefore, it is essential that nutrient data, including trace element contents, are regularly updated to reflect the current data situation and to monitor possible changes (Gerber et al., 2008). These data could be helpful in judging the value of nutrient composition data as a base for dietary recommendations (Leonhardt and Wenk, 1997).

Overall, the most widely used trace elements determination techniques in salmon are Uv-Vis spectrophotometry (Bland et al., 1999), ETAAS (Angelova et al.,2006; Gosslim et al., 2007; Dospatliev et al., 2008), X-ray fluorescence, ICP-OES (Farias et al., 2002;

Dospatliev et al., 2010; Dospatliev et al., 2011) and ICP-MS (*Forrer et al., 2001; Matsuura et al., 2001*).

The aim of this study was to examine the proximate composition and the concentration of essential elements like iron (Fe), potassium (K), sodium (Na), calcium (Ca), phosphorus (P), selenium (Se), magnesium (Mg), copper (Cu), and zinc (Zn).

MATERIALS AND METHODS

Fish

The farmed Atlantic salmon (Salmo salar L.) were grown in aquaculture base of Eurocorrect Ltd., Vacha Dam Lake, Bulgaria comprising duplicate 8m×8m×6m net cages. The fish were fed a commercial ration of the Soprofish® series (Subotica Ltd., Subotica, Serbia). The diets consisted of 13/45 (fat/protein levels) for the 3 mm, pellet sizes.

Sample Preparation

Immediately after catching, they were anaesthetized (el stunning) and stored on ice in an insulated box and transported to the Central laboratory of Trakia University on the next day. The mean weight and length of the salmon were 89.32±1.42 g and 15±0.85cm. There fish was beheaded. Spinal meat samples (35 grams) without skin from all fish specimens were taken and examined. They were prepared for the experiment in standard ways dried at 105° C in a fan oven and stored in dark plastic bottles.

Reagents

Reagents were qualified as pure (Merck® and Fluka®). The standard solutions for ETAAS determination of K, Na, Ca, Fe, Mg, Zn and Cu with concentration of 1000 mg/l were supplied by Merck (Darmstadt, Germany). Double-distilled water was used for all the procedures. The samples were analyzed with Perkin-Elmer A Analyst 800 atomic absorption spectrometer (Norwalk, CT).

Mineralization of samples

We weighed 3.0 g of air-dried salmon to the nearest 0.01 g in a round-bottomed 100 ml flask and added 22.5 ml of HCI and 7.5 ml of HNO_3 acid. We connected the flask to a reflux condenser and let it stand for no less than 16 hours at room temperature, then heated gently to boiling for 2 hours. After cooling and flushing the condenser with 25 ml of 12.5% nitric acid the sample was filtered and 100 ml of 12.5% nitric acid was added to the part of it in liquid phase.

Proximate Analysis

The samples were prepared AOAC (2006; method 983.18) and subjected to moisture analyses using air drying AOAC (1997; method 950.46). Crude protein content was calculated by converting the nitrogen content by multiplying by 6.25 due to the fact protein is 16 percent nitrogen (100/16=6.25), determined by Kjeldahl′s method using an automatic Kjeldahl system (Kjeltec 8400, FOSS, Sweden). Lipid content was determined by the method of the Soxhlet using an automatic system (Soxtec 2050, FOSS, Sweden). Crude ash was determined by incineration in a muffle furnace (MLW, Germany) at 550° C for 8 h. Crucibles were brought about the room temperature and weighted.

Statistical analysis

Statistical analyses were performed using STATISTICA 6. The accuracy of the measurements was assessed by standard deviation (SD) and relative standard deviation (RSD) for n = 12.

RESULTS AND DISCUSSIONS

Table 1. Proximate composition of the muscle of Atlantic salmon (Salmo salar).

Proximate composition				
Element	Technique	n	Mean	SD
Moisture	AOAC 950.46	12	73. 65	0.076
Crude protein	AOAC 988.05	12	18.81	0.056
Crude fat	AOAC 960.39	12	4.46	0.158
Crude ash	AOAC 942.05	12	0.96	0.013

The results of proximate analysis in the flesh of salmons are shown in Table 1. Moisture, protein and lipid and ash contents of the salmon meat averaged 73.65, 18.81, 4.46 and 0.96%, respectively. However, the present values are favorably comparable with the published reports in different salmon species (USDA 2005; Exler, 2007; Stancheva and Merdzhanova, 2011).

The concentration ranges and averages of macro elements in the salmon fishes analyzed are summarized Table 2.

Table 2. Mineral contents of Atlantic salmon (Salmo salar).

Macro elements (mg/100g)					
Element	Technique	n	Mean	SD	RSD
Ca	ETAAS	12	5.45	0.11	0.86
P	ETAAS	12	118.20	7.02	2.04
Na	ETAAS	12	195.05	6.29	2.18
K	ETAAS	12	244.7	5.6	1.18
Mg	ETAAS	12	32.69	0.70	1.53

Calcium is an essential macro element and it is present in the structure of bone. Calcium is present in a healthy human body at about 2% of body weight (Öksüz, 2012). The daily requirement of calcium of human, salmon can be seen as a poor source of calcium because raw tissue contained between 3.5 and 8.7 mg/100g.

Phosphorus is structural component of hard tissues such as bone and scales, as well as a constituent of various coenzymes, phospholipids and nucleic acids. Fish can obtain a substantial number of required minerals directly from their rearing water, but phosphorus is one essential mineral that must be supplied by the diet (Lall, 2002). Fish meat is a rich source of phosphorus and can be on the order of 140-200 mg per 100 g of product. This element occurs in almost all species of fish (Stanek and Janicki, 2011). In our study was determined 118.20 ± 7.02 per 100 g.

Sodium is a natural ingredient in raw animal products. Sodium content in fish varies a great deal, depending on the species and variety. Especially, Atlantic salmon contain fewer than 60 mg of sodium per 100 gr. In our study was determined 195.05 ± 6.29 per 100 g at the result significantly different from what was expected.

Potassium is macro mineral, which is important for building muscle, metabolizing protein and carbohydrate, balances water and acid in the blood and body tissues. Salmonoids have the highest amounts of potassium, ranging between 375 to 628 mg. In farmed Atlantic salmon have only 384 mg, while wild Atlantic salmon have 628 mg, Sockeye has 375 mg, Chinook has 505 mg, farmed Coho has 460 mg, wild Coho has 434 mg and Pink salmon has 414 mg per 100 g. In this case, again observed numbers were so different from the expected (244.7 mg).

Magnesium is an essential element for organisms for oxidative phosphorylation and activates many enzymes (Öksüz, 2012). He is widely distributed among the foods, and it ranges from 25 to 76 mg in each raw edible 100 g of salmonoids (Silva and Chamul, 2000). Magnesium content of farmer salmon was determined as 32.6 mg/100g.

Table 3. Trace elements of Atlantic salmon (Salmo salar).

Trace elements (mg/100g)					
Element	Technique	n	Mean	SD	RSD
Fe	ETAAS	12	2.6	0.21	4.32
Zn	ETAAS	12	1.96	0.04	0.97
Cu	ETAAS	12	0.98	0.02	0.67
Se	ETAAS	12	0.34	0.01	0.26

The trace element contents of the salmon are given in Table 3. They were determined in the raw muscle. A lot of metals are necessary in low concentration for human, because they are essential elements, such as Fe, Cu and Zn.

The iron content of fish is very low compared to that of mammals (Watanabe et al., 1997). But according to the U.S. National Library of Medicine and the National Institutes of Health, salmon is a good source of iron, along with tuna for adults and children. The iron concentrations (mg/100g wet weight) in the samples analyzed ranged from 1.9 to 3.4 mg/100g with a mean of 2.6 ± 0.21.

Zinc is another important essential element and it is present active sites of many enzymes. It is also a natural component of many sea foods (Ikem and Egeibor, 2005). The zinc content of farmed salmon was about 1.96 mg/100 g., similar to cultured and wild coho salmon reported by Felton et al., (1994).

Copper and selenium are essential trace elements for fish metabolism and important micronutrients in the human diet (Ames, 1998). Copper is a cofactor in a wide range of enzymes including cytochrome oxidase, superoxide dismutase and lysyl oxidase (Watanabe et al., 1997). Copper is essential for good health but very high intake can cause adverse health problems such as liver and kidney damage (Ikem and Egeibor, 2005). Muscle copper concentrations in salmon were below the MAFF guideline value of 3.0 mg /100g (MAFF, 1995).

Selenium is important for a strong immune system and helps regulate the thyroid hormones. He is an integral component of glutathione peroxidase which protects cells

from oxidative damage (Watanabe et al., 1997) and is an important nutrient for Atlantic salmon (Johnston et al., 2006). Most seafood is rich in the trace minerals selenium, and salmon is no exception, providing nearly 40 micrograms. Muscle selenium levels in the salmon were 0.34 within the range reported for healthy farmed salmon (Johnston et al., 2006).

CONCLUSIONS

The investigation focused on chemical composition of Salmon fish and provided in particular information on the both macro elements and trace elements in meat. Most of elements (Mg, Na, Fe and Cu) in the Bulgarian diet are derived from plant foods. From the other side, the macro elements Ca and P, and the trace elements Zn and Se, were derived mainly from meat and meat products. This well known fish meat is a very good source of minerals like phosphorus, potassium, sodium, magnesium, zinc and iron with the exception of calcium. Sodium was the highest in all macro elements, followed by P, K and Ca. The variation recorded in the concentration of minerals in salmon meat from another investigations and results, could have been be influenced by a number of factors such as seasonal and biological differences (size, age and sexual maturity), food source and environment (water chemistry, salinity, temperature and contaminants). However, we conclude that salmon cultivate from the Vacha Dam Lake is recommended for human consumption as a good source of macro elements and trace elements.

This investigation provides practical and useful information on the chemical composition of salmon, which is the first time cultivated in Bulgaria. It can be concluded that this study contributes to a description of the chemical and proximate composition of salmon meat which could be use to extend existing information. Furthermore, the mineral composition of salmon meat are presented, some of elements for the first time, in order to establish a database on the nutrient composition on salmon meat for further use in research on human consumption of this relative unknown type of meat in Bulgaria. These results will be important for the nutritionists and researchers for improving processing. It is also helpful for similar academic studies and to prepare tables of compositions of food.

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DETECTION OF *NEOSPORA CANINUM* ANTIBODIES IN MILK ON DAIRY CATTLE

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Abstract

Serodiagnostic of Neospora caninum infection in cattle is generally based on using the enzyme-linked immunosorbent assay (ELISA) method for detection of specific antibodies in serum samples, but the use of milk is also possible. The present study was undertaken to assess the potential of an ELISA kit for testing individual and pooled milk samples in dairy farms. Pairs of milk and blood samples (n = 60) were collected from 3 dairy farms (A, B, and C) in southern Romania. Additionally, four pooled milk samples were obtained (one pooled milk sample for each farm and a total sample as a pooled sample from all three farms). Skimmed milk was obtained and tested by using a commercially available ELISA kit (HerdChek N. caninum Antibody Test Kit, IDEXX Lab.). The optimized cut-off value of S/P> 0.704 was determined by receiver operating characteristic (ROC) analysis, using serum results as 'gold standard'. The sensitivity and specificity of the assay at this cut-off were 70.4% and 100.0%, respectively and the agreement with classic serology, expressed as kappa values, was good (K=0.723). When samples with low positive response on sera were excluded, the correlation obtained was even better (K= 0.921). For pooled milk samples a lower cut-off was necessary in order to identify as positive all dairy farms with a 15% or higher within-herd seroprevalence.
The results of this study demonstrate that the prevalence of N. caninum in dairy farms can be estimated by using this indirect ELISA kit on individual and pooled milk samples.

Key words: *ELISA, milk, Neospora caninum, Romania.*

INTRODUCTION

N. caninum is one of the main primary etiologic agents of abortion in cattle (Dubey, 2003) causing significant economic losses around the world. Congenitally infected bovine fetuses may die in uterus, may be born dead or alive with clinical signs or apparently healthy, but with persistent chronic infection that can be later transmitted by females to their progeny (Dubey and Schares, 2011). *N. caninum* is one of the most effective vertically transmitted pathogens in cattle (Bjorkman et al., 1996).
Many serosurveys have been reviewed recently showing worldwide distribution of *N. caninum* infection (Dubey and Schares, 2011).
N. caninum infection can cause repeated abortions in some cows (Anderson et al., 1995) and seropositive cows are more susceptible to abortion than seronegative ones (Dubey et al., 2007). Studies from U.S., but also from Europe, calculated significant economic losses due to reproductive problems associated with *N. caninum* infection in cattle (Dubey et al., 2007).
The routine diagnosis for *N. caninum* infection in cattle is based on detection of specific antibodies in serum samples, but also milk samples can be used for lactating cows (Conraths and Gottstein, 2007).
Testing of milk samples presents some advantages over testing of blood samples, like easily and lowered costs of collecting samples, noninvasiveness of the method with reduction of some disease transmission by needle and reduction of productions losses caused by stress (Schares et al., 2004).
In previous studies conducted in different regions of the world some ELISAs for detection of *Neospora caninum* antibodies in cattle were adapted for use in individual or bulk milk samples (Bjorkman et al., 1996; Schares et al., 2004; Bartels et al.2005; Frossling et al., 2006; Hall et al., 2006; Wapenaar et al., 2007 González-Warleta et al., 2011), but different test characteristics were obtained. In a recent study whole and skim milk samples were analyzed with a commercial serum ELISA test and both were equally suited as a screening tool (Byrem et al., 2012). However, no studies have

been performed in South Romania to evaluate characteristics of testing milk samples for *N. caninum* antibodies.

In a serological survey performed in 13 dairy farms from south of Romania seroprevalence rates of *N. caninum* infection ranged between 6.3% and 80%, with a medium of 40.3% (Mitrea et al., 2012; Enachescu et al., 2012). *N. caninum* infection in cattle has been also reported in west with 27.7% prevalence (Imre et al., 2012), north-west and center with 34.6% prevalence (Gavrea et al., 2011) of Romania.

In the context of an already existing milk quality testing, testing milk rather than sera would be a cost-effective approach for evaluating *N. caninum* exposure in dairy farms.

This study was undertaken to assess the potential of an ELISA kit for testing individual and pooled milk samples in order to determine the *N. caninum* infection status in dairy farms from southern Romania. Therefore, the aim of this study was to evaluate use of skim milk samples for estimation of *N. caninum* prevalence by two commercially available indirect ELISAs in dairy farms from the southern Romania, compared with serum samples. This study also investigated the potential use of pooled milk samples with an indirect ELISA to determine the *N. caninum* infection status of the cattle herds.

MATERIALS AND METHODS

Serum and milk samples

The study was conducted in south area of Romania comprising three counties (Ilfov, Olt and Dambovita). A total of individual 60 pairs of milk and blood samples were collected in 2010 from 3 dairy farms (A, B, and C) as a part of a larger serological survey (Mitrea et al., 2012; Enachescu et al., 2012). Animals were randomly selected. Additionally, pooled milk samples were obtained by homogenization of all milk samples for each farm and a total sample as a pooled sample from all three farms. About 5 ml of blood and 5 ml of milk were collected in plain vacutainer tubes and rapidly transported to the laboratory in cold conditions. Blood and milk samples were centrifuged at 2,500 rpm and 8000 rpm respectively for 10 min in order to separate serum and skimmed milk. Serum and skimmed milk samples were aliquoted and stored at-20°C until used.

Antibody analyses

Skimmed milk and serum samples were analysed by using a commercially available indirect ELISA: HerdChek *Neosporacaninum* Antibody Test Kit, IDEXX Lab. Manufacturer's instructions were strictly followed, with an exception: skimmed milk was diluted 1:2 in the dilution buffer delivered with the ELISA kit, as per recommendation of Schares et al. (2004). Plates were read at 620 nm and the test results were expressed as an S/P ratio obtained by an equation provided by the manufacturer. Serum samples with an S/P ratio equal or higher than 0.5 were considered positive.

Optimized cut-off values were calculated for skimmed milk samples with serum results considered as "gold standard" (see *Results*).

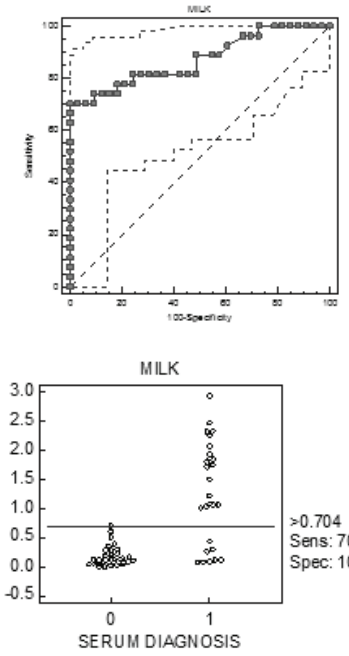
Data analysis

Receiver operating characteristic (ROC) curve analysis, test agreement, sensitivity, specificity, 95% confidence intervals, positive and negative likelihood ratio, positive and negative predictive value, Youden index, and significance levels were calculated using a statistical software program (MedCalc for Windows, version 12.4.0.0, MedCalc Software, Mariakerke, Belgium). Statistical significance was assumed at $P< 0.05$.

The agreement between classic serology and milk ELISA (Inter-rater agreement) was quantified by Weighted Kappa (*K*), interpreted as follows: < 0.20 poor; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 good; 0.81-1.00 very good (Altman, 1991).

RESULTS AND DISCUSSIONS

When the serum results were considered as the gold standard, ROC curve analysis revealed the associated criterion of S/P? 0.704, with 100% specificity ($CI_{95\%}$=89.4 – 100.0) and 70.34% sensitivity ($CI_{95\%}$=49.8-86.2), AUC of 0.873 ($P < 0.0001$) and Youden index of 0.7037 (Figure 1).



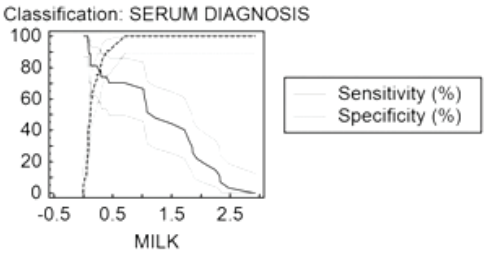


Figure 1. Results of analyzing milk by IDEXX ELISA, with serum results as „gold standard": (a) ROC curve for milk IDEXX ELISA with results shown as dots representing sensitivity/specificity pairs; (b) Interactive dot diagram for milk IDEXX ELISA results classified after serum diagnosis (0=negative, 1=positive); (c) Plotting of milk IDEXX ELISA sensitivity and specificity with 95% confidence intervals.

The associated criterion is the value with the highest specificity and sensitivity and was chosen as cut-off for using IDEXX ELISA on skimmed milk samples.

Other important criterion values revealed by ROC curve analysis on milk samples are presented in Table 1.

Table 1. Criterion values revealed by ROC analysis for milk samples. with serum sample as the true status

Criterion	Sensitivity		Specificity		Likelihood ratio (%)		Predictive values (%)	
	Percentage (%)	$CI_{95\%}$ (%)	Percentage (%)	$CI_{95\%}$ (%)	Positive	Negative	Positive	Negative
=-0.004	100	87.2-100.0	0	0.0-10.6	1		45	
> 0.079	100	87.2-100.0	27.27	13.3-45.5	1.37	0	52.9	100
> 0.08	96.3	81.0-99.9	27.27	13.3-45.5	1.32	0.14	52	90
> 0.088	96.3	81.0-99.9	33.33	18.0-51.8	1.44	0.11	54.2	91.7
> 0.09	92.59	75.7-99.1	39.39	22.9-57.9	1.53	0.19	55.6	86.7
> 0.092	88.89	70.8-97.6	42.42	25.5-60.8	1.54	0.26	55.8	82.4
> 0.118	88.89	70.8-97.6	51.52	33.5-69.2	1.83	0.22	60	85
> 0.122	81.48	61.9-93.7	51.52	33.5-69.2	1.68	0.36	57.9	77.3
> 0.232	81.48	61.9-93.7	75.76	57.7-88.9	3.36	0.24	73.3	83.3
> 0.267	77.78	57.7-91.4	75.76	57.7-88.9	3.21	0.29	72.4	80.6
> 0.293	77.78	57.7-91.4	81.82	64.5-93.0	4.28	0.27	77.8	81.8
> 0.301	74.07	53.7-88.9	81.82	64.5-93.0	4.07	0.32	76.9	79.4
> 0.398	74.07	53.7-88.9	90.91	75.7-98.1	8.15	0.29	87	81.1
> 0.439	70.37	49.8-86.2	90.91	75.7-98.1	7.74	0.33	86.4	78.9
> 0.704	**70.37**	**49.8-86.2**	**100**	**89.4-100.0**		**0.3**	**100**	**80.5**
> 2.935	0	0.0-12.8	100	89.4-100.0		1		55

Because area under the ROC curve (AUC) is significantly different from 0.5, milk IDEXX ELISA has the ability to distinguish between positive and negative bovines, regarding *N. caninum* infection. When the variable under study cannot distinguish between the two groups the AUC will be equal to 0.5 and the ROC curve will coincide with the diagonal, but when there is a perfect separation of the values of the two groups the AUC equals 1 and the ROC curve will reach the upper left corner of the plot (Zweig & Campbell, 1993).

Milk ELISA classified 19 of 60 samples as positive (31.7%, $CI_{95\%}$ = 19.55 – 43.78) at ?0.704 cut-off value while serum ELISA classified 27 of 60 samples as positive (45%, $CI_{95\%}$ = 32.04 – 57.96). The agreement between

serum and milk was *K*=0.723, corresponding to a good agreement.

The within-herd prevalence of dairy farms with serum and milk ELISA is presented in Table 2.

Table 1. The within-herd prevalence of dairy farms with serum and milk ELISA

	A	B	C	TOTAL
Serum prevalence (% (+/n))	80% (16/20)	40% (8/20)	15% (3/20)	45% (27/60)
Milk prevalence (% (+/n))	70% (14/20)	15% (3/20)	10% (2/20)	31.7% (19/60)
K-value	0.737	0.419	0.773	0.723
Intense serum results*	87.5% (14/16)	62.5% (5/8)	66.7% (2/3)	77.8% (21/27)
K-value**	1.00	0,679	1.00	0.921

Previous studies have used IDEXX ELISA to test milk samples for *N. caninum* infection (Schares et al., 2004; Bartels et al., 2005; Byrem et al., 2012) and revealed also a relative high sensitivity and a good agreement with serum results. Schares et al. (2004) reported a TG-ROC determined cut-off value of 0.261, with 90% sensitivity (Se) and specificity (Sp) and *K* = 0.80, for individual skim milk samples compared with serum samples. Bartels et al. (2005) determined a cut-off value of 0.6, Sp of 92% and Se of 61% for testing bulk milk samples. Byrem et al. (2012) calculated a cut-off value of 0.3 for skim milk with Sp of 95%, Se of 77.0% and *K*=0.77.

The sensitivity of 70.34% obtained for skimmed milk samples by IDEXX ELISA was lower than that of 100% reported for using this kit on serum samples (Wu et al., 2002). Others also previously reported a lower sensitivity on milk than on serum of different ELISAs for detecting *N. caninum* antibodies (Bartels et al., 2005; Schares et al., 2004; Byrem et al., 2012).

Differences in cut-off values reported for milk ELISAs may be caused by different commercial or in-house ELISAs that were evaluated or different laboratory techniques, such the use of manual or automated washing steps. Moreover, the stage of lactation in which the paired samples were taken may have played a role. IgG concentration in milk can vary depending on the stage of lactation, so that in late lactation milk quantity decreases but milk protein concentration increases, including IgG (Caffin et al., 1993). Lactation stage was identified as a factor associated with increasing agreement between milk and serum result in individual paired samples in animals (Schares et al., 2004).

The association between seroprevalence level and risk for reproductive losses may be different in distinct dairy industry situations (Wapenaar et al., 2007), involving unknown factors with influence in choosing the appropriate cut-off value and explaining the variance in parameters of milk ELISA.

Based on the value of the S/P ratio, the positive serum samples were divided into 2 categories: low positive (0.5< S/P< 1) and high positive (S/P?1). Milk ELISA performed better when samples with low positive result on sera were excluded (Table 2). A higher intensity of positive reaction (higher S/P values) can be correlated with a higher titer of antibodies indicating increasing performance of milk ELISAs with increasing antibody titer in analyzed samples.

In farm A, reproductive history of animals was known and permitted a correlation of diagnostic performance with abortions. All seropositive bovines with history of abortions were also positive on milk samples and presented a high positive reaction both on serum and milk. Thereby, high positive reaction both in serum and milk from animals that have aborted may be an additional clue for neosporosis as a cause of abortion, but require confirmation by other diagnostic techniques.

Schares et al. (2004) found that using IDEXX ELISA on milk samples, more aborting animals were identified positive than using the same kit on serum samples, but in that study tested cattle farms presented recent history of epidemic or endemic abortions, statistically associated with *N. caninum* infection.

Subsequently, we also analyzed four pooled milk samples of three herds with known seroprevalence of *N. caninum* infection.

When the optimized cut-off value for individual skimmed samples was used also for pooled milk samples only the two batches of samples with higher seroprevalence – farm A and total pooled sample with seroprevalence of 80 and 45%, respectively – were correctly classified, but the test failed to classify as positive the other two batches – farm B and C with seroprevalence of 40 and 15%, respectively (Table 3).

Table 2. Classification of pooled milk samples according to different cut-off values

Pooled samples	Cut-off values			
	> 0.704[a]	**> 0.61**[b]	**> 0.51**[c]	**> 0.398**[d]
A S/P=1.754	+	+	+	+
B S/P=0.687	-	+	+	+
C S/P=0.562	-	-	+	+
Total S/P=**1.573**	+	+	+	+

[a]Se =70.37%, Sp = 100%
[b]Se =70.37%, Sp = 96.97%
[c]Se =70.37%, Sp = 93.94%
[d]Se =74.07%, Sp = 90.91%

The relatively low sensitivity for pooled milk samples in the present study may be the consequence of the cut-off values chosen for the test. According to Bartels et al. (2005) the IDEXX ELISA performed satisfactorily in bulk milk samples at a calculated cut-off value of 0.6 – with 61% sensitivity and 92% specificity – to detect a within-herd seroprevalence of *N. caninum* in lactating cows of at least 15%. When calculated this cut-off value, Bartels et al. (2005) were based on previous studies in the Netherlands which suggested that a within-herd *N. caninum* seroprevalence of 15% can be associated with increased risk for reproductive losses.

When the interpretation by Bartels et al. (2005) of bulk milk IDEXX ELISA results with respect to seroprevalence levels was used, 3 of the 4 herds were classified correctly, at a similar cut-off value of 0.61, but with higher sensitivity and specificity (Table 3). The herd classified as negative had a seroprevalence of 15%, making the interpretation challenging. The cut-off value that classified correctly all pooled milk samples in the present study was > 0.51, with 93.94% specificity and 70.37% sensitivity. A lower cut-off value can be chosen, with increasing sensitivity but decreasing specificity. When a test is used either for the purpose of screening or to exclude a diagnostic possibility, a cut-off value with a high sensitivity may be selected, but when the test is used to confirm a disease, a higher specificity may be required (Zweig & Campbell, 1993).

Testing pooled milk samples may represent an alternative to testing individual milk samples, especially when a high prevalence is suspected.

CONCLUSIONS

When evaluating a diagnostic test it is essential to consider its future utility. The diagnostic performance of the IDEXX ELISA for individual milk samples regarding *N. caninum* infection creates opportunities for implementing an economical and reliable testing scheme for dairy farms, while the diagnostic performance for pooled milk samples justify further consideration.

When considering these aspects, from the present study following conclusions can be drawn:

The *Kappa* value of 0.723 suggests that the use of optimized cut-off value (S/P> 0.704) is adequate for testing bovine milk for *N. caninum* infection in southern Romania.

The specificity for milk IDEXX ELISA was 100% and the sensitivity was 70.4%.

Milk ELISA performed better when low positive sera were excluded (K=0.921) indicating increasing performance with increasing antibody titer in analyzed samples.

Testing pooled milk samples with the IDEXX ELISA may represent an alternative to testing individual milk samples for *N. caninum* infection in laboratory conditions, identifying dairy farms with a 15% or higher within-herd seroprevalence at the cut-off value of S/P> 0.51.

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RESEARCH REGARDING CHEMICAL COMPOSITION AND NUTRITIONAL VALUE OF *LENS CULINARIS* MEDIK. SPECIES (LENTIL) IN ORGANIC AGRICULTURE

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Abstract

Foods for human consumption fall into two categories as vegetable and animal origin. Raw materials of vegetable origin foods are divided into three sub-groups as cereals, oil seed and grain legumes. Grain legumes occupy a crucial place in human diet among these foods. Common bean, broad bean, chickpea, lentil, pea, soya and black eyed peas are the species of grain legumes. Lentil is one of the legume species that have been used successfully in human diet for a very long time.
The paper presents the results of the research regarding chemical composition and nutritional value of Lens culinaris Medik. species (lentil) promoted in organic agriculture.
The experiment was carried out in Moara Domneasca Experimental Field, during 2007-2009. It was studied 7 lentil genotypes: Beluga (France), Sorte du Puy (France), Laird (Turkey), Richlea (France), Masoor (Turkey), Eston (Greece) and a local genotype „Moara Domneasca".
In average, the chemical composition of the lentil genotypes cultivated in Moara Domneasca Experimental Field was the following: 22.18% proteins, 3.03% fats, 63.29% carbohydrates, 4.00% minerals, while the energy value was 259.97 kcal.
The protein yields ranged between 259 kg/ha and 335 kg/ha, the average being 297 kg protein/ha. The highest proteins yields, of more than 300 kg protein/ha, were obtained from Laird and Richlea genotype

Key words: *lentil, chemical composition, nutritional value.*

INTRODUCTION

Foods for human consumption fall into two categories of vegetable and animal origin. Raw materials of vegetable origin foods are divided into three sub-groups as cereals, oil seed and legumes.
Grain legumes occupy a very crucial place in human diet among these foods. Common bean, broad bean, chickpea, lentil, pea, soybean and black eyed peas are main species of grain legumes. Lentil is one of the legume varieties that have been used successfully in human diet for a very long time (Özer et all, 2010).
The lentil plant, *Lens culinaris* L., is a member of Fabaceae family and constitutes one of the most important traditional dietary components. FAO reported that world production of lentils was about 2.83 million metric tons for 2008, primarily coming from Canada (36.9%) and India (28.7%), followed by Nepal, China and Turkey (Truta, 2008).
Lentil plants grow to around 0.5 m in height as a slender bush or twining vine. Lentil plants flower from the bottom of the plant, and the flowering progresses upward. The flowers range in color from white to pale blue. Seed pods usually hold one or two seeds.
Numerous cultivars vary in the seed size and texture, and colors range from green to yellow to orange to red and brown (Roman et al., 2009).
Lentils have been incorporated into different world cuisines throughout the globe, especially the Mediterranean and Indian regions. Lentils have been classified among soft seed-coated pulses that require shorter cooking time, and thus have smaller losses in nutrients as compared to those with hard seed coat (Satya et all, 2010). The shorter cooking time of lentils (23–26 min.) in comparison with most other

pulses makes lentils very convenient for human consumption (Solanki et all, 1999).
Lentil is an important legume since it has higher protein amount/quality than cereals; it is also rich in vitamins and mineral constituents in addition to being a protein source. Furthermore, it provides amino acid balance when consumed with cereals. It is rich in respect of fiber and thus it helps overcoming hunger and balancing the appetite. Lentil is rich in calcium (Ca) (which is necessary for the development and overall health of bones), iron (Fe) (which forms blood in the metabolism) and vitamin B (which helps the nervous system to work efficiently) (Özer et al., 2010).

MATERIALS AND METHODS

The paper presents the results of the research regarding chemical composition and nutritional value of *Lens culinaris* Medik. (lentil) species promoted in organic agriculture.
The experiment was carried out in Moara Domneasca Experimental Field, located near Bucharest, during 2007-2009 and it was organized based on the multi-stage block method with randomized variants in 4 replications.

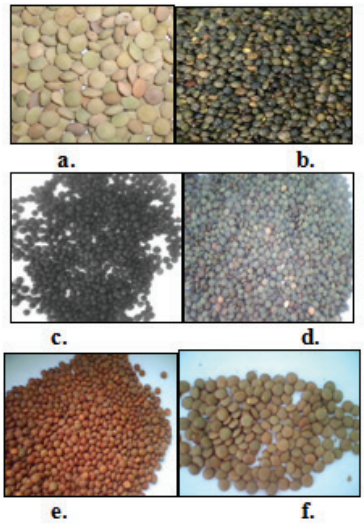

Figure 1. Biological material (genotypes) used in the lentil experiment, a-Laird,Turkey; b-Sorte du Puy, France; c-Beluga, France; d-Eston, Greece; e-Masoor, Turkey; f-Richlea, France.

The biological material used in the experiment came from organic crops and was represented by 7 lentil genotypes (Figure 1) : Beluga (France), Sorte du Puy (France), Laird (Turkey), Richlea (France), Masoor (Turkey), Estonia (Greece) and the local population 'Moara Domneasca' (Romania).
The biochemical compounds (glucides, starch, proteins, fats and minerals) have been analysed by using the common chemistry laboratory methods: for carbohydrates, Bertrand Method; for proteins, Kjeldahl Method; for fats, Soxhlet Method; for minerals, Spectrophotometer Method.

RESULTS AND DISCUSSIONS

Chemical composition of lentil seeds was as follows: 22.18% proteins, 3.03% fats, 63.29% carbohydrates, 4.00% ash, while the energy value was 259.97 kcal.
Regarding protein content, from Table 1 resulted values between 21.14% and 22.85%, the average being 22.18%.
The highest protein contents were determined for "Laird" and "Richlea" genotypes seeds-22.85% and 22.67%-and the lowest protein content was found at "Sorte du Puy" genotype, with 21.14%.
Higher fats content was observed at Sorte du Puy genotype with 3.40%, Beluga genotype with 3.25%, Masoor and Eston genotypes with 3.06% and 3.02% fat content. The lowest values?? were recorded in Moara Domneasca and Richlea genotypes, with 2.78% and 2.81% lipid content.
Carbohydrates content was on average 63.29%, with little difference between the variants; the highest content being recorded at "Laird" genotype with 63.98% and the lowest at "Eston" genotype with 62.87%.
Minerals content presented values ranging from 3.84% for Moara Domneasca genotype and 4.13% for Masoor genotype and the experiment average was 4.00%.
Energy values?? were at least 255.86% kcal from genotype Sorte du Puy and maximum of 265.00% kcal for genotype Laird.
The production data resulted in three experimental years illustrates the favorability of natural conditions for lentils and a high productivity of the tested biological material.

Table 1. Seeds chemical composition of lentil genotypes (% d.m.) (Moara Domneasca Experimental Field, 2009)

Genotype	Protein	Fats	Carbohydrates	Minerals	Energy value (kcal%)
Beluga	21.78	3.25	62.98	4.11	259.02
Sorte du Puy	21.14	3.40	63.57	4.04	255.86
Laird	22.85	2.95	63.98	3.94	265.00
Richlea	22.67	2.81	63.21	3.91	259.77
Masoor	22.27	3.06	63.43	4.13	263.28
Eston	22.34	3.02	62.87	4.07	258.92
Moara Domneasca	22.21	2.78	63.02	3.84	256.74
Average	**22.18**	**3.03**	**63.29**	**4.00**	**259.97**

Table 2. Protein yields of lentil genotypes (Moara Domneasca Experimental Field, 2009)

Genotype	Protein yields		Differences (kg/ha)	Significance
	kg/ha	%		
Beluga	259	86.71	-38	ooo
Sorte du Puy	276	9336	-21	ooo
Laird	335	113.99	38	***
Richlea	317	106.99	20	***
Masoor	287	99.30	-1	o
Eston	309	99.30	12	**
Moara Domneasca	302	99.65	0.5	-
Average	**297**	**100**	Mt	-

$DL_{5\%}$= 7.1 kg/ha
$DL_{1\%}$= 10.7 kg/ha
$DL_{0,1\%}$= 17.2 kgha

Table 2 presents protein yields calculated based on seed yields and protein content.

Seeds yields for the experiments with different genotypes of lentil were on average of 1291 kg/ha, the limits ranging between 1143 kg/ha at "Beluga" genotype and 1427 kg/ha at "Laird" genotype.

The protein yields ranged between 259 kg/ha and 335 kg/ha, the average being 297 kg/ha.

Most productive genotypes were determined to be "Laird" with 335 kg protein/ha, exceeding the average by 38 kg/ha, and "Richlea", with 317 kg/ha and an increase of protein yield with 20 kg/ha.

The lower protein yields were recorded in "Beluga" and "Sorte du Puy" genotype, which produced 259 kg/ha, respectively 276 kg protein/ha.

It can be noticed that "Richlea" and "Laird" genotype gave the highest seed yields, had the highest seeds protein content and gave the highest protein yields

CONCLUSIONS

Chemical composition of the lentil seeds was as follows: 22.18% proteins, 3.03% fats, 63.29% carbohydrates, 4.00% minerals, while the energy value was 259.97 kcal.

Fats content ranged from 2.78% at "Moara Domneasca" genotype to 3.40% at "Sorte du Puy" genotype.

Carbohydrates content was 63.29% on average with low differences between genotypes, the highest content being recorded at "Laird" genotype with 63.98% and the lowest at "Eston" genotype with 62.87%.

The results obtained in the three experimental years illustrate the favorability for lentils of the experimental area natural conditions, which offer favorable prerequisites for the

achievement of successful crops and high quality production.

Lentils is one of the alternative crops promoted by the organic agriculture system and can be a potential alternative crop for organic farms in the area.

However, introduction and expansion in culture of these species may contribute to diversification of human nutrition and animal feeding.

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HEMATOLOGIC PROFILE AS STRESS INDICATOR IN FISH

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Abstract

Hematologic parameters are considered important indicators of the fish health status, providing useful information for assessing their welfare. Fish response to stressors is similar to higher vertebrates, being represented by the quick release of catecholamines, followed by the corticosteroids release.
The study aims to establish stress level in common carp (Cyprinus carpio) by obtaining an overview of the morphology of blood cells, their number and percentage distribution in peripheral blood. The biological material was represented by a group of 5 fish weighing between 2.5 and 3 kg. From fish there were collected blood samples using heparinized syringes, after which the samples were transferred into Li-heparin containers. Leukocyte count was made on smears with Diff-Quick staining. The study of cells characteristics was performed by optical microscopy. Obtained results were compared with hematologic reference ranges for carp.
Analyzing the results, it was noticed the presence of erythrocytes and platelets and, from the white series, of lymphocytes, monocytes, eosinophils and heterophils. Among leukocytes, 80% were represented by lymphocytes, eosinophils were reduced in number and basophils were not identified, this aspect being in accordance with other authors' findings. The results obtained in this study framed for most parameters within the reference ranges for carp. In fish, according to literature, lymphocytes have a role in specific immune response, neutrophils in the inflammatory response and in phagocytic function, eosinophils have a role in phagocytosis of parasites, monocytes migrate into tissues to become macrophages. Action of stress factors upon the white line cells is manifested as lymphopenia with relative granulocytosis.
Given the results, it can be concluded that there is a low level of stress for the fish in study and they are reared under appropriate, welfare-friendly conditions.

Key words: *fish, stress, hematologic profile, welfare.*

INTRODUCTION

An extensive part of knowledge in the field of vertebrate haematology is based on mammalians′ study. The concept of non mammalian vertebrate blood analysis is relatively new (Claver J.A. and Quaglia A.I.E, 2009), starting to develop successfully as the data concerning the physiology of blood cells and standardized techniques became available (Hrubec T.C. and Smith S.A., 2010).
The above authors (Hrubec T.C. and Smith S.A., 2010) argued that bony fish are "more closely related to other bony vertebrates (e.g. mammals) than they are to their cartilaginous counterparts".
Azevedo et al. (2006) showed that an important health indicator in fish is represented by hematologic profile, which could help the researcher in assessing their welfare.
This approach is also supported by Gabriel et al. (2004), who demonstrated in their study that the environment may have a significant impact upon the haematological parameters in fish.
The environment, especially water quality, can influence the following parameters: packed cell volume (PCV), red blood cells count (RBC), immature erythrocyte count, white blood cells count (WBC) and haemoglobin (Hb).
Leonard J.B.K. and McCormick S.D. (1999) noticed the increasing of the PCV and RBCs number in American shad during upstream migration due to the decreasing of the dissolved oxygen concentration.
El-Sherif et al. (2008) revealed in their study that haemoglobin and PCV decreased in relation with the increasing of the unionized ammonia nitrogen (UIA-N).
Wedemeyer (1996) stated that the exposure to nitrite can affect the thymus by inducing hemorrhagic and necrotic lesions. According to Bowden et al. (2005) the thymus is involved in the development of the adaptive immune system.

Das et al. (2004) described that immature erythrocyte count increased while nitrite − nitrogen concentration increased up to 4 mg/l. The count decreased to zero when nitrite − nitrogen ranged within 8 to 10.4 mg/l, value at which the erythrocytes appeared shrunken and tapered. WBC′s count increased along with the increasing of the exposure period.

Mishra S. and Srivastava A.K. (1979) reported the following haematological changes in a fresh water teleost (*Colisa fasciatus*) exposed to zinc sulphate: decreased RBCs, WBCs count and PCV, with increased erythrocyte sedimentation rate.

Kaoud et al. (2011) found that the exposure of Nile Tilapia (*Oreochromis niloticus*) to cadmium can lead to a significant decrease in PCV, haemoglobin and RBC′s count.

Bozorgnia et al. (2011) studied the effect of temperature on common carp blood parameters and found that the RBCs and WBCs counts increased at 32°C and decreased at 15°C.

The present study aims to analyse the number, percentage distribution and characteristics of the blood cells in order to gain an overall picture of the stress response in carp, with respect to the haematological parameters.

MATERIALS AND METHODS

The biologic material consisted in five individuals of common carps (*Cyprinus carpio*), weighing between 2.5 and 3 kg, collected from a fishpond in Plataresti-Calarasi County. Water temperature was approximately 27°C.

The fish were macroscopically examined for clinical diseases or gross lesions. No visible lesions or gross abnormalities were detected.

Blood was collected from the caudal vessels, as described by Branson. E.J. (2008), using heaprinised sterile syringes with 20-21 gauge needles, depending on fish size. Blood was transferred into Li-heparin containers and refrigerated. Smears were prepared within 4 hours from sampling, air dried and prepared for staining.

Total RBCs count was performed manually using a Burker-Turk chamber and optical microscopy, as described by Dumitru C. Curca. (2005).

The prepared blood smears were stained using Diff-Quick protocol. There were also performed the 100 cell differential count and blood cell morphology examinations.

RESULTS AND DISCUSSIONS

The following cells were identified: erythrocytes, erythrocyte precursors, thrombocytes, and from the white series-lymphocytes, heterophils, monocytes and eosinophils.

The erythrocytes had the higher occurrence in the blood smears. They presented most frequently an oval shape, but round shaped cells were also detected. The nucleus was centrally positioned with an oval to round shape.

The erythrocyte precursors were smaller in size with a round centrally located nucleus. They presented a grater nuclear:cytoplasmic (N:C) ratio than the mature ones.

The thrombocytes were oval, round or spiked cells, smaller than the erythrocytes. They presented centred round to elongated nuclei.

The lymphocytes were the most common detected leukocytes and were classified as small and large ones. The small lymphocytes presented a nucleus that occupied most of the cell space in comparison with the large lymphocytes that presented a higher quantity of cytoplasm. Their shape was round. A small number of lymphocytes with a distorted shape was also found.

The heterophils presented as round shaped cells with a kidney shaped nucleus or having two or three lobes.

The eosinophils were rarely observed in the blood smears. The cells were relatively round with an irregular shaped nucleus and red cytoplasmic granules.

The monocytes were the largest observed white blood cells. They presented an indented nucleus and often vacuolated cytoplasm. A lower nuclear:cytoplasmic ratio and cytoplasmic vacuolas aided in differentiating monocytes from large lymphocytes.

No basophils were detected, but their existence in the peripheral blood is not excluded (Hrubec T.C. and Smith S.A., 2010).

The white blood cells functions and the stress influence on this kind of cells are also described by authors like Hrubec T.C. and Smith S.A., (2010) who stated that the WBC play the following immune roles: lymphocytes participate in innate and specific immune T and B cells response, neutrophils participate in the inflammatory response and possess phagocytic functions, eosinophils protect the fish against parasites being also capable of phagocytosis, monocytes migrate to sites of infection into

tissues and differentiate into macrophages. There are also authors like Clem L.W. et al. (1991) and Ruane N.M et al. (2000) who argue that in fish, the stress response is represented by lymphopenia and granulocytosis.
The percentage distribution and total RBC count is presented in tables 1 and 2.

Table 1. RBC complete count and percentage distribution of white blood cells: samples (S) 1-3

Parameter	S.1	S.2	S.3	Reference values (Hrubec & Smith 2010; Svobodova, Vykusova 1991)
Red blood cells x 10^6/µl	**2.11**	**1.98**	1.69	1.69-1.91
Lymphocytes%	78	85	**73**	76-97.5
Heterophils%	**11**	8	**19**	2-10
Eosinophyls%	1	**2**	1	0-1
Monocytes%	**10**	5	**7**	3-5
Basophils%	0	0	0	0-0.5

Table 2. RBC complete count and percentage distribution of white blood cells: samples (S) 4-5

Parameter	S.4	S.5	Reference values (Hrubec & Smith 2010; Svobodova, Vykusova 1991)
Red blood cells x 10^6/µl	**1.57**	1.73	1.69-1.91
Lymphocytes%	91	86	76-97.5
Heterophils%	7	9	2-10
Eosinophils%	0	1	0-1
Monocytes%	**2**	4	3-5
Basophils%	0	0	0-0.5

Thus, according to the results above, the 5 studied individuals presented a reduced number of parameters slightly exceeding or below the reference ranges.

CONCLUSIONS

The study showed that the fishes presented a low level of stress and that the rearing conditions are adequate in terms of welfare.

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EVALUATION OF FUNGAL INCIDENCE IN BROILER FARM

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Abstract

This research aimed the isolation and identification of mycoflora from forage, water, air, litter and sanitation swabs collected during the year 2011. Collecting and processing of samples was done according with the literature data and current standards. Therefore the air samples were collected and processed by method of sedimentation and the forage, water, litter and sanitation swabs samples have been harvested and processed according to ANSVSA Order no. 25 of 19 March 2008, SR EN ISO 6887-1/2002 and ISO 7218 /2007. Through the qualitative mycological exams have been identified 11 genres (Aspergillus spp.*, Penicillium* spp.*, Mucor* spp., *Absidia* spp., *Rhyzopus* spp., *Alternaria* spp., *Ulocladium* spp., *Cladosporium* spp., *Fusarium* spp., *Candida* spp., *Rhodotorulla* spp.*) and 12 species (Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Absidia corymbifera, Fusarium oxisporum, Rhodotorulla rubra, Candida albicans, Candida sake, Candida rugosa, Candida famata and Candida lusitaniae).*

Key words: *broiler,fungi,incidence.*

INTRODUCTION

A permanent objectiv in broiler farms is monitoring hygiene status. In parallel with hygienisation programs, for efficient fight against pathogenic microorganisms is necessary periodic evaluation of the microbial species existing in farm. The diseases caused by pathogenic fungi, like all of infectious diseases, causes the economic losses through high morbidity and mortality and decreased the production indicators. Among the fungal infection of poultry, the most frequent reported in the literature are: aspergillosis and candidosis. Aspergillosis is the most important fungal infection, being frequent caused by *Aspergillus fumigatus* and rarely by *Aspergillus flavus* or the *Aspergillus niger*. The disease mainly affects respiratory tract of broilers and develops as a bronchopneumonia (Milos C. et al, 2011; Stoenescu Virginia., 1964). Candidosis is encountered frequently in respiratory mucosa and air sacs of poultry. The pathogenic fungi (in the presence of mucosal injury) such as *Penicillium* spp.*, Mucor racemousus*, may cause especially at hens, pseudotubercles nodules and clinical signs similar to those of aspergillosis (Stoenescu Virginia., 1964). Other authors (Coman I., 1985) have shown that fungi of the Penicillium spp., *Alternaria* spp., *Aspergillus* spp., or *Fusarium* spp., get into the organism of poultry through feed contaminated with spores, thus becoming toxic for poultry to which triggers a hemorrhagic syndrome.

MATERIALS AND METHODS

From January 2011 to September 2011, a total of 50 samples were taken according to ANSVSA Order no. 25 of 19 March 2008, from commercial broiler farm. Of all 50 samples, 10 samples were represented by the feed, 10 by the water, 10 by the air, 10 by the litter and 10 by the swabs. All samples were aseptically transported to the laboratory and were stored at 4°C for fungal analyses. The air samples harvested through the Koch method (Coman I., 1997) on the surfaces of Sabouraud Chloramphenicol Agar were immediately thermostated, for 7-14 days at 25°C. After that the fungal strains develop were identified. The samples of feed, water, litter and swabs were processed according to current standards (SR EN ISO 6887-1/2002; SR EN ISO 7218 /2007). Of the inoculum resulting from the processing of samples were performed dilutions from which were apply each 0,1ml to the center of a dry plate with Sabouraud Cloramphenicol Agar and spread with loop of the entire surfaces for a

better absorption. Then plate was incubated at 25°C for 7-14 days. To obtain pure culture were transferred onto Malt Extract Czapek for the identification of isolate fungi. Most filamentous fungi can be identified based on a combination of colonial morphology and microscopic features in accordance with keys (http://www.mycology.adelaide.edu.au/Keys, http://www.doctorfungus.org,http://www.cbs.knaw.nl). Slides were prepared for identification of mycelium and macroconidia with lactophenol blue staining method. Yeasts were subcultured and identified with the Api 32 C system (BioMerieux France).

RESULTS AND DISCUSSIONS

In Table 1. is shown the incidence of fungal genres and species isolated from broilers farm.
Table 1. Fungal incidence in forage, water, air, litter and swabs

Table 1. Fungal incidence in feed, water, air, litter and swabs

	Feed No.samples/10		**Water** No. samples/10		**Air** No. samples/10		**Litter** No.samples/10		**Swab** No.samples/10		**Grand total** No.Total samples/50	
	Strains		Strains		Strains		Strains		Strains		Strains	
Genus / Species	nr.	%	nr.	%	nr	%	nr.	%	nr.	%	nr.	%
Aspergillus niger	3	30%	0	0%	3	30%	3	30%	2	20%	11	22%
Aspergillus flavus	2	20%	1	10%	2	20%	2	20%	1	10%	8	16%
Aspergillus fumigatus	2	20%	1	10%	7	70%	5	50%	5	50%	20	40%
Aspergillus glaucus	1	10%	0	0%	0	0%	2	20%	1	10%	4	8%
Penicillium	4	40%	3	30%	3	30%	7	70%	5	50%	22	44%
Mucor spp.	3	30%	0	0%	1	10%	3	30%	2	20%	9	18%
Absidia corymbifera	1	10%	0	0%	0	0%	2	20%	1	10%	4	8%
Rhyzopus spp.	0	0%	0	0%	1	10%	1	10%	1	10%	3	6%
Alternaria spp.	3	30%	1	10%	1	10%	3	30%	2	20%	10	20%
Ulocladium spp.	2	20%	0	0%	1	10%	1	10%	1	10%	5	10%
Cladosporium spp.	2	20%	1	10%	2	20%	2	20%	1	10%	8	16%
Fusarium oxisporum	3	30%	1	10%	1	10%	2	20%	1	10%	8	16%
Total molds	**26**		**8**		**22**		**33**		**23**		**112**	
Rhodotorula rubra	2	20%	2	20%	0	0%	4	40%	2	20%	10	20%
Candida albicans	3	30%	2	20%	0	0%	3	30%	1	10%	9	18%
Candida sake	0	0%	0	0%	0	0%	1	10%	0	0%	1	2%
Candida rugosa	0	0%	0	0%	0	0%	1	10%	1	10%	2	4%
Candida famata	0	0%	1	10%	0	0%	0	0%	0	0%	1	2%
Candida lusitaniae	0	0%	0	0%	0	0%	1	10%	0	0%	1	2%
Total yeasts	**5**		**5**		**0**		**10**		**4**		**24**	
Grand total strains	**31**		**13**		**22**		**43**		**27**		**136**	

Form the data analysis presented in Table 1 and Figure 1., follows that of the total samples taken been identified 136 strains of fungi out of which 112 (82,35%) were molds and 24 (17,65%) yeasts.

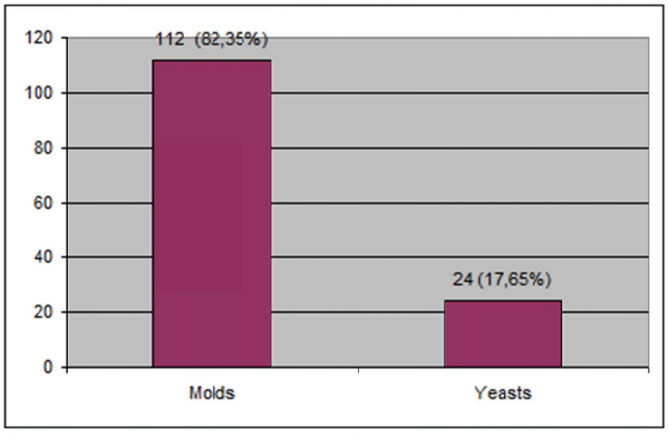

Figure 1. Incidence of molds and yeasts in broilers farm

More precisely of the 50 samples taken from the farm were found 11 strains (22%) of *Aspergillus niger*; 8 strains (16%) of *Aspergillus flavus*; 20 strains (40%) of *Aspergillus fumigatus*; 4 strains (8%) *Aspergillus glaucus*; 22 strains (44%) of *Penicillium* spp.; 9 strains (18%) of *Mucor* spp; 4strains (8%) of *Absidia corymbifera*; 3 strains (6%) of *Rhyzopus* spp; 10 strains (20%) of *Alternaria* spp; 5strains (10%) of *Ulocladium* spp; 8 strains (16%) of *Cladosporium spp*; 8 strains (16%) of *Fusarium oxisporum*; 10 strains (20%) of *Rhodotorulla rubra*; 9 strains (18%) of *Candida albicans*; 1 strain (2%) of *Candida sake*; 2 strains (4%) of *Candida rugosa*; 1 strain (2%) of *Candida famata* and 1 strain (2%) of *Candida lusitaniae*. From the data presented in Table 1, it results that samples of litter are the most intense contaminated (43 strains), being followed by the feed samples (31 strains) and the sanitation swabs (27 strains). From the feed, water and litter samples the most frequent isolated fungi species is *Penicillium* spp. (Figure 2, 3) *Aspergillus fumigatus* (Figure 4, 5) is the most fequent isolated fungi species from the air and litter samples. The yeasts *Rhodotorulla rubra* (Figure 6, 7) and *Candida albicans* (Figure 8, 9) were most frequent isolated from the litter samples.

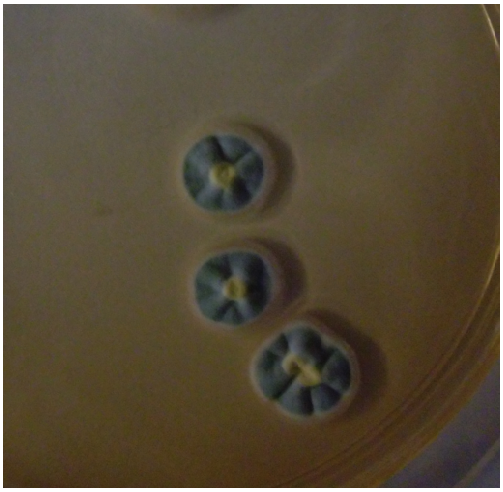

Figure 2. *Penicillium* spp. macroscopic

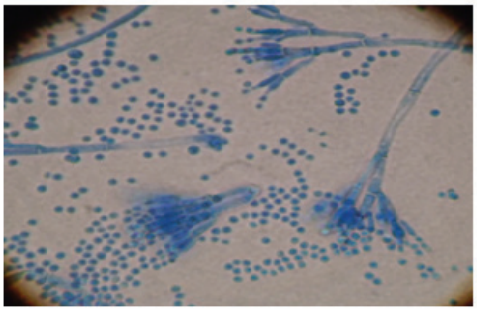

Figure 3. *Penicillium* spp. microscopic

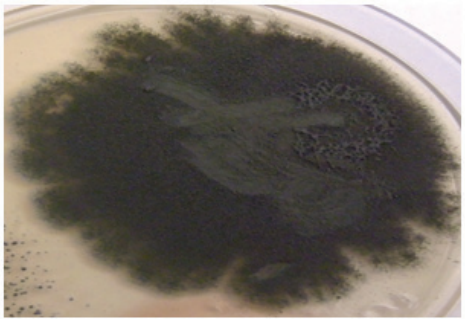

Figure 4. *Aspergillus fumigatus* macroscopic

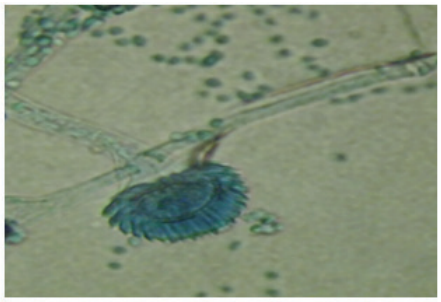

Figure 5. *Aspergillus fumigatus* microscopic

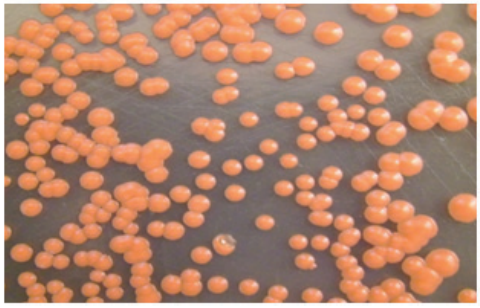

Figure 6. *Rhodotorulla rubra* macroscopic

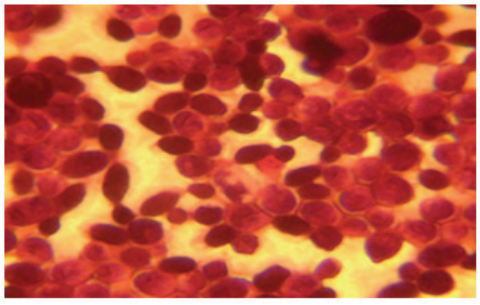

Figure 7. *Rhodotorulla rubra* microscopic

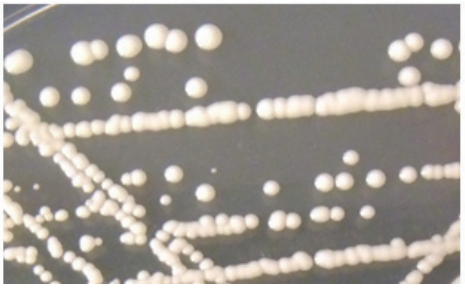

Figure 8. *Candida albicans* macroscopic

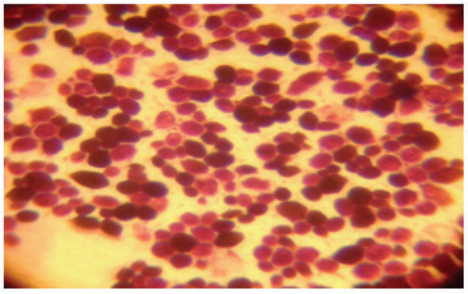

Figure 9. *Candida albicans* microscopic

Analyzing the results obtained of the mycological research about the incidence of fungi, it follows that are in accordance with the data in the literature. Therefore, Arne (Arne P. et al, 2011) show in his analysis that *Aspergillus fumigatus* grows in litter of poor quality, in feed stored in poor conditions and in the air from broilers farm. Furthermore, inadequate ventilation increases the risk of exposure of poultry to inhalation of spores causing high morbidity and mortality. The results of present study are also in agreement with the findings of Azarakhsh (Azarakhsh Y. et al, 2011) who reported a higher incidence of *Aspergillus spp* in feed.

CONCLUSIONS

The fungal flora isolated and identified from forage, water, air, litter and swabs samples was various. Also were have identified some pathogenic fungi can exert immuno-suppression on broilers.

Through the macroscopic, microscopic and biochemical tests were isolated and identified 11 genera and 12 species of molds and yeasts. Presence of opportunistic pathogens from *Aspergillus* poses a risk of invasive aspergillosis in poultry farm.

Compared to the rest of the samples, the litter have proved to be the most intensely contaminated (43 strains), the most common fungi isolated were *Penicillium* spp., *Aspergillus fumigatus, Candida albicans* and *Rhodotorulla rubra*.

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EFFECTS OF DEFICIENT NUTRITION ON THE REPRODUCTION OF THE MILK COWS

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Abstract

In the past decades, the downsize of the fertility in the milk cows stock farm has become a more and more acute problem. This downsize being caused by a series of factors and reasons such as: genetic improving, inadequate nutrition (deficient or excessive diet), deficient management of reproduction, raise of diseases rate due to immunity abasement, growth technology wrongly applied and the overall well-being of the animal.

In the paper the following are presented: causes, etiology, cause-related mechanisms of under and over feeding, vitamins and minerals substances deficient, involved in causes which determine nutritional infertility. Of the highest importance is ensuring the corresponding proportion of each feeding principle. Cows with large milk production are predisposed to genitalia diseases due to metabolic overload.

In zootechnical exploitations over 60% of the infertility cases are due to unbalanced diets, so that the protein deficiency from food disturbs the hypothalamic-hypophyseal activity, which leads to the deficit of gonadotropic hormones creation, causing hypoesthesia, apparition, anovular heat.

Infertility determined by insufficient energetic substances: the lack of fibrous from food causes the decrease of inferior vermilion fat acids, which are the base for steroid hormone synthesis.

Infertility caused by the lack of Vitamin A occurs by the retention of fetal tectorium, uterine under growth, anovular heat apparition. The Vitamin D insufficiency can cause dysgravidism.

Vitamin E insufficiency can cause placental degenerative processes. Also, Vitamin C is involved in stress related annihilation problems.

Microelements insufficiency such as: Mg,P,Ca,Na,K,Cl,S,Fe,Mn,Cu,Zn, will cause several affections such as: anovular heat set up, luteal cysts, placental retentions, uterine involution, anoestrum, weak or ailing conception products.

Key words: *genital apparatus, gonadotropic hormones, ovarian cysts, anovular heat, infertility, luteal cysts, uterine involution, anoestrum.*

INTRODUCTION

The genetic selection of milk cows has proven that there is a connection between the activity of hemadens and the nutritional metabolic balance, so that the reproduction is compromised in the periods when the diet is insufficient, especially at the beginning of lactation. The necessary energy demand to synthesize and release hormones, to keep an embryo in early stages of development, is probably minimum compared to the energy necessary to keep lactation.

In any case, the and endocrine metabolic signs associated with the Negative Energy Balance (NEB)affect the ovulatory cycles, oocytes, the quality of the embryo as well as keeping the fetation.

The organism requirements to synthesize milk are higher, the reproduction function may be affected when the nutritional absorption is insufficient to ensure the necessary energetia, mineral and minor elements substances, which are extremely important for the organism in this period.

The milk production rate grows fast in the first week after birth.

In the period when milk synthesis rises, the reproduction function may diminish, if the compensatory necessary absorption of nutritive substances level is not reached. Several recent studies show that the milk production rises at a very fast pace in regard to the energy absorption (Villa –Godoy et al., 1988, Vasconcelos et al., 2003).

When cows suffer from a Negative Energy Balance (NEB), the free fat acids concentration from blood rises, registering at the same time low values of IgF-1, glucoses and insulin. These changes in metabolites and blood

hormones may compromize ovarian functions and fertility.

Also there has been reported that the Negative Energy Balance (NEB) and the dry matter substance intake (DMS) may affect the plasma concentrations in progesteron (Villa –Godoy et al., 1988, Vasconcelos et al., 2003) fact that may interfere in the evolution of ovarian folicules and keeping the fetation. Over the last decades genetic selection and cow population managing have increased extraordinarily the production of milk cows, at the same time decreasing fertility. (Butler, 2003).

This was also due to the fact that the endocrine profile of the milk cow was modified so that the somatotrophin and galactin from the bovines blood have also increased, while insulin has decreased. (Bonczeck et al., 1988).

These hormonal changes and the grown nutrient necessary for the milk production may negatively affect the reproduction of the milk cows.

In any case, adequate diets and proper administration have shown an equilibrium between production and fertility decrease within the cows populations with an ordinary milk production, larger the 12,000 l/cow/year, (Nebel and McGiliard, 1993; Jordan and Foudraine ,1993).

Several nutritional strategies were proposed to enhance the milk cow reproduction, without affecting the milk production performance. Maximizing the dry matter consume in this period of transition determined a minimization of the postpartum problems incidents, diets which have higher insulin concentrations from the beginning of the lactation, as well as adding additional fats, manipulating fat acids consume (FA), fat resources, it is expected to bring benefits to the milk cow reproduction. (Santos 2010).

The economic desideratum in milk cows farms represent on one side obtaining a milk production where the milk is rich in protein, and on the other side obtaining a calf every year.

In this regard there must be a good functioning of the axis level: hypothalamus-hyphophysis-uterus-ovary.

The main hormones in the oestral cycle, ovulation and conception are progesterone and estrogen, whose production varies according to several nutritional causes and unbalanced diets. Progesterone is a steroid hormone induced by the ovarian yellow body cells, and during the gestation period is also produced by the placenta.

Its role is to prepare the uterine mucosa to receive the fertilized ovule. The estrogen is produced by the ovaries with the role of maintaining the structure and function of the vagina wall, and is responsible with the nutrition of the fertilized ovule and later of the development of the fetus.

A good functioning of this mechanism leads to an uterine involution, to a normal ovary rhythm, to an eloquent manifestation of heat and finally to the installation of a new gestation.

There must be ensured a balanced diet from the quantity and quality point of view, any unbalance between these factors or disrespect of the proportion between the necessary nutrition elements will cause the organism to look to the accumulated reserves and the decrease of reproduction will be installed, infertility, decrease of vitality of the new born. The scarce diet of the cow determined faint heat, the reduction in volume of the ovaries (ovarian underdevelopment), affecting the follice genesis and ovigenesis or even the total lack of heat. All these abnormalities are installed before the apparition of clinical loss of weight signs (Birtoiu et al., 2006)

The effects of the milk cow scarce diet

Infertility determined by lack of protein

The protein insufficiency affects the metabolic processes, diminishes the plastic and cellurar recovery processes, the hepatic reduction oxidation processes, it interrupts the hypothalamus-hyphophysis activity, the hyphophysis being deprived of the necessary amino acids and as a consequence the gonadotropic hormones elaboration, causing hiposympathicotonia and ovarian inactivity. The antehypophyseal hipofunction induces the shut-off of follicule and ovulation maturation. In some follicues, oocytes present modifications characterized by the contraction of the propoplasma and nucleus pyknosis. In ovaries the progestative bodies are missing and the ovaries become half-grown, while the parenchyma becomes dense and lack of elasticity.(Birtoiu et al.,2006).

Infertility determined by lack of protein

Lactating cows need large quantities of metabolized amino acids necessary for the synthethys of the milk protein.

It is necessary that the administered rates to contain between 16-18% of raw protein. Feedstuffs which have reduced quantities of raw protein may compromise microbial development and fermentation processed from the vermilion, also causing the milk production decrease.

On the other hand feedstuffs which contain excess protein cause higher concentrations of ammoniac and urea in the blood, causing the decrease of fertility. (Butler, 1998).The decrease of fertility of milk cows on excess protein diets is due to the decrease of uterin pH at the beginning of the luteal phase of the estral cycle.(Butler, 1998).This effect seems to limit itself only to the first stages of development of the embryo. (Rhoads et al.,2006).

Because high-producing lactating dairy cows are more efficient in utilizing protein sources when diets are moderate in crude protein and are balanced for the supplies of metabolizable protein and limiting amino acids (Noftsger et al., 2003), it is not justified to feed diets with protein concentrations that will increase urea N and harm fertility.

Excess protein may cause the aggravation of the energy balance, which may cause the delay of the first ovulation after birth. The ammoniac excess in the vermilion causes a high level of urea in the blood which has a toxic effect on the embryo and ovulation too. The large urea concentration in the blood has negative effects on the progesterone secernment too. (Rhoads et al.,2006).

The lack of lipids from the diet has a bad influence on the reproduction. The accumulation of large quantities of ketonic bodies in the organism resulted from incomplete and abnormal adjusting of lipids associated with the lack of carbohydrates influence the hiphosis and ovarian functions, causing the hipofunction of the antehyphophyseal. The lipids excess in the diet will lead to adiposis of the animals, which negatively affects fertility. (Şara.A., 2007).

Table 1. The inadequate and excessive influence of the nutrient diet input, on the bovine reproduction (Bearden et al,1992).

Nutrient intake	Consequences
Excess energy intake	Decreased conception rate, abortion, dystocia, placenta retentions, libido decrease
Inadequate energy intake	Late puberty, suppression of the oestrus, ovulation and libido and suppression of sperm production
Excess protein intake	Low conception rate
Inadequate protein intake	Suppresses estrus, decreased conception rate, fetal absorbtion, early birth, week fetuses
Vitamin A deficiency	Affects spermatogenesis, anoestrum, low conception rate, abortion, week progenies, placenta retentions

Infertility caused by energetical substances

Cow fertility is tightly connected to the feedstuffs input, both raw and fiber. The lack of fiber leads to the decrease of the fat content in milk, to ruminal oxydosis, genital ketosis and genital secretion causing a decrease of inferior fat acids in the vermilion, which are the basis of steroid hormones synthesis, causing this way the decrease of sexual steroids and as a consequence the apparition of fertility affections. (Birtoiu et al., 2006)

Tetany is an extension of a normal metabolic process which happens to milk cows with large milk productions. The glucoses (or sugar) deficit in the blood and tissue leads to the apparition of ketosis.

The glucoses is synthesize by cows from carbohydrates which are found in plats from grazing fields or in administered foodstuffs diets. During late gestation the glucose is mainly used for the normal development of the fetus. During the lactation period glucose is essential to form lactosis and milk fat. The glucose requirement being at a high level will determine the decrease of its concentration in the blood setting for hypoglycaemia.

Fifty grams of glucoses are necessary to produce a liter of milk which has 4,8% lactosum and thirty grams of glucoses for litre

of milk which has a fat content of 4%. To satisfy this necessary the diets can be succenturiated with different quantities of carbohydrates.
In case the glucide quantity of the diet is not enough to satisfy the glucoses necessary which the cow needs then the liver will produce glucoses from other basic compoundsof the organism, usually mobilizing the fat reserves. As a consequence of these metabolic processes the cetone are resulted (Ballarat, 2007).

The primary cetone is installed when the cows with large milk productions are not ensured with diets which have a sufficient quantity of carbohydrates.
The secondary cetone may be installed when primary problem or desease appears which determines digestive or carbohydrate failures. (Ballarat, 2007).
Hypoglycaemia, during artificial insemination, but even 3-4 days prior to it suppresses the oestrus or inhibits fecundation.

Table 2. The main biochemical modifications seen in phleboi blood during ketosis at milk cows in mg/dl (after J. Brugere P. ,1978)

Concentration	**Normal**	**Clinical ketosis**
Glucoses	40-70	30 (20-40)
Cetonic bodies	< 10	10-100
Free plasmatic fat acids	8	30

The values of glycaemia for cows are between 40-70 mg/dl of blood.
In clinical ketosis the blood glucoses level decreases between 30-20 mg/dl.
In case of secondary ketosis glycaemia is always superior to the value of 40 mg/dl, often being over the average of 50 mg/dl of blood.
In hiperacetonemia the normal blood level of the cetonic bodies, which is lower than 10 mg/dl, becomes higher (from 10-100 mg/dl of blood).In case of secondary ketosis, the acetonemia is rarely superior to the value of 50 mg/dl.

In acetonemia there is registered the rise of the AGLP level, from 8 mg/dl-30 mg/dl, this rise demonstrating a lipomobilization.
The value of cetonic bodies in milk varies between 3 mg/dl, at a healthy cow and 40 mg/dl, at a cow with ketosis. (J. Brugere, 1978)
The negative energy balance present after fecundation may determine early embryonarmortality. To avoid this process a stimulating diet must be applied post oestrus (flushing postestral). The energy excess is as damaging as the lack of it causing a higher frequency of quiet heat and affecting nidation. (Birtoiu et al., 2006)

Table 3. Metabolic load (ML) causes negative energy balance (NEB) and increase metabolic rates impairing Reproduction in lactating, high-producing dairy cows. (Knight et al. 1999

Metabolic load (ML) causes:	**Categories of changes and consequences**			
	Metabolic/endocrine	**Ovarian/endocrine**	**Functional/clinical**	**Breeding consequences**
Negative Energy Balance (NEB)	- Reduced synthesis and secretion of GnRH and LH - Reduced glucose - Reduced insulin - Reduced IGF-I	- Low or absent production of estrogens - Delayed LH-peak - Delay or absence of ovulation	- Poor estrous signs - poor oocyte quality - Failure of fertilization - Early embryo death - Shorter estrous cycles - Anestrus	- Repeat breeding - Low pregnancy rates - Low calving rates - Extended calving interval - Economic losses - Poor animal welfare
			- Impaired liver function - Impaired endometrial function (less favourable for embryo development) - Impaired immune function (increased propensity to endometritis and retained placenta)	
Increased metabolic hepatic rates	Increased catabolism of estradiol and progesterone	Lower levels of estradiol and progesterone in circulating blood	- Poor or absent estrous signs - Failure of fertilization - Early embryo death	

Vitamin A insufficiency

Vitamin A or retinol is the most important vitamin in the bovine diet. It is the only vitamin which must be added to the bovine diet. The beta-caroten is found in large quantity in green plants, this being converted in the animal organism in vitamin A. In cows, such a deficiency may occur only towards the end of winter, and then only if no green forage is available at that time.The Holstein-Fries breed can convert better the beta-caroten in vitamin A as the Jersey breed. Vitamin A is stored in the liver and is necessary to the good development of the bones, sight, maintaining epitalial tissue and has a very important role in reproduction.

The symptoms of vitamin A deficiency are scouring, low resistance to bacterial infection.The fertility of cows is always affected. A shortened period of gestation, a high incidence of retained placentas, stillbirths and abortions are common symptoms. Often calves are born blind and their movements are unco-ordinated. .(Bredon et al., 2005)

Vitamin A insufficiency may negatively influence gametogenesis, sexual cycles, anuvulate heat apparition, involution of the heat luteal body, apparition of the persistent luteal body. Vitamin A directly or indirectly participates to the biosinthesys of the progesterone. (Bîrțoiu et al., 2006).

Vitamin D insufficiecy

Vitamin D is found in plants of the provitamin form which is converted in the animal body in vitamin D when these are exposed to the sun. At cows the vitamine D insufficiency may also appear in cases of low quantities of calcium and phosphorus in the body. Because of the lack of vitamin D the bony cage of calves become very weak, with rachitis phenomena. At adult cows the vitamin D insufficiency will cause the decrease of the reproductive function as well that of the milk production. (Blezinger, 2004)

Vitamin E insufficiency

Vitamin E was identified as an essential nutritive element for animals 60 years ago. Rats fed with diets without vitamin E cannot reproduce; lots of studies demonstrated similar results for other species.

In case of vitamin E insufficiency a major problem for milk cows is mammitis, especially clinical mammitis or infections of the mammary gland. The plasma concentrations of the tocopherol (the chemical active form of vitamin E)are connected with the vitamin E input. During the peripartum period the plasma concentrations of tocopherol are significantly lower than during the gestation and lactation period.

At milk cows with plasma concentrations of the tocopherol less than 3 mg/l , it has been observed a grater incidence of clinical mammitis. Vitamin E interacts with selenium together with the two nutrients prevent the deterioration of the tissues. The vitamin E or selenium insufficiency may cause the decrease of the reproduction performance, the increase of placenta retentions, it increases the risk of early abortion.(Blezinger, 2004)

Vitamin C insufficiecy

Vitamin C decreases the organism immunity and has an indirect action on reproduction neutralizing sterility problems related to stress, intervenes in the development of and maturment of ovarian follicule and in the synthesis of steroid hormones. (Bîrțoiu et al., 2006)

Microelements insufficiency It is know that diets lacking of some mineral substances can cause infecundity states at animas.

Calcium insufficiency

Milk fever generally appears at cows with large production starting from the third lactation. Tetany is usually associated with birth, installing at 72 hours after birth. Because of the calcium request and due to the large volume of milk and poor calcium diets, the level of calcium from the blood drops, and tetany is installed.

Calcium is essential for the muscular activity. The calcium insufficiency may affect different groups of muscles, causing the apparition of hypomotility of the digestive system. Also it can affect the genital system muscles. The cervix may be dilated, atonic, uterine contractions are weak in intensity or can even be missing, the uterus is atonic, which may cause dystocia, late births which last very long, affecting both the cow as well as the calf, placenta retentions, uterine involution. Due to the tardive involution of the uterus the apparition of the first estrus after birth is prolonged, increasing the risk of clinical and subclinical uterine infections which may

influence negatively the reproductive function. Tetany cows have a high level of cortisol in the blood which may decrease immunity facilitating infections such as mammitis or hysteritis. (Macky, 2007.)

Table 4. Consequences of subclinical hypocalcaemia (after Kamgarpourr et al, 1999)

Digestive apparatus	Decrease of vermillion and rennin
Genital apparatus	Decrease uterine contractions Placenta retention Decrease of uterine and cervical involution speed Increase the birth-fecundation interval Decrease of fertility Decrease of the follicle number Decrease of the yellow body size Decrease of the progesterone

Phosphorus insufficiency

Phosphorus is essential for the normal functioning of the tissue. In the situation of a diet poor in phosphorus the animal compensates the phosphorus insufficiency in the blood by mobilizing it from the bones reserves. In the conditions of a long term diet poor in phosphorus there will appear growth failures, bone failures, decrease of appetite, the milk production and fertility are affected. The phosphorus insufficiency determined a low conception rate, quiet heat, oestrous irregular cycles and infertility. (Brendon et al., 2005).

The phosphorus excess blocks the calcium absorption, in the same time affecting also the reproduction function.

Magnesium insufficiency

Magnesium is necessary in the bovine diet especially to form bones. Cows need 1,9 g Mg/kg/dry matter.(Hamlyn-Hil, 2012) .The magnesium insufficiency may cause dystocia and placenta retention.(Rotaru, 2009)

Sodium insufficiency

Sodium is an essential macroelement. The subclinical sign of the sodium insufficiency are similar to those of the phosphorus insufficiency. The sodium insufficicency from the diet causes the decrease of the milk production. (Hamlyn-Hill, 2012).

Diets poor in sodium may cause sexual cycles affections, decrease of fertility and placenta retentions.(Bîrțoiu et al.,2006)

Potassium insufficiency

Potassium has a large number of functions, it facilitates glucoses and neutral amino acids absorption, maintaining the balance of basic acids in the body The Potassium necessary in that of 5 g /Kg/dry matter. (Hamlyn-Hill, 2012).

Hipopotassemia was associated with muscular weakness, decrease of muscular trophism. In the K insufficiency the plasma value is that of 2,5 mmoli/l(Goff, 2006).

The extracellular concentration of K is that of 3,9-5,8 mmoli/l, Potassium having an important role in maintaining the basic acids balance. Potassium insufficiencies lead to the decrease of fertility by affecting sexual hormonopoiesis, causing the impossibility to synthesise steroid hormones. Diets with a high level of Potassium may delay ovulation affecting the luteal body, delaying growth and development of heifers as well as installing anoestrus.(Smith et al., 1979)

Trace elements insufficiency

Iodine insufficiency

Iodine is essential for the normal body development, half of the total Iodine quantity being present in the thyroid gland. The Iodine insufficiency at milk cows shows placenta retentions, decrease of reproductive performance, stopping fetus development which will cause fetus mortality, abortions, hairless descendants, irregular oestrous cycles or stoppage of these.(Brendon et al.,2005)

Manganese insufficiency

Although manganese insufficiencies rarely appear at ruminants, they can cause multiple problems, affecting development and reproduction.

Heat in cows and heifers are hard to observe (quiet heat) and the decreased conception rate. The Manganese insufficiency at cows with calf will cause weak calves with extremities problems.(Brendon et al.,2005).

Zinc insufficiency

Phosphorus insufficiency causes growth problems, parakeratosis, hair and skin traumas. The calcium excess in the diet may affect Zinc absorption although the zinc deficit at ruminants is rarely seen. (Brendon et al., 2005)

The zinc insufficiency causes a n underdevelopment of the genital apparatus and infertility by affecting ovulation. (Bîrțoiu et al., 2006)

Copper insufficiency

Phosphorus insufficiency in the diet may have rather serious consequences for the health of the animals. There is a strong correlation between hypocuprosis and abortion. A recent study showed that 87% of anoestrus heifers are due to cuprum deficit.

A new born calf from a cow with copper deficit will suffer bone marrow affections which will determine muscular trembling, ataxia and paralysis.(Sakhaee, 2011)

A low level of cuprum in the body causes ovarian inactivity, increase of the placenta retentions, decrease of fertility and instalment of anoestrus. (Brendon et al. 2005),

Selenium insufficiency

Usually selenium insufficiency is found in animals with feedstuffs diets, from soils poor in selenium. The selenium deficit may cause a series of muscular affections, reproduction problems, decrease of fertility, cardiac insufficiency at young animals. At old bovines it causes the retention of fetal tectorium, ovarian cysts, anoestrus, embryo mortality, mammitis and the increase of somatic cells number in milk. (Heather, 2009)

CONCLUSIONS

Genetic selections at cows in the past decades have concentrated mainly on the increase of milk production. By increasing production performances a series of problems have appeared, causing health problems of the animals and the decrease of fertility.

Studies have shown that nutrition management during the transition period may improve reproduction, minimizing metabolic affections during the postpartum period and even later on.

Managing the negative energy balance and increasing the energy intake improve the reproduction function. The mineral elements present in the animal body are characterized by properties favourable to circulation and chemical mobility necessary to life. There still are many mineral interrelationships and interdependencies probably many unknown facts until present time where excess or deficit of a mineral will influence the absorption or usage of another mineral.

It is very clear that diets are strictly connected to the milk cows reproductive function. The basic issue is the deficit level, the unbalance or excess in the diet of animals with large productions.

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EUROPEAN LEGISLATION ON OTA IN FOOD AND FEED AND THE RISK OF ITS PRESENCE ON HUMAN AND ANIMAL HEALTH

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Abstract

Studies on ochratoxin A (OTA) showed that this mycotoxin is nephrotoxic, hepatotoxic, carcinogenic, immunesuppressive and teratogen.
Consumption of food contaminated with OTA affects the health of farm animals and their productivity leading to its presence in animal products.
Early identification and removal of feed and food chain products contaminated with OTA can be achieved by control strategies. This paperwork aims to present the impact on human and animal health, the probable risk of OTA residues in animal products and control strategies that apply in the feed industry.

Key words: *Ochratoxins , human toxicity, animals toxicity, control strategies.*

INTRODUCTION

Food safety and security, safe food procurement remain essential in most countries.
In the recent years intense national and international efforts have been made regarding food security. There are taken in consideration both microbiological and chemical risks.
World Health Organization (WHO) recognizes the chemical risks of food and feed contamination by mycotoxins (toxic metabolites of fungi), fishery products (toxins produced by algae) and edible plant species by plant toxins as an important source of food originated disease WHO, 2002a).
Mycotoxins have received the most attention in many parts of the world, as they are a major issue in food safety.
Many countries have adopted regulations regarding the mycotoxins in food and feed due to the severe effects that they can have on people and animals. The mycotoxin regulations have a major role in the protection of the human and animal health and economic interests of producers and traders.
Ochratoxin A is a fungus mycotoxin that aroused worldwide interest in terms of economic losses due to the effects on human health and animal productivity and the national and international trade.
The disease caused by the presence of this mycotoxin is called ochratoxicoza. The main target of this mycotoxin is the kidney.
Following epidemiological studies, it was demonstrate that OTA is involved in the pathogenesis of many forms of human renal disease, including kidney cancer (Marquardt and Frohlich, 1992; Ringot et al., 2006; Pfohl-Lenszzkowicz et al., 2007).
Due to high toxicity to human and animal body, ochratoxins are extensively studied in recent years.
The conclusions of these studies was that these toxins are nephrotoxic, immunotoxic, neurotoxic, myelotoxic and carcinogenic (Group 2B toxicity) according to the CIRC classification (CIRC, 1993).
The OTA elimination ways through kidney and in part by through may explain the degenerative changes in the epithelial cells of the kidney and liver. (Koynarski et al., 2007).
The genotoxicity and the oxidative way can be taken into account for the occurrence of nephrotoxic and carcinogenic effects as shown by research conducted in recent years. (Pfohl-Lenszzkowicz et al., 2007).

The attention paid to these mycotoxins is motivated by the many signs of food contamination: cereals, coffee, dried fruit, spices, chocolate, wine, cocoa (Bayman and Baker, 2006).
OTA can be found in animal tissues and products because mycotoxins can be transferred through the food chain.

OTA in feeds

OTA is a toxic by-product produced by species of Aspergillus and Penicillium.
The factors affecting ochratoxinogeneza are: temperature, humidity, water activity, degree of aeration, substrate biocoenosis. (Tabel 1)

Table 1. Growth conditions for ochratoxin production.

Growth conditions	Aspergillus ochraceus	Penicillium verrucosum
Optimum temperature for growth	24-37 C	20°C
Optimum temperature for ochratoxin production	31°C	20°C
Optimum growth	3-10	6.0 – 7.0
Minimum wather activity for ochratoxin production	0.8	0.86

OTA production is influenced by environmental conditions (temperature and water) as well as the seed's type and integrity. Aspergillus ochraceus grows better in oilseeds (soybeans, peanuts), Penicillium verrucosum grows better in cereal crops: wheat, corn. (Madhyastha et al., 1990).
The main source of ochratoxin in the vineyards of France, Spain and Italy A.carbonarius.
In cooler regions, OTA is produced by Penicillium and in warmer regions by Aspergillus (Pohland et al.,1992; Varga et al., 1996). Ochratoxin formes on acidic foods (Cuero et al., 1987).
Large amounts of ochratoxin occur during storage of agricultural products due to the humidity (18-24%) favoring mycotoxigen fungal growth (Shotwell et al., 1969; Zimmerli and Dick, 1996; Campbell et al., 2003).
The highest amounts were reported in Northern Europe and North America (World Health Organisation, 2002).
OTA formes mainly coffee during beans' storage.
Rapid drying of agricultural products after harvesting can reduce the production of OTA. (Levi et al., 1974; Urbano et al., 2001; Samson et al*.,* 2004; Frisvad et al., 2004).

OTA effects on animal health and production

After consumption of OTA contaminated food, major economic impact can be observed on monogastric animals, birds and pigs. The ruminants are more resistant to OTA contaminated food. The consumption of OTA contaminated food reduces the growth rate and thus lowers animal productivity.

Nephrotoxic action

The effects of consumption of OTA contaminated on animal health depend on dose, animal species and the amount ingested.
It is believed that pigs are the most sensible to OTA. (European Food Safety Authority-EFSA, 2006). Nephrotoxicity of ochratoxin is different from animal to animal. Consumption of food containing OTA in concentration of 1 to 3 mg/kg resulted in the appearance of nephropathy and kidney cancer in pigs and humans after the installation of the tubular degeneration. The immunosuppressive activity in 'natural killer' cells could explain tumour growth (Pfohl-Lenzkowicz et al., 1993; CIRC, 1993; Pfohl-Lenzkowicz et al., 2002).
The installed kidney necrosis can be explained as a result of increased lipid peroxidation demonstrated for OTA in vivo and in vitro. (Meki AR et al.,2001).
OTA inhibits the activity of many enzymes from the Krebs cycle, resulting in the decrease of the ATP production and the inhibition of the mitochondrial respiration. (Wei et al.,1985).
The ingestion of OTA produces polyuria and polydipsia. The increased blood urea and creatinine levels draw attention upon the renal impairment.
The consumption of food containing OTA in concentrations higher than 1 mg / kg leads to leukocytosis, increased neutrophils / lymphocytes ratio and decreased hemoglobin and erythrocytes levels. It was observed an impairment in the immune function associated with lymphocyte development and production of interleukins IL-2. (Harveyet al., 1992).

On necropsy there are found kidney discoloration and hypertrophy, atrophy and degeneration of proximal convoluted tubules, interstitial fibrosis and sometimes hyalinisation glomeruli. The impairment of the function renal was observed after consumption of OTA contaminated food in concentration of 200-4000g/kg. (Stoev et al., 2002).

On histology examination there were found proximal tubular lesions and interstitial fibrosis.

After consumption of OTA contaminated food in concentrations higher than those that cause nephrotoxicity, there are found embryotoxicity, immunotoxicity and teratogenicity. (Benforg et al., 2001).

On pigs, OTA ingestion lowers resistance to infection. (Stoev et al., 2000).

On poultry, it was observed a decrease of immunoglobulins levels and phagocytic capacity of monocytes, and decreased antibodies. (Thuvander et al., 1996). Many European countries have experienced episodes of porcine nephropathy. The pigs intoxicated with OTA showed biochemical lesions: glucosuria, proteinuria, enzimurie, reduced urine concentration, renal insufucienta. (Petkova et al., 1991; Pfohl et al., 2002).

The poultry are also affected by contamination with OTA. Chicken duck, turkeys are sensible to OTA. Ingestion of OTA contaminated food leads to decreased egg production, poor quality shelled eggs, decreased feed conversion, reduced weight gain, nephrotoxicity.

The administration of OTA contaminated food (2 mg/kg) on laying egg hens significantly decreases food consumption per day, egg and serum triglycerides production and increases liver weight. (Denli et al., 2008).

On chickens receiving OTA contaminated food (2 mg/kg) there were observed weight loss, diarrhea, excessive urine and kidney damage (Dwivedi et al., 1984). It was also observed a decrease a tocopherol concentration in the liver of chicken that consumes OTA contaminated food (2.5 mg/kg). (Hoehler et al., 1996).

On poultry, there were found the same biochemical and histological lesions as pigs.

Carcinogenic action

OTA administration on rodents causes kidney, breast, liver and testis tumors (IARC, 1993). Because of the genetic sensibility related to biotransformation, male rats are more sensible to OTA (Pfohl-Leszkowicz et al., 1998).

The administration of OTA contaminated food for 2 years in a group of female pigs has led to kidney cancer. This is due to metabolism and excretion ochratoxins relatively quickly with an RL50 (disposal) in pigs for several days. (Krogh and Role, 1992).

The OTA contamination of animal products

The contamination of animal products may occur after consumption OTA contaminated food or by direct contamination with fungi.

After the consumption of OTA contaminated food there is a rapid absorption of toxins into the bloodstream followed by relatively slow elimination through urine and feces. (Galtier, 1991; Mantle,2008).

A team of researchers found that after oral administration of a single dose of 500g/kg of OTA peak plasma concentration at 2 hours is about 30% of the OTA intake. (Vettorazzi et al., 2009).

The persistence of OTA in plasma is due to enterohepatic circulation and resorption in renal tubules. (Roth et al., 1988; Marquardt and Frohlich, 1992).

On pigs that were fed during the growth with OTA contaminated diet in concentration of 25 g/kg, residue in pork was up to 1 g/kg ((Malgutii et al., 2005).

On ruminants, OTA residue does not accumulate in a significant level, because this toxin is rapidly detoxified in the rumen in less toxic metabolites (Muller et a., 1998).

The porcine nephropathy, which originally appeared in Denmark, has been reported in many European countries. The main cause of this renal disease is the consumption of OTA contaminated food. (Stoev et al., 2002).

Macroscopic changes observed after examining the pig kidney is an indirect method of determining the level of OTA in carcass in Denmark (Jorgensen and Petersen,2002).

At an OTA level of 25g/kg in pig kidney the meat is checked to ensure that it does not exceed the value of 10 g/kg. After some studies, it was established that the OTA content in carcass is 40% in the case of pig kidney (Buchmann and Hald, 1985).

When the quantity of OTA varies between 10 and 25 g/kg in kidney, liver and pig kidney are rejected.

There was a correlation between OTA content in organs (kidney, liver, whole blood and plasma) and various forms of nephropathy after a study on two pigs in Sweden. (Rutqvist et al., 1978).

OTA concentration in the feed might be used to specify residues in pig tissues and organs. This can be done following correlations established between the consumption of foods with OTA and debris from kidney, liver, muscle and adipose tissue (Krogh et al., 1974; Krogh, 1976; Rutqvist et al., 1978).

After some studies, many researchers have tried to establish a relation between the average concentration of OTA in serum and concentration of OTA in pig's feed (Stoev, et al., 2002; Malgutti et al., 2005; Jarczyk et al., 2008**;** Aoudia et al., 2009), ar depicted in Figure 1.

Knowing that OTA content in blood serum reaches a plateau after 10-13 days, there have been experiments where exposure to OTA lasted at least 14 days. The equation in the figure below confirms the relation between OTA content in feed and its residues in blood serum (Hult et al., 1979; Aouila et al., 2009).

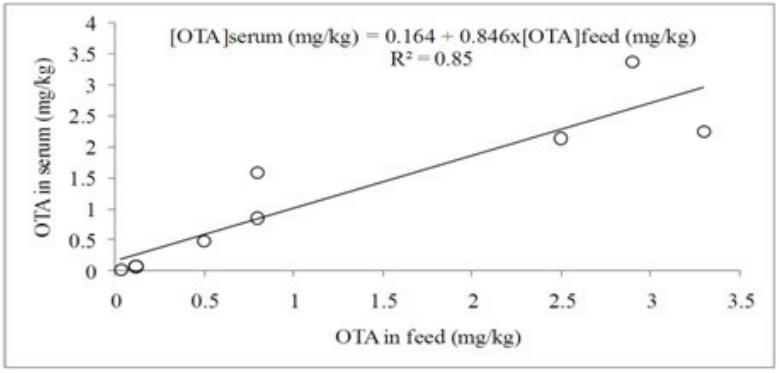

Figure 1. Relation between the concentration of OTA in the diet and its concentration in pig blood serum.

The consumption of OTA contaminated food led to accumulation of OTA residue in renal and hepatic tissues and growth of the organs which are involved in detoxification and elimination processes (Stoev, et al., 2002; Aoudia et al., 2009). A weight gain of these bodies was observed in experiments in which OTA contamination level exceeds the recommendation of 0.05 mg/kg indicated by the European Commission (Stoev, 2010).

There was detected OTA residues in chicken muscle and eggs (Marquardt and Frohlich, 1992).

At a consumption of OTA contaminated food in concentration of 2 mg/kg, there was an increase in the content of OTA in the liver (15.1 g/kg). (Denli et al.,2008). OTA did not exceed the limit of detection (0.05 micrograms/kg) in the analyzed eggs. After studies by several researchers concluded that intake of OTA by eating eggs is not a concern. (Tangni et al., 2008). On birds which were fed with an OTA contaminated diet in concentration of 10 mg/kg an contamination in concentration of 0.7-1.3 g/kg was observed (Neimiec et al., 1994).

OTA was not detected in Japanese quail eggs when administrated a diet of 1 mg OTA/kg (Piskorska and Juszkiewicz, 1990).

Toxic action of OTA on the human body

OTA is considered as a possible causative agent for two chronic diseases: Balkan endemic nephropathy (NEB) and chronic interstitial nephropathy (North Africa).

In Balkan region ochratoxicosis were confirmed in humans. The appearance of Balkan nephropathy was associated with nephrotoxic effect of OTA in humans.

In 1956, this disease was described, for the first time, in a study conducted on a group of 664 hospitalized patients for kidney diseases, in Bulgaria. The Balkan endemic nephropathy was diagnosed in Romania with a spreading area of five outbreaks in Oltenia and one in Banat. (Gluhovschi et al., 1994).

On patients in Bulgaria, suffering from the Balkan endemic nephropathy, there were

observed renal and bladder tumors similar to those obtained experimentally in rat kidney.
The link between exposure to ochratoxin and renal and bladder cancer incidence was found on patients in the region of Midi-Pyrenees. Several researchers argue OTA involvement in Balkan endemic nephropathy etiology (Petkova and Kernozemsky, 1988; Phofl et al., 1999; Abarca, 2001; Pfohl, 2002).
It was established that nephrotoxic Ochratoxin A is a mycotoxin, immunotoxic, myelotoxic and carcinogenic according to data from (CIRC, 1993).
The toxic action of ochratoxin occurs through several mechanisms: effects on lipid and carbohydrate metabolism on mitochondrial respiration and changes in the transcription and transduction.
Following epidemiological studies which demonstrate that OTA can cause in humans a higher incidence of renal tumors and nephropathy, the European Scientific Committee indicates for human alimentation a tolerable food consumption lower than 5 mg/kg/day.
After oral administration, OTA is present in the blood for 35 days. (Petzinger, and Weidenbach, 2002).
After some studies it was observed that renal tumors often appear on food consumption of 70 g/kg/day of OTA (Phofl et al,1993, Phofl et al,2007; Phofl,2009).

Legislation on OTA in food

Attempts to eliminate mycotoxins in animal nutrition and the food is impossible (Bennett et al., 2003).
Many countries and organizations have established levels of OTA in feed and food.
There were established regulations and guidelines establish maximum limits for mycotoxins that the: US Food and Drug Administration (FDA), Food and Agricultural Organization of the United Nations (FAO), European Union (EU), the Institute of Public Health of Japan.
The 1881 regulation issued in 2006 the European Commission has set maximum limits for mycotoxins (Regulation (EC) No 1881/2006). (Table 2) to basic products: cereals, cereal based products, dried fruits and wine, baby food, coffee.
Limits of different mycotoxins in feed, cereal and cereal products for animal feeding recommended guidelines for the maximum tolerable the Commission of the European Communities (Table. 2). (Recommendation 2006/576/EC)

Table 2. European Union Maximum level of ochratoxin permitted in foodstuff

Commodities	Maximum level (μg/kg)
Raw cereals	5.0
Cereal products	3.0
Infant based food	0.5
Dried vine fruit	10.0
Soluble coffee	10.0
Roasted coffee beans	5.0
Wine and grape juice	2.0

Table 3. Guidance values for OTA in feeding stuffs with a moisture content of 12%, as set in the Commission Recommendation 2006/576/EC

Products Intended for Animal Feed	Guidance Valuein mg/kg
Feed materials	0.25
Cereals and cereal products	
Complementary and complete feedingstuffs	
Complementary and complete feedingstuffs for pigs	0.05
Complementary and complete feedingstuffs for poultry	0.1

There were used several strategies to reduce the risk of ochratoxins appearance in food industry, as a result of the transfer or the food chain.
Public health issues are justified on the basis of demonstrated toxic effects caused by contamination with ochratoxin.
Mycotoxins can contaminate feed materials (cereals) before arriving in feed mills due to weather conditions.
It is necessary to develop the capacity determination of mycotoxins level in whole food chaine: plant-animal-animal originated food products. (Savu et al., 2004).
As a precaution, the quality control on each lot is in order. OTA is a hard to break mycotoxin when it appears in feed. Temperatures up to 250°C on a extensive time of a few minutes are demanded in order to destroy OTA compounds in foodstuffs. (Boudra, H.; Le Bars, P.; Le Bars, J).
If the OTA is considered to be a feed hazard, there can be used specific absorbents to block the mycotoxin in the digestive contents or microorganisms capable of transforming it into non-toxic metabolites. (Denli, 2008; Schatzmayr et al.2006).

Several studies indicate that antioxidants play a role in reducing the toxicity of OTA in several species. Abdel-Wahhap et al. and Ozcelik et al. concluded that melatonin shows a preventive effect of OTA-induced oxidative stress. (Özçelik et al.,2004; Abdel-Wahhab et al., 2005).
The use of a tocopherol in the diet decreased by 57% overall DNA adduct in the kidney caused by a single administration of OTA in mice and rats. (Grosse et al., 1997).
The ochratoxins' suppressive effect on egg production and the toxic effect of OTA in different organs lowered by adding a plant extract (artichokes) in laying eggs hens' diet (Stoev, 2010).
The official controls carried out in order to ensure the verification of compliance with feed and food under EC Regulation no. 882/2004 had a major role in ensuring food safety.
There were developed laws that establish sampling analyzing methods for the official control of feed and food:
• EC Regulation no. 401/2006 established sampling and analyzing methods for the official control of mycotoxins in food;
• EC Regulation no. 152/2009 established sampling and analyzing methods for the official control of feed.
Feed business operators have to be licensed in accordance with European Commission Regulation no. 183/2005 and to conduct a risk analysis and critical control points in HACCP implementation.
The application of HACCP system in all units that are parts of the the food chain is required.
Hazard analysis and critical control points (HACCP) is a scientific and systematic apparatus used to identify:
• Risks associated to a food product regarding food safety;
• Risk monitoring to ensure food innocuousness.
The legislation in food industry aims to reduce, eliminate and prevent a risk to human and animal health. The three components of risk analysis: risk assessment, risk management and risk communication lead to the establishment of efficient and accurate measures of health protection.
European Commission Regulation no. 178/2002 establishes safety requirements regarding feed and feed business operators' responsibilities. Food and feed traceability shall be established at all stages of production, processing and distribution.
European Food Safety Authority (EFSA) is endowed with a number of important tasks regarding: independent scientific advice on all aspects of food security, early warning systems and collaboration with national agencies, thus ensuring a high level of protection and consumer confidence.
EFSA is a decentralized organism of the European Community in food security and safety functions.
The Food and Veterinary Office Commission (FVO), as guardian of the Treaties of the European Community is responsible for ensuring that Community legislation on food safety, animal health, plant health and animal welfare is properly implemented and enforced. As a service of the Commission, The Food and Veterinary Office (FVO) plays an important role in this task.
The Food and Veterinary Office mission, through its audits, inspections and related activities contribute to:
• European Community development policy in food safety, animal health and welfare and plant health sectors;
• Development and implementation of effective control systems in food safety, animal health and welfare and plant health sectors.
The control strategies for OTA in food consist in: early identification and elimination of the contaminated products from the food chain.

MATERIALS AND METHODS

We used the test kit with competitive enzyme immunoassay for the quantitative analysis of *Ochratoxin A (OTA)* in fodder and foods.
The determination is made based on working kit protocol used is based on the reaction of antigen-antibody. ELISA kit (Enzyme-linked immunosorbent assay-enzyme immunoassay, or EIA). After the sample preparation the test procedure, the measurement is made photometrical at 450 nm.
Reagents: - 1n HCl, 5 n HCl; CH_2Cl_2 ; 0,13M buffer ($NaHCO_3$) with pH=8,1
Equipment: - microtiter plate spectrophotometer (450 nm), centrifuge, magnetic stirrer, paper filter, gradual pipette, micropipettes, purification columns OTA.

All reagents required for determinations had adequate quality according and the determinations were made using modern equipment from Sanitary-Veterinary and Food Safety Direction-laboratory of Brasov. This laboratory applies a GPL system and a quality system.
To avoid contamination of samples was taken into account the observance of rules, namely:
-when entering the laboratory, samples were pureed;
- it was a laboratory sample is stored in the freezer representative until determination;
To obtain valid results has been considered subject to the following precautions:
- all reagents were brought to temperature 20-25°C and were mixed before use;
-these steps were imposed by the kit work in compliance with time forced;
- to work in the solvent extract preparation-70% methanol (OTA);
- were observed using working volumes: 50, 100, 500 and 1000 µl-micropipets;
All kits must be certified according: detection limit (LOD), recovery rate, sample preparation and specificity (Table 3).

Table 4. Performance criteria for ELISA kit

Mycotoxin	Recovery%	LOD	Matrices
Ochratoxin A ***RidaScreen***	85	625 ppt*	Cereals, feed, food

(ppb= ng/mL= µg/Kg; ppt = ng/Kg)

Table 5. The results of determinations made are shown in the table below

Matrices	Nr. Samples			OTA, (µg/ Kg)		
	2010	2011	2012	2010	2011	2012
Mixed fodder for pigs	3	3	10	Ned... 0.478	Ned.	Ned.
Corn beans	6	4	11	Ned.	Ned.	Ned.
Bran	3	4	9	Ned.	Ned.	Ned.
Ground grain	6	5	8	0.35.. 0.74	0.12.. 0.21	0.12.. 0.21
Pig kidney	-	-	6		-	Ned

Ned.-undetectable

Values obtained from determinations were performed according to the European legislation: Recommendation 2006/576/EC and Regulation (EC) No 1881/2006

RESULTS AND DISCUSSIONS

The strategies for reducing the risk of ochratoxins appearance in food as a result of transfer through the food chain consisted in quality control (test mycotoxicological) feed and animal products batches.
For that matter, between 2010 and 2012 there were analyzed feed samples (grains, pig feed, bran).
In 2012 there were analyzed 6 samples (matrix-kidney) for the determination of ochratoxin A.
The results of the performed determinations were interpreted according to the European legislation and are thus presented in the tables below.

CONCLUSIONS

This paperwork approaches about the problem of ochratoxin A in relation to toxicity and the mechanisms by which it exerts its toxicity to human and animal health and control strategies used in the feed industry.
The strategies used to reduce, eliminate or avoid the risk of ochratoxins are justified by the demonstrated toxic effects caused by contamination with ochratoxin.
Official controls performed to ensure the quality of feed and food under Regulation (EC) 882/2004, led to the conclusion that the EU legislation on food safety is observed.
The feed and animal products (pig kidney) samples that were collected and analyzed for the determination of ochratoxin A and compliance results show that they are not harmful to humans and animals

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THE INFLUENCE OF REFRIGERATION ON *SALMONELLA*

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Abstract

This study aimed to analyse the influence of refrigeration on Salmonella.Two register strains have been used – S. Enteritidis (D) (ATCC 13076) and S. Typhimurium (ATCC14028), each having a known load of $28*10^7$, respectively $40*10^7$. The two strains have been subjected to temperature variations as such: 1.-18°C for 1 h 45 min; 2. 2-4°C for 6 h after the first stage; 3. 2°C for 10 h after the second stage.The collected samples have been analyzed according to ISO 6579:2002. The results have shown that after quick refrigeration (-18°C for 1 h 45 min), the S. enteritidis and S. typhimurium microorganisms at the load of 22 log ufc/cm^2, respectively 44 log ufc/cm^2 could not be noticed. The other two variations (2-4°C for 6 h and 2°C for 10 h) did not have any effect on the two Salmonella strains, no matter the UFC number used. We can affirm that the temperature of-18°C prevent the multiplication of microorganisms only if the microbial load is reduced, and once with increasing temperature the microorganisms have been highlighted.

Key words: *multiplication, refrigeration, Salmonella, strains, temperature.*

INTRODUCTION

Refrigerated semicarcasses are kept at a temperature of 4°C, under which circumstances *Salmonella* spp. does not multiply itself (Bolton et al., 2002).

Most producers begin to process the refrigerated carcass the next day after slaughter, in order to allow the carcass to reach a temperature of 7°C, by subjecting it to refrigeration for 14 to 16 h. All the studies on refrigeration show a decrease in the number of microorganisms (Greer and Dilts, 1998). Gill and Landers (2004) discovered that refrigeration reduced the presence of *E. coli*, but not the number of aerobic germs found on the carcasses. Variations of the cooling of carcasses depend on intrinsic causes (weight of carcass, how thick the fat layer is, the initial temperature of the carcass) and on extrinsical causes (temperature, air speed, relative humidity, distance between carcasses) (Sheridan, 2000).

Spescha et al. (2006) evaluated in a study the effect of refrigeration on carcasses in two slaughter houses in the EU. The results refer exclusively on refrigeration, not on a combination of treatments (application of organic acids). The authors observed a reduction in the total number of aerobic germs and in the number of *Enterobacteriaceae* as a result of refrigeration. A reduction by one log of the number of aerobic mesophilic germs has been noticed, excepting the cervical area. It is possible for the residual waste water on the surface of the carcasses to lead to contamination of the surface of the neck with pathogen agents. It is also possible for the refrigeration and drying process to be less effective because of low air movement from pavement.

The study on risk evaluation conducted by Delhalle et al. (2008) established that 15 to 24 h were needed to obtain an inner temperature of 7°C of the carcass. The time of refrigeration affects the total number of germs, but the medium raise was only of 0.005 log UFC cm^2, so its influence was low.

Mafu et al. (1989) observed an increase in the presence of *Salmonella* spp. (12,5%) on the pavement in the refrigeration area. The increase was caused by activities of the staff. Therefore, the staff's hygiene and discipline is important in the reduction of contamination during refrigeration of carcasses.

MATERIALS AND METHODS

Two register strains have been used – *S. enteritidis* (D) (ATCC 13076) and *S. typhimurium* (ATCC14028), each having a

known load of $28*10^7$, respectively $40*10^7$. The two strains have been subjected to temperature variations as such:-18°C for 1 h 45 min; 2-4°C for 6 h after quick refrigeration; 2°C for 10 h after quick refrigeration.

The methods used are presented in the following: (Table 1). Samples were analysed according to ISO 6579:2002.

Table 1. The methods used

Strains	Load	Sampling hour		
S. enteritidis	$28*10^7$	1h 45 min	6 h	10h
	$14*10^7$			
	$28*10^6$			
	$28*10^2$			
	28 ufc/cm^2			
S. typhimurium	$40*10^7$	1h 45 min	6 h	10h
	$20*10^7$			
	$40*10^6$			
	$40*10^2$			
	40 ufc/cm^2			

RESULTS AND DISCUSSIONS

The analysis performed on the collected samples revealed the following results:

Following the fast refrigeration process (maintaining the carcasses at a temperature of-18°C for 1h 45 min), no microorganisms could be noticed on the samples having a load of 28 ufc/cm^2, respectively 40 ufc/cm^2. This result was obtained because the load was low, as well as the temperature. Therefore, the two strains of *Salmonella* could not multiply themselves.

After the next step of the refrigeration, at a temperature of 4°C and after 6, respectively 10 h after the firs and second stage, the samples revealed microbial load. The fact that the samples tested positive is caused by the high load of germs reported to the surface. Such values are not usually found on the surface of the carcass. The samples did not test positive at a low load of 28 ufc/cm^2 respectively 40 ufc/cm^2 after fast cooling because of its′ numerical effect.

Carcasses according to demands can be obtained by respecting hygiene and specific slaughter procedures by the staff.

CONCLUSIONS

The temperature of-18°C during 1h 45 min prevent evidence of *S. enteritidis* and *S. typhimurium* strains.

A microbial load whose value exceeds two ciphres increases the occurrence of the disease.

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INTERACTIVE E-LEARNING FOR VETERINARY MEDICINE STUDENTS AND PRACTITIONERS

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Abstract

Innovative methods in education, including in medicine, either human or veterinary contribute to enhance the learning effectiveness. The aim of this paper is to present the results that have been obtained by implementation of 3 e-learning platforms in veterinary undergraduate and post-graduate education and training. The e-learning platforms were developed through three projects, ***Higher Education at European Level in the Field of Veterinary Medicine, ID: HRDSOP 86/1.2./S/63654, Labour Market Integration of Veterinary Medical Students – Practical Training, ID: HRDSOP/90/2.1/S/63915*** *implemented by the Faculty of Veterinary Medicine Bucharest in partnership with the other faculties of veterinary medicine in Romania and* ***Improving the Quality of Human Resources in Veterinary Medicine, ID: HRDSOP/81/3.2./S/58833,*** *implemented by the College of Romanian Veterinary Physicians in partnership with the Faculty of Veterinary Medicine Bucharest. These projects are cofinanced by the Social European Fund through the Human Resources Development Sectorial Operational Programme 2007 – 2013. A number of 3,000 students from all the faculties of veterinary medicine from Romania have accessed the e-learning platform developed by the first mentioned project, that introduced a modular curriculum with 4 new courses: Veterinary Medical Deontology and Professional Ethics, Development of Professional Abilities and Career Guidance, Quality Management of the Veterinary Medical Act, Quality Management in Veterinary Higher Education. The second project e-learning platform presents 5 modules. Farm Animals Breeding Units, Clinics and Hospitals, Laboratories (research, diagnosis, food control), Slaughtering Houses, Rural Veterinary Clinics are the five domains for which platform presents scenarios that introduce the students to real-life situations. 2,500 students accessed the platform before the effective practical training. The third e-learning platform was intended for 1,000 veterinary practitioners in order to help them to update their knowledge in ten thematic areas: Advanced Veterinary Diagnostic Imaging, New Technologies in Laboratory Diagnosis, New Technologies in Large Animals' Pathology and Clinic, New Technologies in Companion Animals' Pathology and Clinic, Modern Technologies in Clinical Biochemistry and Molecular Biology, Modern Devices Used in Animal Hygiene, Use of New Technologies for Food Control and Expertise, Informatics Technologies Dedicated to Food Safety and Quality Management, Modern Devices Used in Reproduction Pathology, Animals' Selection and Amelioration and New Technologies Introduced in Emergency Veterinary Therapy. The project's success is proved by the fact that a number of 2,488 public and private veterinary practitioners have already accessed the platform.*

A positive feedback of the e-learning platforms was recorded from all the members of the target groups, students and practitioners.

Key words: *veterinary medicine education, e-learning, lifelong learning in veterinary medicine.*

INTRODUCTION

Computer aided learning in veterinary medicine is not a novel attempt to provide valuable resources for education in this field. In 1993, Teaching and Learning Technology Programme – TLTP represented a successful project in the United Kingdom (Dale et al. 2005). Internet - based medical education advantages, limitations and impact as well as the opportunities and challenges for students, teachers and practitioners in veterinary medicine are largely discussed in veterinary education literature (Simões, 2010, Choules, 2007 and Ruiz *et al.*, 2006). Tenhaven et al., (2013), have published a richly documented comparative study on the use of the internet and Web 2.0 by students and the veterinary profession, entitled with a question "Is there a next generation in veterinary medicine?", conclude that both students in veterinary medicine and veterinarians are comparable with other professional groups

in terms of online media usage. These authors highlight that it is important to train students but also teaching staff and practitioners and to illustrate both risks and opportunities of getting media information. The aim of the present paper is to present the results obtained after developing 3 e-learning platforms dedicated to under and postgraduate veterinarians in order to provide valuable resources for students and longlife learning in veterinary education.

MATERIALS AND METHODS

The e-learning platforms were developed through 3 projects, **Higher Education at European Level in the Field of Veterinary Medicine, ID: HRDSOP 86/1.2./S/63654, Labour Market Integration of Veterinary Medicine Students – Practical Training, ID: HRDSOP/90/2.1/S/63915** implemented by the Faculty of Veterinary Medicine Bucharest in partnership with the other faculties of veterinary medicine in Romania and **Improving the Quality of Human Resources in Veterinary Medicine, ID: HRDSOP/81/3.2./S/58833**, implemented by the College of Romanian Veterinary Physicians in partnership with the Faculty of Veterinary Medicine Bucharest. These projects are cofinanced by the Social European Fund through the Human Resources Development Sectorial Operational Programme 2007 – 2013.

First project introduced a modular curriculum with 4 new courses: Veterinary Medical Deontology and Professional Ethics, Development of Professional Abilities and Career Guidance, Quality Management of the Veterinary Medical Act, Quality Management in Veterinary Higher Education. The second project e-learning platform presents 5 modules. Farm Animals Breeding Units, Clinics and Hospitals, Laboratories (research, diagnosis, and food control), Slaughtering Houses, Rural Veterinary Clinics are the five domains for which platform presents scenarios that introduce the students to real-life situations. The third e-learning platform was intended for veterinary practitioners in order to help them to update their knowledge in 10 thematic areas: Advanced Veterinary Diagnostic Imaging, New Technologies in Laboratory Diagnosis, New Technologies in Large Animals′ Pathology and Clinic, New Technologies in Companion Animals′ Pathology and Clinic, Modern Technologies in Clinical Biochemistry and Molecular Biology, Modern Devices Used in Animal Hygiene, Use of New Technologies for Food Control and Expertise, Informatics Technologies Dedicated to Food Safety and Quality Management, Modern Devices Used in Reproduction Pathology, Animals′ Selection and Amelioration and New Technologies Introduced in Emergency Veterinary Therapy. All the developed courses were provided by experts in the field, and processed in interactive presentations by specialized IT companies. At the end of each module/ course/ thematic area different tests enable participants online evaluation.

RESULTS AND DISCUSSIONS

3,000 students from the faculties of veterinary medicine from Bucharest, Cluj, Iasi and Timisoara accessed the e-learning platform developed through the project Higher Education at European Level in the Field of Veterinary Medicine. In fig. 1 there is represented the distribution, in %, of the students' options for the 4 courses offered. The student's interest in the module Quality Management in Veterinary Higher Education is decreased as compared to the others. The Quality Management of the Veterinary Medical Act module was also less visited than modules Veterinary Medical Deontology and Professional Ethics, Development of Professional Abilities and Career Guidance. The e-learning platform of the project Labour Market Integration of Veterinary Medicine Students – Practical Training has five modules that covers the practical needs and allow the application of the acquired theoretical knowledge: *Animal Farms and Breeding Units, Veterinary Clinics and Hospitals, Laboratories (research, diagnosis, food control), Slaughtering Houses, Rural veterinary Clinics.*

The platform presents 360° scene simulations for these five domains that introduce the students to real-life situations. Students accessed the platform before the effective practical trai-

ning, e-learning process and outcomes being on-line evaluated, master teachers and tutors having access to the results obtained by the students.

The outcome of this project is presented in fig. 2 and it is important to specify that students could access more than one module.

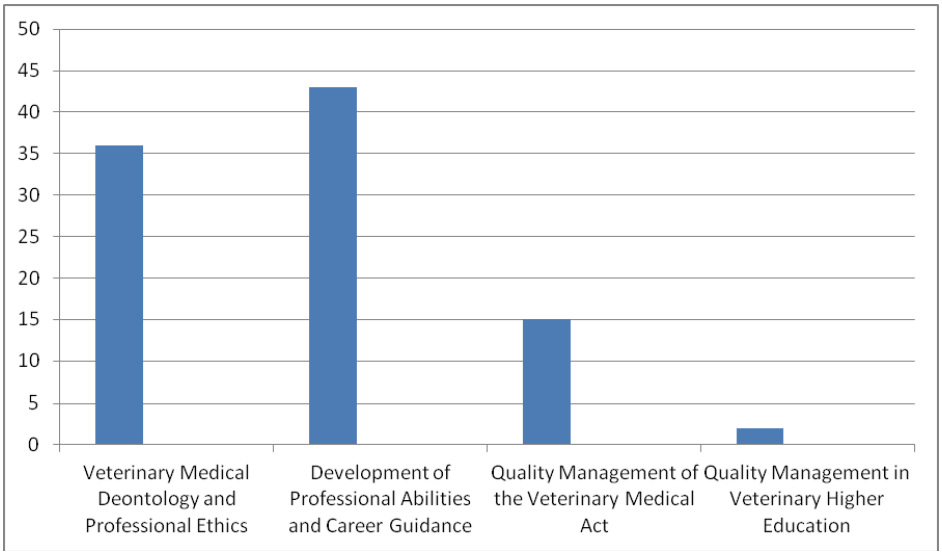

Figure 1. Students' options (%) for the courses from the e-learning platform of the project Higher Education at European Level in the Field of Veterinary Medicine

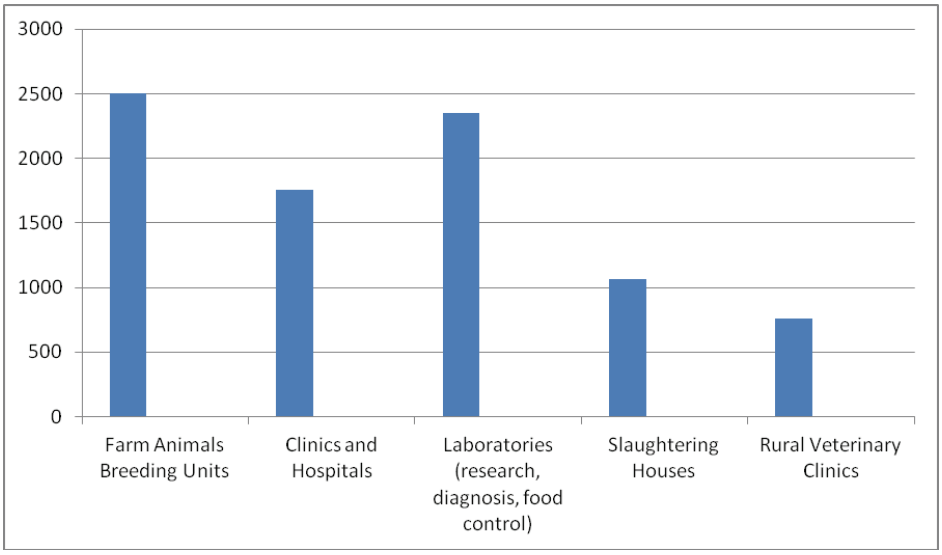

Figure 2. Number of students accesses on the e-learning platform of the project Labour Market Integration of Veterinary Medicine Students – Practical Training

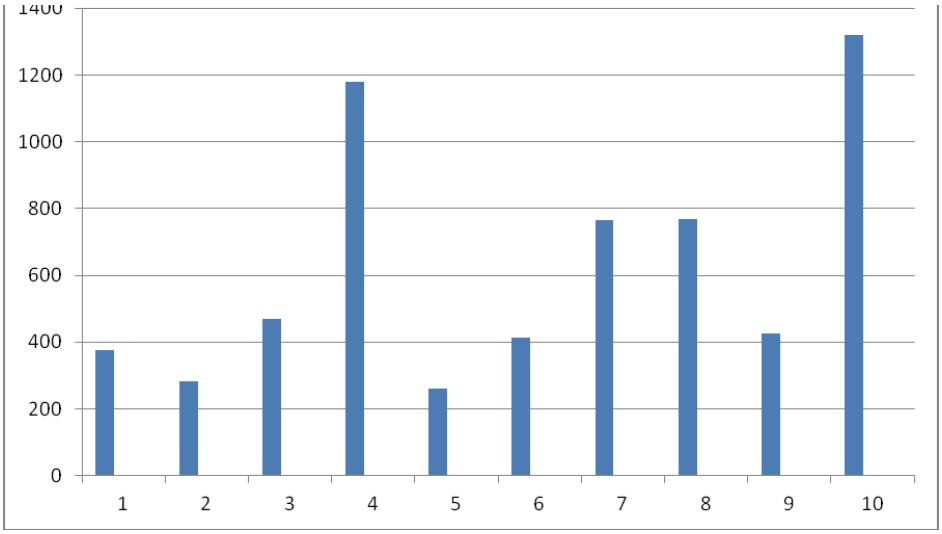

Figure 3. Post-graduate options for the 10 thematic areas of the e-learning platform of the project Improving the Quality of Human Resources in Veterinary Medicine

(1- Advanced Veterinary Diagnostic Imaging, 2- New Technologies in Laboratory Diagnosis, 3-New Technologies in Large Animals′ Pathology and Clinic, 4- New Technologies in Companion Animals′ Pathology and Clinic, 5- Modern Technologies in Clinical Biochemistry and Molecular Biology, 6- Modern Apparatus Used in Animal Hygiene, 7- Use of New Technologies for Food Control and Expertise, 8- Informatics Technologies Dedicated to Food Safety and Quality Management, 9- Apparatus Used in Reproduction Pathology, 10-Animals′ Selection and Amelioration and New Technologies)

The practitioners involved in the project Improving the Quality of Human Resources in Veterinary Medicine could also access more than one thematic area. It was very well received by veterinary professionals community. The number of participants, 2,488 is more than double than the expected number of 1,000 members of target group. This project includes both e-learning activities for each thematic area and face-to-face courses. All the mentioned projects aimed to meet changing professional and social demands.

CONCLUSIONS

E-learning represents a useful tool for distance and longlife learning, contributes to a better comprehension of information, enhance communication among professionals and students.

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