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APPLICABILITY OF ASSISTED REPRODUCTION TECHNIQUES IN CONTEMPORARY SMALL RUMINANT FARMING

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ABSTRACT

Sheep and goat breeding has long tradition in the Balkan countries, making it leading trade in animal husbandry. Dynamic changes in global agriculture production from traditional to industrial livestock technologies, has also impacted the small ruminant farming systems. The reproduction management is considered to be a crucial point for good farming practice among other animal husbandry factors (housing, nutrition, selection, healthcare, etc.). In the last few decades, our sheep and goat farming systems have introduced various assisted reproduction techniques, such as estrous and ovulation synchronization, laparoscopic intrauterine insemination, embryo production (MOET and IVF), semen cryoconservation, photoperiod manipulation etc. This article reviews the current novelties in this field, presenting worldwide scientific reports and our personal experiences in research and translation to everyday farm practice.

Ovine and caprine species have been considered as typical seasonal breeders, becoming sexually active as result of pineal gland and day-length alterations in late summer/early autumn. Lambing/kidding and milk production follow established seasonal patterns. In order for farmers to yield these productive traits in various seasons of the year, they are able to use different hormonal combination of progestagens, prostaglandins, exogenous gonadotropins (eCG) or "natural" methods: light control or exposure to a male after period of isolation ("ram/buck effect"). The hormonal treatment by vaginal sponges is applicable throughout the year, resulting in pregnancy and higher lambing rates compared to seasonal breeding (see review Dovenski and Gvozdic 2012).

The implementation of most recent Artificial Insemination techniques in small ruminants, suggests that the transcervical insemination success rates could be improved by intracornual deposition of semen (laparoscopy), bypassing the "cervical barrier". Our results indicate high pregnancy rates could be obtained by Intrauterine Laparascopic Insemination in sheep (45% out of season, 60% during the breeding season) and even higher in goats 70-80% (Dovenski at al. 2012). Some attempts for pharmacological relaxation of uterine cervix in sheep with unsatisfactory success have been also reported by Candappa et al (2009).

Survival rate of buck's spermatozoa has been dramatically improved by using the "egg-yolk free" extenders, based of soya lecithin as cryoprotectant. Conversely, ram semen cryopreservation did not make substantial progress in past decades, despite the great research work in testing of novel media supplemented by various antioxidants (oxidized glutathione, reduced glutathione, cysteine), and the peculiar in-vivo trials which has shown optimistic results.

Key words: assisted reproduction, sheep, goats, artificial insemination, estrous synchronization.

Introduction

The small ruminants are seasonally polyestrous animals and they show signs of sexual activity at regular intervals during the breeding season. The season of sexual activity in a moderate climate zone lasts from mid-summer to mid-winter, i.e. in the period when duration of the day light become shorter, therefore they are called "short day breeders". The whole process is dependent upon the excretion of melatonin from epiphysis during night time, which is under the influence of

genetic and environmental factors. The season of kidding and lambing lasts from January until early summer, with the most prominent period in spring when climate and nutrition conditions are optimal for surviving of offspring.

According to the study of Abecia et al. (2012), the economic impact may be influenced by several factors, such us the breeding models, reproduction management and reproductive efficiency at the farm. Apart of these basic factors, the author suggests several other means by which the economic outcomecould be altered,

- Providing continuous supply of markets with lamb meat throughout the year
- Providing a greater exploitation of facilities and equipment used for sheep farming
- Even distribution and utilization of the labour force during the year
- Reducing the risks of adverse conditions of production and products sales, which could appear in some period of the year
- Providing a stable source of income to producers throughout the year

The reproductive management on small ruminant farms is based on application ofhormonal and non-hormonal methods.

Hormonal methods for oestrus synchronization Applying progestagen impregnated vaginal sponges is the most common method which needs to be used in strictly defined protocols.

The protocol for goats involves the application and maintaining ofvaginal sponges for 11 days. Forty-eight hours prior to the sponge removal, the veterinarian should administer IM prostaglandin injection (PG) to ensure luteolysis of existing corpus luteum on the ovaries, as well as injection of equine chorionic gonadotropin (eCG), which will initiate the final follicular development and ovulation (Gonzalez de Bulnes et al. 1999).

In sheep, the protocol takes 12–14 days and the eCG is applied on the day of the sponge removal. Dose of the eCG depends on the period of the year, the level of milk production and the parity of the animal (nulliparous or multiparous) (350–600 IU).

The heat detection should be performed 24–30h after the sponge removal and in goats insemination should be carried out around 43 h (36-47 h) after sponge removal (in sheep 55 h, 52 h – ewe lambs). The success of the method is around 70% of kidding/lambing. Goats in which oestrus was not positively detected 24–30 h after sponge removal, have fertility of only 30% after the AI. It should be noted that the occurrence of vaginal discharge following sponge removal does not adversely affect the results.

If this protocol is frequently used, it becomes less and less effective because of the creation of eCG antibodies that reduce the effectiveness of the protocol and the female does not ovulate at the expected time. Furthermore, the European Union regulative is becoming more strict regarding maximum allowed levels of hormone residues (MRLs) in milk (96/22/EC) (Grizelj et al. 2008).

Non-hormonal methods are becoming more interesting for modern sheep and goat breeders, especially when farmers desire to follow modern market trends for organic food production and increasingly stringent legislation imposed by the European Commission. These methods include the "photoperiodic treatments" and the "male effect".

By applying the photoperiodic treatments (manipulation of the day length, aiming to change the period of sexual activity), it is possible to control the sexual activity of sheep and goats and cause the occurrence of sexual activity during the non-breeding season (spring) or transition period (so-called "breeding season advancement").

The "buck effect" is a phenomenon based on the fact that females in the transitional period, could be provoked, synchronized and introduced into sexual activity by presenting males among

the group of receptive females, which have been previously isolated for 2 months. The males should be housed at the remote objects, at proper distance, thus preventing olfactory, visual and/or acoustic contact (total isolation). The deliberate introduction of male among the group of females (to assure permanent contact and ratio 1 male to 10 females) will result in synchronous appearance of fertile oestrus peaks in the goats 7–11 and then 27–35 days after introduction of the male. In sheep this effect appears 20–25 days following the introduction of the ram (Grizelj et al. 2014).

The "ram effect" has been studied and observed in a large number of breeds. However, the efficiency of the method looks to be influenced by the "depth" of anoestrus. Thus, the Merino sheep in which anoestrusis relatively shallow, reactbetter to the deliberate introduction of male then breeds with deepanoestrus. But, even within Merino breed all females will not ovulate after the introduction of the ram; the exact percentage could vary from 40–90% (Martin et al., 1983).

Responsiveness to the "ram effect" differs significantly among various breeds, depending on theseason in which the stimulation is performed. Merino sheep react at any time of the year, while the Romney breed react only if the stimulation is performed in the late anoestrus period. In our climatic conditions, the ram effect during the early anoestrus (February to May) has better response in crossbred Tsigai x Wurttenberg (27.1% in oestrus) than in Tsigai breed (13.3%) (Sahinovic and Stancic, 1991).

A large percentage of corpora lutea formed by oestrus synchronization and "male effect" introduction may be insufficiently functional and could regress within the first 6 days (up to 50%). This effect can be avoided by single dose injection of progesterone (20 mg) prior to introducing of the males among the females.

Flock synchronization with non-hormonal methods is most commonly performed by combining the afore mentioned methods. After submitting the animals (males and females) to the long-daylight period (70–90 d, 16 h light/day), a short-day-light treatment needs to be applied (8–12 h of continuous lighting or melatonin subcutaneous implant application). During this time males should be isolated from females. Sixty days after the "short daylight treatment", the introduction of males among females is performed. This introduction among the receptive females will provoke synchronous appearance of fertile estruses.

Artificial Insemination

Crucially important method in assisted reproduction is Artificial Insemination (AI). It is usually applied in selection programs for farm animals. In sheep satisfactory results were not obtained by using transcervical insemination, especially during non-breeding season and using deep frozen-thawed semen. Low fertilization rates are probably due to difficult transport of spermatozoathrough the cervix (Boland et al., 1983). However, this difficulty could be resolved by using laparoscopic intra-uterine AI, where semen is deposited directly into the uterine horns (Dovenski et al., 2012). Significantly higher pregnancy and lambing rates are achieved by IUAI in compared to the intracervical AI (80.95% vs. 67.48% in goats; 61.36% vs. 45.24% in sheep).

Artificial insemination is a routine practice among goat breeders in some countries (out of 1.300.000 goats in France 400.000 are under AI program). The cervix does not present a major barrier during the semen deposition in goats, so the use of straws with frozen semen is also possible. Freezing resistance of buck spermatozoa is much higher than the rams' spermatozoa. However, prior to dilution with Tris-egg-yolk extender, the spermatozoa should be washed with a sodium Ringer lactate solution and subjected to the centrifugation process for removal of the seminal

plasma (Mukul et al., 2017). This procedure is necessary to prevent reaction of Egg yolk with some bulbourethral gland's glycoproteins that have triacylglycerol hydrolase activity, resulting in decreased sperm motility and disruption of the cell membrane (Pellicer-Rubio and Combarnous, 1998). Recently, Chelucci et al. (2015) reported that soy-lecithin can be a suitable alternative to egg yolk extender for goat semen cryopreservation, due to its cryoprotective effects on the sperm membrane which obtains higher fertilization rates after AI.

Results of induction and synchronization of oestrus in a large sheep flock

Application of hormonal reproduction control method by induction and synchronization of oestrus outside the breeding season, has yielded significant results on a large size farm in Macedonia (>5000 sheep). Induction and synchronization of oestrus was performed byapplying intravaginal sponges impregnated with a synthetic progestagen. They were removed12 days following the application, and an eCG dose of 500 IU/sheep was applied. Table 1. shows the results of induction and synchronization of oestrus during 2008.

Table 1: Results of the implementation of the hormonal control of reproduction through the induction and synchronization of oestrus outside breeding season on a large farm in the Republic of Macedonia in 2008 (Dovenski and Gvozdic, 2012).

Date of sponge application	Inserted sponges Sheep No	Removed sponges d. 12.		Pregnancy rate		No of lambs	Lambing rate	Twining	Dead born		Abortus	
	N	N	%	N	%	N	%	%	N	%	N	%
25.Jan.	300	298	99.33	225	75.50	326	144.89	33.89	4	1.3	10	3.4
09.Feb.	300	297	99.00	218	73.40	329	150.92	37.37	3	1.0	11	3.7
08.March	300	300	100.00	209	69.67	354	169.38	48.33	3	1.0	8	2.7
04.May	300	300	100.00	244	81.33	345	141.39	33.67	5	1.7	12	4.0
05.0 May	300	300	100.00	232	77.33	369	159.05	45.67	2	0.7	5	1.7
12. May	300	299	99.67	251	83.95	407	162.15	52.17	0	0	4	1.3
13. May	300	298	99.33	233	78.19	372	159.66	46.64	4	1.3	10	3.4
20. May	300	300	100.00	247	82.33	387	156.68	46.67	4	1.3	7	2.3
Total	**2400**	**2392**	**99.67**	**1859**	**77.71**	**2889**	**155.51**	**43.06**	**3.1**	**1.1**	**8.38**	**2.8**
Jan.-March	**900**	**895**	**-**	**652**	**72.86**	**1009**	**155.06**	**-**	**-**	**-**	**-**	**-**
May	**1500**	**1497**	**-**	**1207**	**80.63**	**1880**	**155.79**	**-**	**-**	**-**	**-**	**-**
Breeding season	**5120**	**5120**	**-**	**4528**	**88.44**	**5343**	**104.36**	**15.92**	**38**	**0.7**	**77**	**1.5**

Out of 2.400 ewes included in the program of oestrus induction and synchronisation, out of the breeding season, 1.500 ewes have been included in May, 2008, whereas 900 ewes were included in the period between January and March, 2008. Average lambing rate in sheep synchronized outside of the breeding season has beenhigher (155%) then in ewes during the breeding season (104%), although pregnancy rate has been insignificantly lower (77.7% vs. 88.4% respectively).

Results of the systematic application of hormonal reproduction control on the farm during the period 2008–2011 are shown in Table 2. These data indicate that the systematic implementation of this method on a farm with over 5.000 sheep could yield more than 7.900 lambs per year, which in 4-years period of continuous trend, can reach up to 31.000 lambs. Probably the best indicators of the success are the in-season and out-of-season-breeding lambing rates which range 118–137% and 155–162%, respectively. Induction and synchronization of oestrus outside the breeding season

promote the occurrence of twinning in sheep; the percentage of twinning outside the breeding season varied between 43–48%, while in the breeding season the twinning rate was significantly lower (16–21%).

Table 2: Results of the systematic implementation of the hormonal control of reproduction through the induction and synchronization of oestrus outside breeding season on a large farm in the Republic of Macedonia during the period 2008–2011 (Dovenski and Gvozdic, 2012).

Year	Season	Sponges Sheep No	Removed sponges d. 12.		Pregnancy rate		No of lambs	Lambing rate	Twining	Dead born	Abortus
		N	N	%	N	%	N	%	%	%	%
2008	Non-breeding	2400	2392	99.7	1859	77.7	2889	155	43	1.0	2.8
	Breeding	-	5120	-	4528	88.4	5343	118	16	0.7	1.5
2009	Non-breeding	1800	1797	99.8	1385	77.1	2256	162	48	1.1	2.3
	Breeding	-	4578	-	4138	90.4	5109	123	21	0.5	1.2
2010	Non-breeding	1500	1495	99.7	1195	79.9	1867	156	45	0.9	2.1
	Breeding	-	4835	-	4312	89.2	5139	119	17	0.7	1.3
2011	Non-breeding	1800	1800	100.00	1390	77.2	2251	162	48	0.8	2.2
	Breeding	-	4356	-	3561	81.8	4892	137	-	0.5	1.3

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PHARMACOKINETICS OF CIPROFLOXACIN IN PIGS AFTER SINGLE INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION

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ABSTRACT

The pharmacokinetics of ciprofloxacin in pigs after single intravenous or intramuscular application was followed out. Blood concentrations were assayed using HPLC with UV-detection. By means of the TopFit, v. 2.0. software, the pharmacokinetic parameters were calculated by two pharmacokinetic models – compartmental and non-compartmental.

After *i.v.* application, the respective values of calculated pharmacokinetic parameters were as followed: $t_{1/2\alpha}$ = 0.54 h; $t_{1/2\beta}$ = 5.92 h and $t_{1/2\beta}$ = 5.76 h; MRT = 8.74 h and MRT = 8.41h; $AUC_{0\rightarrow\infty}$= 23.14 μg.h/ml and $AUC_{0\rightarrow\infty}$= 22.24 μg.h/ml; V_{ss}= 4.87 l/kg and $Vd_{(area)}$ = 5.14 l/kg.

After *i.m.* application of ciprofloxacin, the respective values of calculated pharmacokinetic parameters were as followed: $t_{1/2\alpha}$ = 0.46 h; $t_{1/2\beta}$ = 4.83 h and $t_{1/2\beta}$ = 4.61 h; MRT = 9.89 h and MRT = 9.68 h; $t_{1/2abs.}$ = 0.56 h; MAT = 1.15 h and MAT = 1.12 h; C_{max} = 0.714 μg/ml and C_{max} = 0.625 μg/ml; T_{max}= 0.76 h and T_{max}= 0.88 h; $AUC_{0\rightarrow\infty}$ = 17.630 μg.h/ml and $AUC_{0\rightarrow\infty}$ = 16.835 μg.h/ml; F = 76.23% and F = 75.69%.

Key words: ciprofloxacin, pharmacokinetics, pigs.

Introduction

Gram-negative infections in pigs cause serious illnesses. The antimicrobial therapy for their treatment includes the use of chemotherapeutics from the group of fluorinated quinolones. In the veterinary clinical practice, the drug enrofloxacin is commonly prescribed for treatment of Gram-negative and Gram-positive infections in animals. Another fluoroquinolone, permitted for use in chickens and pigs is ciprofloxacin. It is widely applied and investigated in human medicine (Müler *et al.*, 1999; Morlet *et al.*, 2000; Hassan *et al.*, 2007). Pharmacokinetic studies with ciprofloxacin were conduced in animals as well – rats (Dautrey *et al.*, 1999; Tung-Hu Tsaj *et al.*, 2001), chickens (Atta Sharif, 1997; García Ovando, 1999; Anadón *et al*., 2001; Raina *et al.*, 2007; Raina *et al.*, 2008), rabbits (Bashir, 2007; Bashir *et al.*, 2007; Bashir *et al.*, 2008), dogs (Albarellos *et al*., 2006; Boothe *et al.*, 2006; Hendrix and Cox, 2008), cats (Boothe *et al.*, 2006), goats (Iqbal *et al.*, 2007; Raina *et al.*, 2008; Javed *et al.*, 2009; Iqbal *et al.*, 2011), sheep (Javed *et al.*, 2009; Iqbal *et al.*, 2012) and calves (Nows *et al.*, 1988; Saini and Srivastava, 2001; Javed *et al.*, 2009).

The antimicrobial activity of ciprofloxacin against various *Pseudomonas* strains is attributed to the pyperazine ring attached to position 7, while the fluorine atom at position 6 confers activity against Gram-positive microbial pathogens (Vancustem *et al.*, 1990).

The activity spectrum of ciprofloxacin is broad, it exhibits activity against Gram-negative and some Gram-positive pathogens, *Mycoplasma* spp., *Chlamydia* spp. and *Rickettsia* spp. (Hannan *et al.*, 1989; Prescott and Yelding, 1990). It is effective against anaerobic bacteria too (Prabhala *et al*., 1984). The antibacterial effect mechanism is mediated through inhibition of the enzyme topoisomerase, aka DNA-gyrase (Gellert, 1981).

Due to its good solubility in lipids and the low degree of protein binding, ciprofloxacin is characterized with a large volume of distribution, thus allowing high concentrations of the drug to reach numerous tissues and body fluids in the different animal species (Neer, 1988).

The physiological differences among the animal species do not permit extrapolation of dose regiments from one species to another. This was the incentive of our study, aiming to follow out the pharmacokinetic behaviour – absorption, distribution, elimination and bioavailability – of ciprofloxacin after intravenous and intramuscular injection to pigs.

Material and methods

Animals and housing. The pharmacological experiment was performed in 8 clinically healthy, sexually intact pigs, Danube white×Landrace crosses, equal number of both genders. The animals were 10 weeks of age and weighed 14.3–18.9 kg.

The pigs were housed freely in 4.5×1.9 m pens with brick floors, with common feeding and watering troughs. The animals were divided into 2 groups depending on the gender (4 pigs per pen). The premise with pens was continuously aerated and with mixed light regimen (dark and light), and air humidity 55%. The ambient temperature was 23–24 °C. The animals were fed compound grower feed, and water was available *ad libitum*.

Drugs. In this study, ciprofloxacin hydrochloridum was used, purchased from Actavis Ltd, Sofia, and stored in a refrigerator at 4 °C until use. It was dissolved *ex tempore* in sterile distilled water for parenteral use to obtain 5% solution for intravenous and intramuscular application.

Experimental design. ***Prior to application, the drug solutions were warmed in a water bath to 37 °C. The intravenous application was in the right ear* v. auricularis*, and the intramuscular – in the neck muscles. The tested quinolone, previously dissolved in sterile distilled water, was applied once, either intravenously or intramuscularly at a dose of 10 mg/kg. A 15-day "wash out" period was allowed between both routes of administration for complete elimination of the drug and its metabolites. Blood for analysis after* i.v. *treatment was collected before the treatment and at post treatment hours 0.08, 0.17, 0.33, 0.50, 1, 2, 4, 6, 8, 12 and 24 h. The sampling intervals after* i.m. *application were: 0 h, 0.17, 0.33, 0.50, 1, 2, 4, 6, 8, 12 and 24 h.***

Blood samples. Blood samples were collected from the orbital sinus (*sinus ophthalmicus*) in capped Eppendorf tubes and left for 2 h at room temperature. The serum was separated by centrifugation at 1500×*g* for 15 min. Blood serum samples was stored in capped Eppendorf tubes frozen at –25 °C until analysis.

Sample analysis. ***For assays, ciprofloxacin hydrochloridum substance was used, as well as acetonitrile HPLC grade (Labscan); triethylamine (TEA; Merck); tetrabutyl ammonium hydrochloride 40% (TBA, Merck); phosphoric acid 85% (Merck) and perchloric acid for analysis 70% (Merck). The water used in analyses was treated through a Millipore purifying system.***

Serum ciprofloxacin concentrations of treated pigs were assayed by a highly sensitive automated technique – high-performance liquid chromatography with UV detection. The analysis protocol was that of Imre *et al*. (2003), already used by us, with a slight modification consisting of substituting blood serum for plasma. Serum concentrations were analyzed on a reverse-phase HPLC system Waters equipped with quaternary pump, fluorescence detector, protected and analytical columns Lichrospher (Beckman).

The mobile phase consisted of acetonitrile and water (1:1), supplemented with 3% potassium phosphate buffer, 2% TEA and 2% TBA. Mobile phase pumping rate was 1.2 ml/min.

The HPLC system was connected to a PC with Waters Empower software.

Blood proteins were precipitated by adding two acids – 85% phosphoric acid and 70% perchloric acid to 100 μl serum. Then, serum was homogenised by a vortex mixer and centrifuged for 10 min at 1500×*g*. One hundred μl of supernatant aliquots were injected in the HPLC system.

Assay was validated by the method of external calibration curves from spiked matrix standards. The limit of quantitation (LOQ) for ciprofloxacin was 0.025 μg/ml, and the limit of detection (LOD) – 0.005 μg/ml.

Pharmacokinetic analysis. The TopFit, v. 2.0. software was used for describing the behaviour of the tested fluoroquinolone after single intravenous or intramuscular injection in this pharmacokinetic experiment (Henzel *et al.*, 1993). Two pharmacokinetic models were used – the compartmental and non-compartmental analysis (Gibaldi and Perrier, 2007). The pharmacokinetic modelling was done according to Akaike's criterion (Yamaoka *et al.*, 1978). Determined pharmacokinetic parameters were presented as means (Mean) ± standard error of means (SEM).

The following parameters were determined: rate constants for the distribution (α) and elimination (β) phases; drug transfer rate constants from the central to the peripheral (k_{12}) and from the tissue to the central (k_{21}) compartment; absorption rate constant for i.m. application ($k_{abs.}$), and elimination rate constant (k_{el}). The distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$), the mean residence time (MRT) and absorption half-life ($t_{1/2abs.}$); the volume of distribution in the central (V_c) and peripheral (V_t) compartments, as well as steady state volume of distribution (V_{ss}) were determined. The total body clearance (Cl_B); back-extrapolated zero serum concentration of the fluoroquinolone (C°p); area under the serum concentration curve from hour 0 to the limit of quantitation ($AUC_{0\rightarrow LOQ}$) and from hour 0 to infinity ($AUC_{0\rightarrow\infty}$); maximum serum drug concentration (C_{max}), and the time to reach maximum serum concentration (T_{max}) were calculated

The absolute bioavailability (F) after intramuscular injection of fluoroquinolone solution was calculated as per the formula:

$$F\ (\%) = [(AUC_{0\rightarrow\infty\ i.m.} \times D_{i.v.})/ [(AUC_{0\rightarrow\infty\ i.v.} \times D_{i.m.})] \times 100.$$

On the basis of mean residence time (MRT) values after *i.v.* and *i.m.* application, individual mean absorption times (MAT) were calculated as:

$$MAT = MRT_{i.m.} - MRT_{i.v.}$$

Results

After intravenous application of ciprofloxacin, serum concentrations were higher than 0.076 μg/ml until the post treatment hour 24. The analysis of individual time *vs* concentration curves showed comparable serum values, usually close to mean values.

Serum concentration curves in both routes of administration corresponded to two-compartmental open pharmacokinetic model of distribution and elimination, with a rapid distribution phase and a slower elimination phase (Fig. 1).

After intramuscular injection, serum ciprofloxacin concentrations appeared very soon – after 0.17 h and persisted in detectable concentrations over 12 hours (Fig. 1). The maximum levels (C_{max}) were attained very soon after *i.m.* application (by the 1st hour) and in 2 pigs – even earlier – after 0.50 h.

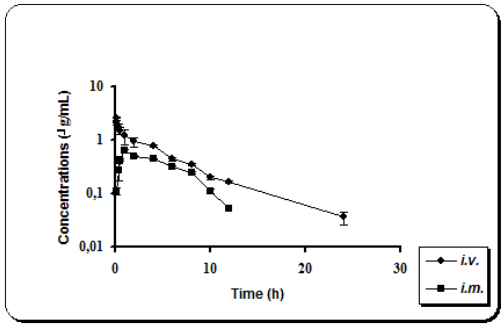

Figure 1: Serum ciprofloxacin concentrations after single intravenous (*i.v.*) and intramuscular (*i.m.*) application in pigs

Table 1: Pharmacokinetic parameters of ciprofloxacin after single intravenous application to pigs at a dose of 10 mg/kg (Mean±SEM)

Parameter	Units	Compartmental method		Parameter	Units	Non-compartmental analysis	
		Mean	SEM			Mean	SEM
$t_{1/2\alpha}$	h	0.54	0.03	$t_{1/2\beta}$	h	5.76	0.09
$t_{1/2\beta}$	h	5.92	0.08	**MRT**	h	8.41	0.08
MRT	h	8.74	0.11	$AUC_{0\rightarrow 24\,h}$	µg.h/mL	21.78	1.04
k_{12}	h^{-1}	2.04	0.08	$AUC_{0\rightarrow\infty}$	µg.h/mL	22.24	2.47
k_{21}	h^{-1}	0.29	0.05	Cl_B	mL/min/ kg	7.16	0.20
k_{el}	h^{-1}	0.26	0.04	V_d	L/kg	5.14	0.09
Cl_B	mL/min/ kg	1.44	0.11	$AUMC_{0\rightarrow\infty}$	µg.h/mL	348.23	5.24
$AUC_{0\rightarrow\infty}$	µg.h/mL	23.14	0.72	r^2	-	0.99	0.08
V_c	L/kg	3.91	0.02				
V_{ss}	L/kg	4.87	0.03				

$t_{1/2\alpha}$ – distribution half-life; $t_{1/2\beta}$ – elimination half-life; MRT – mean residence time; k_{12} – transfer rate constant from the central to the peripheral compartment; k_{21} – transfer rate constant from the peripheral to the central compartment; k_{el} – elimination rate constant; V_{ss} – steady-state volume of distribution; Vc – volume of distribution in the central compartment; Vd – volume of distribution; CL_B – total body clearance; $AUC_{0\rightarrow\infty}$ – area under the serum concentration curve from time 0 to infinity; $AUC_{0\rightarrow 24h}$ – area under the serum concentration curve from hour 0 to hour 24; r^2 – correlation coefficient.

Table 1 presents the pharmacokinetic parameters of ciprofloxacin after its intravenous application to pigs. Similarly to other fluoroquinolones, it is characterised with a long elimination half-life ($t_{1/2\beta}$) and a rapid distribution phase as could be seen from the distribution half-life ($t_{1/2\alpha}$). The fluoroquinolone left rapidly the central compartment and spread at a large volume in body fluids and the other tissues in pigs, as shown by values of k_{12}, k_{21}, V_c and V_{ss}.

The area under the serum concentration curve ($AUC_{0\rightarrow\infty}$) was 23.14±0.18 µg.h/ml (Table 1). The steady-state volume of distribution V_{ss} was rather high – 4.87±0.32 l/kg (Table 1).

Table 2 presents pharmacokinetic parameters depicting ciprofloxacin behaviour after single intramuscular injection. The absorption of the drug from the neck muscles of pigs was rapid and maximum serum concentrations are attained within a short time as seen from $t_{1/2abs.}$, T_{max} and C_{max}. Maximum serum concentrations (C_{max}) for this route of application, determined by the two pharmacokinetic models were 0.714 µg/ml and 0.625 µg/ml, respectively and appeared after 0.76 h and 0.88 h respectively.

The distribution half-life ($t_{1/2\alpha}$) was short – 0.46 h (Table 2).

Table 2: Pharmacokinetic parameters of ciprofloxacin after single intramuscular application to pigs at a dose of 10 mg/kg body weight (Mean±SEM)

Parameter	Units	Compartmental analysis		Parameter	Units	Non-compartmental analysis	
		Mean	SEM			Mean	SEM
$t_{1/2\alpha}$	h	0.46	0.01	$t_{1/2\beta}$	h	4.61	0.16
$t_{1/2\beta}$	h	4.83	0.02	MRT	h	9.68	0.74
MRT	h	9.89	0.13	$AUC_{0\rightarrow 24\ h}$	μg.h/mL	14.23	2.08
$t_{1/2abs.}$	h	0.56	0.06	$AUC_{0\rightarrow\infty}$	μg.h/mL	16.84	1.74
$AUC_{0\rightarrow\infty}$	μg.h/mL	16.63	1.34	C_{max}	μg/mL	0.63	0.12
C_{max}	μg/mL	0.71	0.23	T_{max}	h	0.88	0.26
T_{max}	h	0.76	0.02	$AUMC_{0\rightarrow\infty}$	μg.h/mL	512.34	4.24
MAT	h	1.15	0.23	MAT	h	1.12	0.44
F	%	76.23	2.76	F	%	75.69	1.92
				r^2	-	0.991	0.05

$t_{1/2\alpha}$ – distribution half-life; $t_{1/2\beta}$ – elimination half-life; $t_{1/2abs}$ – absorption half-life; MRT – mean residence time; MAT – mean absorption time; $AUC_{0\rightarrow 24h}$ – area under the plasma concentration curve from hour 0 to hour 24; C_{max} – maximum plasma concentration; T_{max} – time to reach maximum plasma concentration; F – absolute bioavailability; r^2 – correlation coefficient.

It should be noted that biological half-life ($t_{1/2\beta}$) was insignificantly shorter than that after *i.v.* application – 4.83 h and 4.61 h, respectively (Table 2). An opposite tendency was exhibited by MRT, which was 9.89 h and 9.68 h determined by both pharmacokinetic models

Discussion

Data from the present pharmacokinetic experiment suggested that serum concentrations curves for both routes of administration of the tested fluoroquinolone fitted the two-compartmental model. They were comparable to previous investigations in other animal species – cats (Albarelos, 2004), broiler chickens (Atta and Sharif, 1997; Anadón *et al*., 2001), sheep (Javed *et al.*, 2009; Iqbal *et al*., 2012), goats (Iqbal *et al*., 2007; Raina *et al*., 2008; Javed *et al.*, 2009; Iqbal *et al*., 2011), calves (Mohan and Garg, 2003), cows and buffaloes (Javed *et al.*, 2009) and rabbits (Bashir *et al*., 2007; Bashir *et al*., 2008a; Bashir *et al*., 2008b; Parikh *et al*., 2008).

The present study in sexually intact clinically healthy pigs established rapid distribution of *i.v.* and *i.m.* injected ciprofloxacin followed by slower elimination, a trend, observed after application of other fluoroquinolones in pigs (enrofloxacin and pefloxacin) (Dimitrova *et al*., 2009.; Bimazubite *et al*., 2009; Dimitrova *et al*., 2012). A similar tendency was reported by other researchers in other species – broiler chickens (Anadon *et al*., 2001; Bashir *et al*., 2008a; Raina *et al*., 2008; Iqbal *et al*., 2011).

For both used routes of administration in pigs, ciprofloxacin was characterized with a relatively long biological half-life which after *i.v.* injection was 5.92±0.98 h (compartmental model) and 5.76±0.92 h (non-compartmental analysis), and after intramuscular application – 4.83±0.24 h and 4.61±0.16 h, respectively (Tables 1 and 2). The elimination half-life values ($t_{1/2\beta}$) after *i.v.* injection of the quinolone were similar to those reported in broiler chickens (Raina *et al*., 2007), but lower than those in chickens (Atta and Sharif, 1997; Anadón *et al*., 2001) and rabbits (Bashir *et al*., 2007), and higher than values in cats (Albarelos *et al*., 2004), sheep (Javed *et al*., 2009), goats (Raina *et al*., 2008; Javed *et al*., 2009), calves and buffalo calves (Saini and Srivastava, 2001; Mohan and Garg, 2003), buffaloes and cattle (Javed *et al*., 2009).

The V_c and V_{ss} values determined by the two pharmacokinetic models showed that ciprofloxacin penetrated well in all body tissues and was distributed within a large volume, similarly to what was reported by others (Anadón *et al*., 2001; Saini and Srivastava, 2001; Javed *et al*., 2009; Iqbal *et al*., 2011).

The area under the time concentration curve ($AUC_{0\rightarrow\infty}$) for intravenously administered ciprofloxacin in pigs was comparable to that in rabbits (Prikh *et al*., 2008), cats (Albarellos *et al*., 2004) and broiler chickens (Anadón *et al*., 2001).

We demonstrated that the studied gyrase inhibitor was absorbed well and rapidly in the muscles of the neck, as evidenced by values of pharmacokinetic parameters $t_{1/2abs.}$, MAT, C_{max} and T_{max} (Table 2) and HPLC-assayed serum concentrations, which were detected to be sufficiently enough high as early as the 10^{th} min in all pigs after intramuscular application of the fluoroquinolone – over 0.10 μg/ml, and persisted at that level until post application hour 10.

After intramuscular injection, the ciprofloxacin was rapidly absorbed and resulting maximum serum concentrations (C_{max}) were comparable or close to those reported in cats (Albarellos *et al*., 2004), but lower that those in buffaloes, cows and goats (Javed *et al*., 2009; Iqbal *et al*., 2007). The time for attaining maximum serum levels (T_{max}) was similar to values in those ruminant species – within 0.86 and 0.90 h.

In the present investigation, biological half-life values after *i.m.* application ($t_{1/2\beta}$) and mean residence times (MRT) were longer than values reported in buffaloes (Javed *et al*., 2009), cows (Javed *et al*., 2009), sheep (Javed *et al*., 2009) and goats (Javed *et al*., 2009; Iqbal *et al*., 2011). Probable reasons are differences related to the species, hormonal status of animals and the different dosage of the drug.

The absolute bioavailability (F) of ciprofloxacin for this non-venous route of application, determined according to the two pharmacokinetic models – compartmental and non-compartmental analysis (Table 2) was higher than values established in cats (Albarellos *et al*., 2004) but comparable to those observed by Dimitrova *et al*. (2009b) in sheep.

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COINFECTION OF CHICKEN ANAEMIA VIRUS, MYCOPLASMA GALLISEPTICUM, AVIAN METAPNEUMOVIRUS AND AVIAN REOVIRUS IN FANCY CHICKEN BREEDS

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ABSTRACT

In this report, a case of concomitant Chicken anaemia virus (CAV), Mycoplasma gallisepticum (MG), Avian metapneumovirus (aMPV) and Avian reovirus (ARV) infection in fancy chicken breeds is presented. It concerns fancy chickens at the age from 2 weeks to 4 months with repetitive respiratory symptoms such as difficulty breathing, whistling sounds and conjunctivitis. The 5 chickens submitted at the Institute were with depression, rales and dyspnea as 2 of them had anaemia. The necropsy was performed after their humane euthanasia. The 2 older necropsed birds showed fibrinous airsacculitis with gathering caseous exudate and serous tracheitis. The air sacs of the younger chickens were with initial signs of opacity. Atrophy of the thymus was found in one of the examined chickens with anaemia. The 5 serum samples tested by rapid serum agglutination test with MG antigen, were positive for MG. CAV was confirmed in the five fancy chickens by polymerase chain reaction (PCR) and in the both chickens with anaemia histologically. The enzyme-linked immunosorbent assay (ELISA) determined the presence of antibodies to aMPV and ARV in the five tested birds. This study is a case of confirming co-infection of CAV with MG, aMPV and ARV.

Key words: chicken anaemia virus, mycoplasma gallisepticum, avian metapneumovirus, avian reovirus, co-infection, fancy chicken breeds.

Introduction

Raising rare bird breeds is a hobby, practiced in various places in the world. The participation of the fancy bird breeds in exhibitions, while they are getting into contact with birds from other places, is a conducive factor to exchanging various infectious agents. The all-in all-out management system is not applied as fancy birds of different age are mixed, which is precondition for transovarial transmission and persistence of infections. There are taken no other preventive measures such as hygiene improvement and disease monitoring. When fancy chickens are bred, in most cases, no vaccination is performed, which leads to high susceptibility to infections. These are conductive factors to transmitting, persisting and sustaining a number of infectious diseases in them [1, 4]. Therefore from veterinary medical aspect, problems by breeding fancy birds are more different than those that exist in the poultry industry.

CAV is economic important avian pathogen as the infection with it has been described in most countries with a developed chicken industry [10]. Mycoplasma gallisepticum is also an important agent causing chronic respiratory disease in chickens, the infection with which as well as the infection with the Avian reovirus is widespread among the poultry flocks [2, 6, 7]. Avian metapneumovirus causes acute respiratory tract infection and reductions in egg production in various avian species as the infection primary affects the upper respiratory tract of young birds, while also decreases egg production of adult hens [8]. There are data which demonstrate the seroprevalence of these pathogens among fancy poultry [1, 4, 12].

In this report we describe a case of simultaneous confirming CAV, MG, aMPV and ARV in fancy chicken breeds.

Materials and methods

Case presentation. Five fancy chickens at the age from 2 weeks to 4 months originating from fancy chicken-breeding farm, were submitted at the Institute for routine diagnostic examination. According to the owner great percent of the fancy birds in the flock have shown persisting respiratory symptoms such as hard breathing, whistling sounds and conjunctivitis. The birds have been treated with antibiotics Rodotium (Tiamulin), Roxacin (Enrofloxacin) and Doxyvit (Doxycyclin and Ascorbic acid). The chickens submitted to the laboratory were with depression (Fig. 1), rales and dyspnea, while anaemia was found only in two of them (Fig. 1).

Figure 1: Fancy chickens with anaemia and depression

Samples. After blood samples for serological testing were taken, birds were humanly euthanized. Necropsy was performed and tissue samples from thymus and bone marrow were collected for histological and by PCR examinations.

DNA extraction and PCR. CAV DNA was extracted from the thymuses of the five fancy chickens using the Tissue and Cell Genomic DNA Mini Kit (Guangzhou Geneshun Biotech, China) according to the manufacturer's instructions. Amplifications were carried out as described previously [11], but in a total volume of 25 μL (12,5μL mastermix, 2μL of primer S.1.1., 2μL of primer S.1.2., 2μL of target DNA and 6,5μL nuclease-free water), in automatic thermocycler (QB-96, LKB).

The steps and conditions of thermocycling for PCR are presented in Table 1.

Table 1: Cycling parameters of amplification

Steps	Temp.	Time
Initial denaturation	95ºC	2 min
Denaturation	95ºC	1 min
Annaeling	56ºC	2 min
Extension	74ºC	2 min
30 cycles		
Final extension	74ºC	10 min

After the amplification, 7μl of each obtained PCR product was analysed by electrophoresis on a 1.5% agarose gel, and the expected band was visualized by staining with ethidium bromide. A 100-bp DNA ladder served as a size marker.

Serological testing. Sera were tested using commercial ELISAs (Avian Reovirus Antibody Test Kit and Avian Pneumovirus Antibody Test Kit; IDEXX Laboratories). Serological testing was performed according to the manufacturer's recommendations as the optical density (OD) was measured at 450 nm using ELISA Reader LKB 50660-006, Shimagzu. The rapid serum agglutination test for MG was carried out using a MG antigen (Sanofi). A drop of serum was mixed with a drop of antigen suspension and observed for visible agglutination.

For ***histological examination***, thymus and bone marrow samples from the both chickens with anaemia were fixed in 10% neutral phosphate-buffered formalin. They were dehydrated and embedded in paraffin wax, sectioned (4-μm-thick), stained with hematoxylin and eosin (HE) and evaluated under a light microscope.

Results

At necropsy, the most prominent changes were found out in the air sacs as the 2 older birds showed fibrinous airsacculitis with gathering caseous exudate (Fig. 2) and serous tracheitis. The air sacs of the younger chickens were with initial signs of opacity. Thymus atrophy was found in one of the examined chickens with anaemia (Fig. 3).

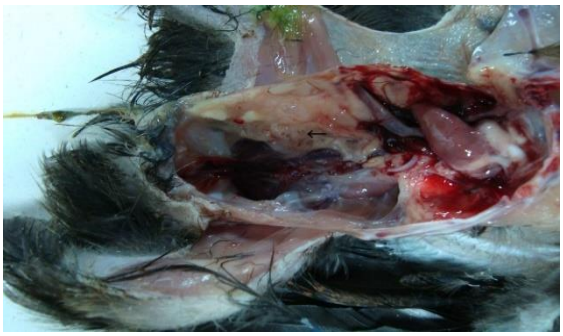

Figure 2: Fibrinous aersacculitis with gathering caseous exudate (arrow)

Figure 3: Atrophy of the thymus (arrow)

The 5 serum samples tested by the rapid serum agglutination test with MG antigen, were positive for MG (Fig. 4).

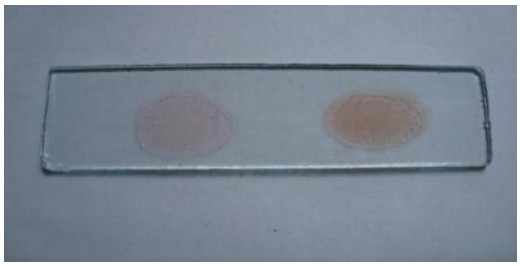

Figure 4: Positive control serum (left) and positive serum sample (right) tested by the rapid serum agglutination test with MG antigen

CAV PCR performed with DNA extracted from the thymuses of the five birds resulted in an amplification of a product with a predictable size of 583 bp (Fig. 5).

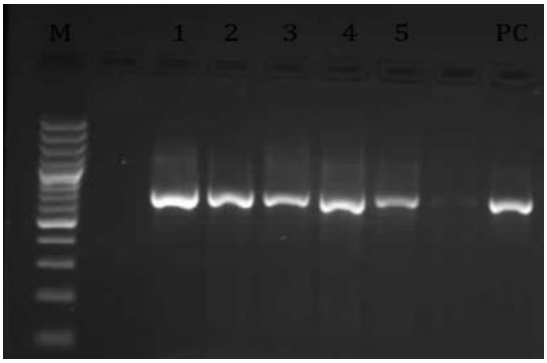

Figure 5: Agarose gel showing PCR product from amplification of thymus samples, as described: Lane M: 100 bp DNA marker; lane 1: positive thymus sample 1; lane 2: positive thymus sample 2; lane 3: positive thymus sample 3; lane 4: positive thymus sample 4; lane 5: positive thymus sample 5; lane PC: positive control

Histologically, hypoplasia of bone marrow (Fig. 6) as well as depletion of lymphocites from subcapsular thymic cortex was detected, which indicated the presence of CAV in the both chickens with anaemia.

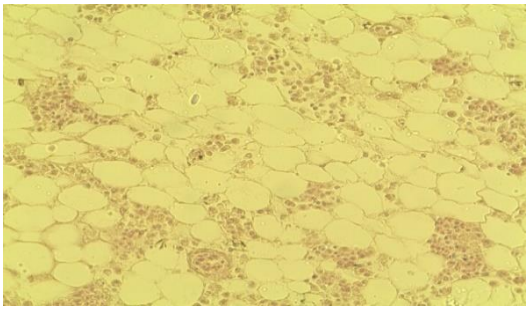

Figure 6: Hypoplasia of the bone marrow: destruction of the haemocytoblasts, which have been replaced by adipose tissue

The ELISA test detected antibodies against Avian reovirus and Avian metapneumovirus in the five tested fancy chickens.

Discussion and conclusions

In this study we confirmed the presence of CAV, MG, aMPV and ARV in fancy chicken breeds in Bulgaria and described a case of co-infection of CAV with MG, aMPV and ARV.

Considering the persisting respiratory symptoms such as difficulty breathing, whistling sounds and conjunctivitis, which have been shown by great percent of the fancy birds in the flock, as well as the observed during the necropsy gross pathological lesions including fibrinous airsacculitis with gathering caseous exudate and air sacs with initial signs of opacity, we suggested that in this case it concerned to MG infection. Thus, we confirmed MG serologically by the rapid serum agglutination test in the five tested fancy chickens. The control of the pathogenic avian mycoplasmas can be carried out in three ways: maintaining the flocks free of infection (all-in all-out management system, good biosecurity including improvement of the hygiene practices and effective monitoring system), vaccination and medication [5]. The practice of maintaining the flocks free of infection, though, by extensively bred birds in yards conditions including fancy birds is as a whole impracticable. That is the reason to rely on treating the sick birds and applying vaccination. The tested by us birds have been treated, but with no effect, because mycoplasma develops antibiotic resistance [3].

The fact that two of the submitted to the laboratoty fancy chickens had anaemia and at necropsy of one of them atrophy of the thymus was found out, turned us to investigation for CAV. By PCR we confirmed the presence of CAV in the five tested birds, that as an immunosuppressive agent causes co-infections with reoviruses (blue wing disease, haemorrhagic anaemia syndrome), adenoviruses (inclusion body hepatitis/hydropericardium syndrome), IBDV-Infectious Bursal Disease Virus, MDV-Marek´s disease virus (early mortality syndrome), IBV-Infectious Bronchitis Virus, Clostridium perfringens (gangrenous dermatitis), Staphylococcus aureus [9]. We assume that the CAV infection together with the MG infection in this case is the reason for the unsatisfied results of the therapy. We claim that the CAV infection is active because we observed anaemia in some of the birds, atrophy of the thymus, though it was found out in only one chicken, and we confirmed the presence of the virus in the five fancy birds by PCR and in the both chickens with anaemia histologically.

The respiratory symptoms of the examined chickens including rales and dyspnea as well as the serous tracheitis which we found out during the necropsy of the two older fancy birds, suggested that other respiratory pathogens might have been involved in the clinical manifestations and lesions, therefor we performed serological testing for presence of antibodies to aMPV. Although blue wing disease was not observed in the five examined chickens, we investigated them for antibodies against ARV. Thus, infections with aMPV and ARV were confirmed serologically by ELISA in the five tested fancy birds. We concider that in this case it concerns to complicated infection of CAV with MG, aMPV and ARV in which the four infectious agents mutually act and enhance their effects as the whole association of viruses exacerbates the clinical picture.

The present study demonstrates that fancy chicken breeds could be reservoir of CAV, MG, aMPV and ARV and play an important role in the transmission of these infectious agents. When persisting respiratory problems conditioned by mycoplasma are found out, a possible infection with CAV should always be considered in the diagnostic plan. The disease complex primarily resulted from the interplay between CAV and MG, subsequently could be aggravated by co-infection with other pathogens such as aMPV and ARV.

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INFLUENCE OF SALT SUPPLEMENTATION – SALT EXCLUSION DIET ON ESTRUS INDUCTION IN NORTH-EAST BULGARIAN MERINO SHEEP

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ABSTRACT

The aim of our study was to investigate the effect of salt supplementation - salt exclusion (SSE) diet on estrus induction in North-East Bulgarian merino sheep (NBMS). It was used three groups consisting of 180 animals each, where one serves as control and two were experimental. The control group was allowed to consume salt *ad libitum*, while the experimental groups had no access to salt for 10 days, after which their diet was supplemented with 17g/per capita for 6 days. During the experiment, teaser rams were used to reveal the animals which are in estrus. It was found that SSE diet doesn't stimulate the estrus onset in the sheep from NBMS.

Key words: sheep breeds, estrus, salt supplementation - salt exclusion diet.

Introduction

There are a lot of research which demonstrates that addition of food supplements or changes in the diet can affect positively many of the physiological functions including the reproductive performance in animals (Kistanova et al., 2012a; Kistanova et al., 2012b; Abadjieva et al., 2013; Marchev et al.,2015; Abadjieva et al., 2016. Kistanova et al., 2016).

However, there are some controversies about the application of certain dietary ingredients for improvement of reproductive traits in female animals.

One of the most debatable questions concerning the management of sheep reproduction is to use salt supplementation - salt exclusion diet as stimulant for induction or synchronization of estrus. In shortly the salt (NaCl) is excluded of the sheep's diet for one or two weeks and after that each animal is supplemented with 15 – 20 g salt daily, for approximately the same time period. In the past many authors proposed the application of this diet as alternative for hormonal treatment for inducing and synchronizing of estrus in sheep (Bratanov et al., 1975; Doichev et al., 1976; Solomonov and Jeliazkov, 1976. Bankov et al., 1989).

The results of the different studies vary a lot, depending on the period of salt supplementation – salt exclusion or the sheep breed which it was applied on. The recent researches of Metodiev et al., (2010a; 2010b) showed week positive effect in stimulating of estrus by salt supplementation – salt exclusion diet in Ile de France breed and positive effect of combined application of this diet with the ram effect in Synthetic Population Bulgarian Milk breed (SPBM). On the other hand Nedelkov et al., (2012) found that there is no significant effect of salt supplementation – salt exclusion diet on induction of oestrus in the ewes from Tsigai, Karakachan and SPBM breeds. The controversial data on that topic imply additional experiments to define whether the salt supplementation - salt exclusion diet can be used for inducing of estrus in sheep or not.

The aim of this study was to investigate the effect of salt supplementation – salt exclusion diet for estrus induction in North-East Bulgarian merino sheep (NBMS)

Material and methods

The experiment was held at the Experimental Station of Agriculture – Targovishte. Only clinicaly healty ewes and gimmers from North-East Bulgarian merino sheep breed were used. The animals (n=540) were divided equally into three flocks. One of the flocks serves as control and the animals from it obtained salt regularly. The animals from the other two flocks serve as experimental group.

The salt was excluded for 10 days from their diet and after that salt lick was returned and additionally each animal received 17 g salt supplemented to their concentrated feed for 6 days. The sheep from control and experimental groups were reared on pastures and supplemented with 300 g combined feed per capita, per day.

The breeding campaign started at the 4th day after salt supplementation was restored in the experimental group. Before the beginning of breeding campaign, the rams were isolated from ewes and gimmers. Each morning 3 teaser rams were released among the flocks in order to locate the ewes and gimmers which are in estrus. Stud rams were used for semen collection according to the individual breeding plan. The semen was collected by the method of artificial vagina by trained technician, evaluated under microscope and diluted with semen extender in ratio 1:3. The so prepared semen was used for artificial insemination which was performed twice in 8 hours interval.

Additionaly the body condition scoring (BCS) was performed according to Russel (1991) for each animal from control and experimental gropus.

Results and Discussion

Since the number of sheep in the control group is 180 and those in the experimental are 360, all the data is presented as percent. The results of the induced estrus in control and experimental groups during each five days of the breeding season are presented in table 1.

Table 1: Induced estrus in control and experimental groups during the breeding season

Days of breeding season	1–5		6–10		11–15		16–20		21–25		26–30		31–35		36–40		41–45	
Group	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Control	12	7.95	13	8.61	12	7.95	27	17.88	34	22.52	30	19.87	8	5.30	9	5.96	6	3.97
Experimental	23	7.14	29	9.00	28	8.69	48	14.91	80	24.84	45	13.97	24	7.45	25	7.76	20	6.21

During the first 15 days of the breeding campaign, the ewes and gimmers with induced estrus was 24.91% in the control and 24.83% of the treatment group. The highest number of sheep with manifested estrus in both groups was between the 16 and 30 day from the beginning of the campaign, which is typical for induction of heat as result of the effect of the ram appearance. In this period there was a manifested estrus in 60.27% of the ewes and gimmers form the control group and 53,72% in the experimental. The obtained data showed that salt supplementation - salt exclusion diet failed to induce estrus in the ewes and gimmers form North-East Bulgarian merino sheep, which is in agreement with the results of Nedelkov et al., (2012) which perform the similar treatment on ewes from Tsigai, Karakachan and SPBM breeds.

Table 2: Dependence of the estrus induction in sheep from their body condition scoring

BCS	N	Experimental				Control			
		1–15 day First Period		16–30 day Second period		1–15 day First Period		16–30 day Second period	
		N	%	N	%	N	%	N	%
2.38	48	18	37.5	30	62.50	11	44.00	14	56.00
3.00	75	26	34.6	49	65.40	10	37.04	17	62.96
3.53	68	19	27.94	49	72.06	6	30.00	14	70.00

The body condition scoring which was performed to part of the sheep from both groups allowed us to define the influence of this index on the effect of salt supplementation - salt exclusion diet and ram effect. The data presented in table 2 showed that during the first 15 days of breeding campaign there was a tendency of higher estrus manifestation in ewes and gimmers with lower than those with greater BCS, both in experimental and in the control groups. During the first period, in the experimental group, estrus was manifested in 37.5%,of the animals with BCS of 2.38, while in those with BCS 3.53 the percent were lower – 27.94% .

Similar tendency was observed in the control group where the percent of animals with manifested estrus was even higher – BCS 2.38 – 44.0%, BCS 3.53 – 30.0%. It can be concluded that the higher number of animals with manifested estrus during the first period of the breeding campaign is not due to salt supplementation – salt exclusion diet.

During the second period of the breeding campaign, in the experimental group the ewes and gimmers with BCS 2.38 manifested estrus with 9.59% lower than those with BCS 3.53. At the same time, in the control group the difference between the animals with BCS 2,38 and those with BCS 3.53 was 14% in favor of the animals with higher BCS.

There was a better response to the "ram effect" of ewes and gimmers with BCS of 3.53 compare to ewes with BCS of 2.38, especially at the second period of the breeding campaign. In experiments with Tsigai, Karakachan and SPBM breeds was also found that ewes with higher BCS had better response to the "ram effect" compaired to those with lower. Nedkov et al (2012).

Conclusion

It can be concluded that the application of salt supplementation – salt exclusion diet alone didn't lead to estrus induction in North-East Bulgarian merino sheep. BCS index doesn't influence the effect of salt supplementation - salt exclusion diet. The ewes and gimmers with higher BCS had better response to ram effect compared to those with lower BCS.

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HEMATOLOGICAL AND BLOOD-BIOCHEMISTRY PARAMETERS OF GUINEA FOWLS IN EARLY STAGE OF NITROSODIETHYLAMINE-INDUCED HEPATOCARCINOGENESIS

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ABSTRACT

In ovo models (avian embryos) are a novel alternative to laboratory animals used in the experimental cancer research. In the present study, the preneoplastic liver lesions induced by N-nitrosodiethylamine in guinea fowls were examined by histopatological methods. The alterations of some hematological and biochemical parameters were examined in guinea fowls hatched from carcinogen-inoculated eggs. Histopathology confirmed the presence of basophilic and eosinophilic foci of altered hepatocytes, strongly resembling the morphology of the preneoplastic lesions previously found in other avian species and laboratory rodents treated with the same carcinogen, as well as in humans with hepatocellular carcinomas. In addition to the focal hepatic lesions, pronounced hyperplasia of cholangiocytes and *spongiosis hepatis* were also detected in treated guinea fowls. The established alterations of hematological and biochemical parameters included thrombocytopenia and an increase of the levels of major liver enzymes and were related to the hepatocarcinogenesis. In addition, changes in the leukogram (leukocytosis, lymphocytosis and granulocytosis), as well as hypoproteinemia, hypoalbuminemia and hypoglycemia were observed.

Key words: in ovo tests, guinea fowl, hepatocarcinogenesis, N-nitrosodiethylamine, hematological and biochemical parameters.

Introduction

Animal experimentation is still one of the major approaches used for safety assessment of chemical substances intended for human or animal use. (Iatropoulos et al., 2001; Pitot, 2007, Marone et al. 2014). With the adoption and implementation of the new Directive 2010/63 / EU of the European Parliament and the Council of EU on the protection of animals used for scientific purposes, sudies aimed at the development of novel alternative models and methods have been gaining an increasing importance. A number of *in vitro* and *in silico* models and methods for evaluation of the carcinogenic potential of the chemical substances have been developed (Anadon et al., 2014). Some of them are now included in the test panels used for chemical risk assessment. The importance of avian embryos as an alternative to the laboratory animals for studies on various pathological processes, including carcinogenesis, is rising steadily (Williams et al., 2014). Moreover, *in ovo* tests for mutagenicity and carcinogenicity (using chicken, turkey or quail embryos) have been proposed and some of them have been subjected to validation studies (Enzmann and Brunnemann, 1997, Williams et al., 2011, Enzmann et al., 2013).

The aims of the present study are to investigate the nature of the liver lesions and some hematological and biochemical parameters in early stage of N-nitrosodiethylamine-induced hepatocarcinogenesis in guinea fowl.

Materials and methods

Avian embryos

Fertilized guinea fowl (*Numida meleagris*) eggs were obtained from birds of diseases-free flock in the animal-housing facilities of IEMPAM-BAS, Sofia, Bulgaria.

Chemical carcinogen and *in ovo* treatment

N-nitrosodiethylamine (NDEA, CAS № 55-18-5; Sigma-Aldrich) was diluted with sterile double distilled water. The *in ovo* carcinogen-treatment was performed as previously described (Enzmann and Brunnemann, 1997, Enzmann et al., 2013). Brefly, NDEA was administered as single dose of 0.2 mg/per egg, with an injection volume of 0.1ml. Control eggs were injected with an equal volume of the vehicle. The eggs were inoculated during the first hours of incubation. After sterilization of the injection site with 70% ethanol, the shell was pierced at the pointed end of the egg, using a needle. Test substance was inoculated into the egg albumen and the opening was sealed with paraffin. The eggs were incubated in an automatic rotating incubator and at the end of the incubation period were transferred to a hatcher.

Experimental birds

Twelve guinea fowls hatched from the treated and control eggs were used in the experiments and each group consisted of six birds. Standard fodder mixtures were used for feeding. Food and water were available *ad libitum*. The experimental birds were exsanguinated at the 45th day after hatching. All experiments were conducted in accordance to the ethical standards of the institutional and national guidelines for care and use of laboratory animals.

Histopathology

Liver samples were taken from the control and treated birds and immediately fixed in 10% buffered formalin for subsequent histopathological examination. Fixed tissues were routinely dehydrated, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Histopathological lesions were identified and documented with microscope Leica DM 5000 B.

Hematology

Venous blood was taken from the wing vein of the treated and control birds 30 days after hatching. Haematological parameters (WBC, 10^9/L; LYM, 10^9/L; MID, 10^9/L; GRA, 10^9/L; HGB, g/L; RBC, 10^{12}/L; HCT,%; PLT, 10^9/L) were measured in whole blood by Veterinary automatic hematology analyzer Hema Screen 18 LIHD 170, (Hospitex diagnostics – Italy).

Biochemistry

Biochemical parameters (total protein, g/L; albumin, g/L; alanine aminotransferase (ALAT), U/L; aspartate aminotransferase (ASAT), U/L; and gama-glutamyl transferase (GGT), U/L) were measured in the blood serum by a semi-automatic biochemical analyzer Screen Master LIHD 113, (Hospitex diagnostics – Italy) and reagent kits for biochemical analyses (Human – Germany).

Statistical analysis

All data are presented as mean values ± standard deviation. The statistical significance of the differences between the control and treatment groups was evaluated by SPSS 16.0 software package

(IBM Corporation) using one-way analysis of variance (ANOVA). Values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

Results

The histopathological examination shows presence of pre-neoplastic liver lesions in all birds treated with N-nitrosodiethylamine in the early stages of their embryonic development. These lesions were classified as foci of altered hepatocytes with an eosinophilic and basophilic phenotype (Fig. 1). In addition, prominent hyperplasia of cholangiocytes has been detected in liver samples from all experimental birds (Fig. 2). Pseudo-acinar structures and *spongiosis hepatis* (Fig. 3) were occasionally found in separate samples.

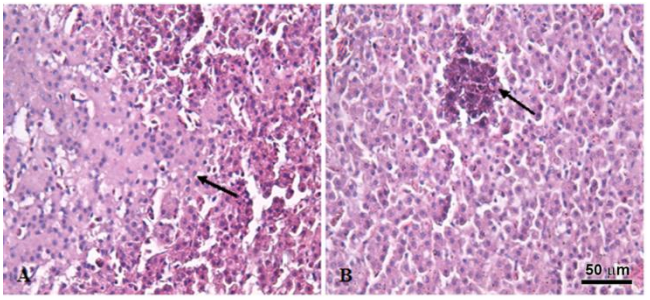


Figure 1: Light microscopy of FAHs in liver samples from guinea fowls, treated with NDEA. A) Focus of eosinophilic altered hepatocytes, H&E; B) Focus of basophilic altered hepatocytes H&E.

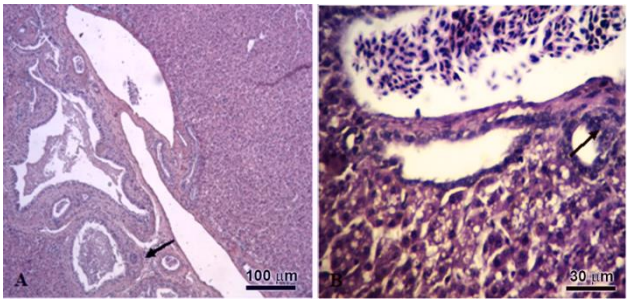


Figure 2: Light microscopy of altered bile ductules in liver samples from guinea fowls, treated with NDEA A) Hyperplasia of cholangiocytes, H&E; B) Pseudopapillary hyperplasia of cholangiocytes, H&E.

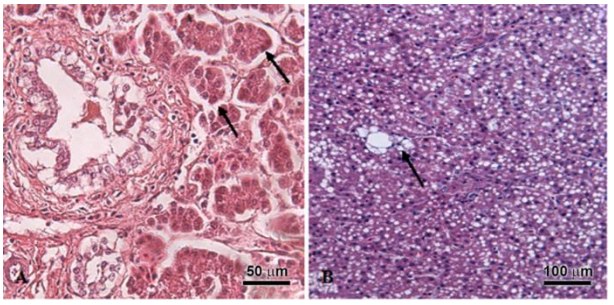
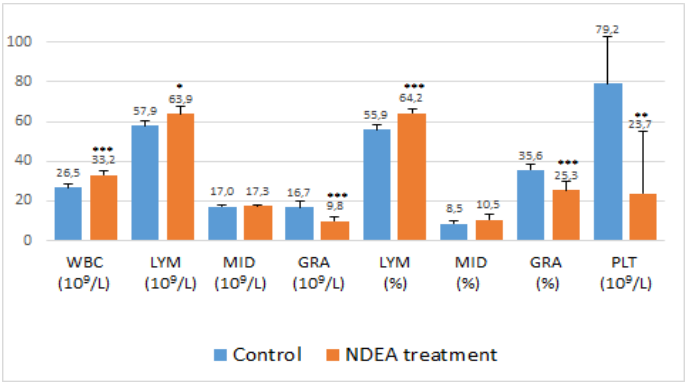


Figure 3: Light microscopy of pseudo-acinar structures (A) and *spongiosis hepatis* (B) (arrows) in liver samples from guinea fowls, treated with NDEA, H&E.

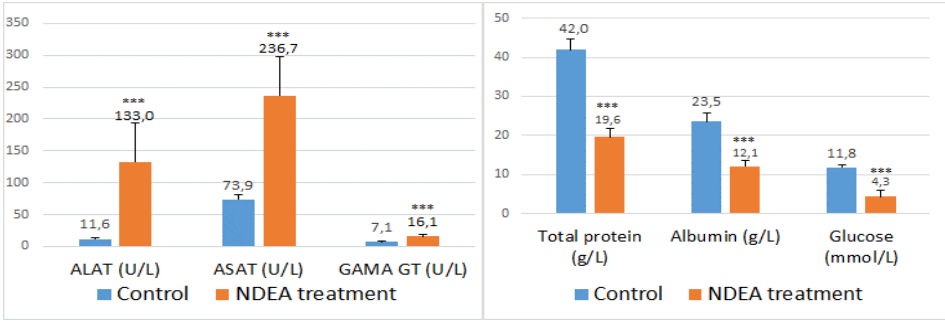
Hematological investigations of white blood cells count revealed a significant ($p \leq 0.001$) leukocytosis with lymphocytosis, accompanied by prominent neutropenia and thrombocytopenia (Fig. 4). The erythrocytes count of the birds from experimental group (2.24±0.05) where also significantly lower ($p \leq 0.001$) compared to red blood cells number in guinea fowls from the control group (3.18 ±0.17). A decrease in hemoglobin and hematocrit has been observed, however values did not reached statistical significance.



*Mean ± SE * p≤0.05; **p≤0.01; ***p≤0.001*

Figure 4: White blood cell and thrombocyte count of treated and control guinea fowls.

The results of the biochemical studies have shown statistically significant increase of the levels of the main liver enzymes - ALAT and ASAT, and a double increase of GGT activity ($p \leq 0.001$) (Fig. 5). In addition, prominent hypoproteinemia, hypoalbuminemia and hypoglycemia ($p \leq 0.001$) have been observed (Fig. 5).



*Mean ± SE * p≤0.05; ** p≤0.01; *** p≤0.001*

Figure 5: Blood serum biochemical profile of treated and control guinea fowls.

Discussion

Histophatology of the livers of 45 days-old guinea fowl, treated with NDEA in the beginning of their embryo development, has shown the presence of preneoplastic lesions, classified as foci of altered hepatocytes with eosinophilic and basophilic phenotype. Foci of altered hepatocytes (FAHs) represent the most prevalent form of hepatic preneoplasia observed in animals for a long time and more recently identified in human chronic liver diseases associated with, or predisposing to,

hepatocellular carcinomas (Bannasch et al., 2003, Su and Bannasch, 2003). A prominent hyperplasia of cholangiocytes was also detected in the liver sections from all experimental birds. In addition, formation of pseudo-acinar structures and *spongiosis hepatis* were occasionally found in some samples from experimental birds. Similar changes in turkey, quail and guinea fowl embryos exposed *in ovo* to N-nitrosodimethylamine, N-nitrosodiethylamine and N-nitrosomorpholine, have been previously described (Enzmann et al., 1992; 1995; 1996; Williams et al., 2011, Nikolov et al., 2015). The development of preneoplastic FAHs has been also found in the liver of chicken embryos after treatment with organic (Georgieva et al., 2011) and inorganic carcinogenic chemicals (Kril et al., 2011), as well as after experimental infection with avian oncogenic retroviruses (Georgieva et al., 2011). In fact, FAHs have been found in all animal species studied, including primates (Bannasch et al., 1997). The striking morphological and biochemical similarities in the cells of FAHs detected in experimental and human hepatocarcinogenesis favors the extrapolation of data obtained in experimental animals to humans (Williams et al., 2014).

In the present study, the liver lesions in guinea fowls hatched from NDEA-inoculated eggs we examined in order to determine whether the preneolaspic lesions observed in the guinea fowl embryos will progress to neoplasia during a 45-days post-hatching period. Our previous results have revealed the presence of hepatocellular carcinoma cells, with clearly expressed signs of malignancy in livers samples from 18-weeks old chikens, exposed *in ovo* to the same carcinogen. The results presented here indicate that the 45-days period was not sufficient for the development of neoplastic alterations in the livers of the experimental birds.

The biochemical profile of the treated and the control birds revealed marked increase of the levels of main liver enzymes (ALAT, ASAT and GGT). Aspartate aminotransferase, alanine aminotransferase and gama-glutamyl transferase are known as a sensitive and reliable indicators for liver failure in other bird species (Harisson et al., 1994). The increased ALAT activity is considered more specific marker for liver damage than the ASAT levels due to the higher concentration of this enzyme in the liver and its longer half-life in blood plasma. In addition, hypoproteinaemia, hypoalbuminemia and hypoglycaemia have been detected. The hematological parameters have shown leukocytosis with lymphocytosis, accompanied by significant neutropenia, thrombocytopenia and erytropenia. The latter results correspond well with the histopathological findings in the livers of the experimental birds, namely the significant alterations of hepatocyte morphology and confirm the marked hyperplasia of the cholangiocytes.

Conclusion

In ovo application of N-nitrosodiethylamine includes preneoplastic and hyperplastic lesions in the livers of guinea fowl hatched from inoculated eggs.

The results of the hematological and biochemical studies complement well the observed morphological changes in the liver.

The established hypoalbuminemia, relative anemia and hypoglycemia show not only changes in the main liver functions, but also represent an essential part of the paraneoplastic syndrome, accompanying hepatocarciogenesis.

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EFFECTIVENESS OF THE GEL CONTAINING CHELATED COPPER AND ZINC FOR TREATING DIGITAL DERMATITIS IN DAIRY COWS

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ABSTRACT

The aim of this study was to evaluate the benefit of topical treatment of copper and zinc chelates to cure digital dermatitis (DD) in dairy cows. The study was conducted between December 2016 and Februari 2017 and involved 38 dairy cows from a dairy farm on which DD was endemic. Lesions were scored in the milking parlor on both hind feet using M0 to M4 scoring system using Relun's method with a headlamp and a mirror on a spatula. The cows were divided into two experimental groups and one control group. Group 1 received topical treatment with gel containing cooper and zinc, on the zero, second and fifth days with bandaging. Group 2 received the same treatment without bandaging. Control group was used to assess the possible effects of surgical debridement and bandaging. The percentage of the lesions recovered from DD in experimental groups was 86.4% and 46.1%. In control group the percentage of the recovered animals was 16.7%. The cure rate of gel containing chelated copper and zinc and bandaging is an excellent option to treat DD.

Key words: digital dermatitis, copper and zinc gel, dairy cows.

Introduction

Digital dermatitis (DD) is a contagious, multifactorial disease involving environmental, management and microbial factors. Clinically, it is manifested with lameness, as well as with restricted or diffuse painful skin lesions in the region of the digit, particularly just above the heel. The disease was first described in 1974 in Italy by Cheli and Mortellaro. Nowadays digital dermatitis has spread all over the world and affects intensively managed dairy cows (Blowey and Sharp, 1988). In our country Lyutskanov and Marutsov (2010) reported prevalence of 18.3% (from 10.3 to 34.0%).

DD is associated with huge economic losses due to decreased reproductive performance, milk production, increased risk for culling in addition to high treatment costs (Bruijnis et al., 2010; Ettema et al., 2010). In addition untreated lesions are very painful and erode hoof and digital skin (Read et al., 1992).

At present etiology is associated with a number of different microbial agents – *Bacteroides spp., Prevotella spp., Fusobacterium spp., Treponema spp., Campylobacter spp.* etc. The infectious factor only is not however sufficient to trigger a clinical disease, but a combination of microbial agent and risk factors must exist (Blowey and Sharp, 1988; Bassett et al., 1990; Frankena et al., 1991; Lyutskanov and Marutsov, 2010). Risk factors related to a high prevalence of DD lameness are cleanliness of the environment, wet floors, replacement stock purchase, restricted grazing, parity, early lactation, heel horn erosion, regular hoof trimming and use of hoof bathing (Rodriguez-Lainz et al., 1996; Wells et al., 1999; Toholj et al., 2008; Barker et al., 2009).

Classical therapy involves treatment with topical antibiotics, which supports the hypothesis of a bacterial etiology (Brizzi, 1993; Graham, 1994; Guard, 1995; Guterbock and Borelli, 1995). Non-antibiotic products have been also reported to be efficacious (Britt and McClure, 1998; Hernandez et al., 1999).

The purpose of this study was to evaluate the benefit of topical treatment of copper and zinc chelates to cure digital dermatitis (DD) in dairy cows.

Materials and methods

Animals. The study was conducted on a dairy farm located in Southern Bulgaria between December 2016 and Februari 2017. The farm was chosen because of its history of digital dermatitis related lameness. Dairy cows of the Holstein-Friesian breed were housed in a concrete floor, free stall barns that were bedded with straw. Concrete alleyway floors were cleaned by automatic scrapers at least five times daily. Cows were milked two times daily using 2 x 12 side-by-side milking parlor. All lactating cows were fed a Total Mixed Ration (TMR) delivered twice daily. Water was freely available at all times. Hoof trimming are conducted biannual on a regular six monthly basis at dry off and around 120 -160 days in milk (DIM).

Foot examination. At the beginning of the study the rear feet were flushed with medium-pressure tap water in the milking parlor and scored for digital dermatitis using Relun's method with sensitivity of 0.90 and specificity of 0.80 (Relun et al., 2011). Lesions were scored after milking on both hind feet using M0 to M4 scoring system (Döpfer et al., 1997), where M0 is claw free or DD lesion; M1 is a lesion up to 2 cm, generally not painful; M2 is classical ulcerative stage, with diameter > 2 cm and painful to digital pressure; M3 is healing stage covered by a scab; M4 is the chronic stage and is characterized by dyskeratosis or proliferation of the surface that is generally not painful. Further, a new variant of M4 lesions is described by Berry (2012) as stages M4.1 characterized as a chronic lesion with a new M1 lesion within its perimeter.

Study design and treatments. All cows with erosive M2 lesions were included in the trial and randomly divided into two treatment groups (G1 and G2, each group with an equal number of 13 cows) and a control group (CG with 12 cows). Cows that received antibiotic treatment for other reasons like metritis or mastitis were excluded from the trial.

All DD lesions were evaluated in a hoof trimming chute. The data recorded were the affected claw, the size, type and location of ulcers, and the level of pain in response to firm pressure with a finger. All lesions were cleaned with water and brush and then dried with a paper towel. Surgical treatment involved removal of all necrotic tissue and if necessary prophylactic functional hoof trimming was done. In group G1, 5g of the gel was applied with a brush on the zero, second and fifth days followed by bandaging in the first two treatments. Group G2 received the same treatment without bandaging, while the control group surgical debridement and bandaging. The gel used for the treatment was Intra Hoof fit gel® (IntraCare), with active ingredients copper chelate (40 mg/g) and zinc chelate (40 mg/g), aloe vera and alcohol.

Cows from different groups were restrained in a hoof-trimming chute and examined 18 days after the beginning of treatment. The degree of healing was determined as transition of DD lesions from M2 to M3 stage.

Statistical analysis. Statistical analysis was performed using Statistica 5.0 (StatSoft, USA). Significances were tested using the T-test of dependent samples, with confidence intervals 95 and 99%.

Results

In Group 1 the combination of cleaning the hoof and surrounding skin, treatment with gel containing chelated copper and zinc and bandage resulted in the most significant recovery of DD

lesions 86.4% (Table 1). The recovery in Group 2 was significantly lower 46.1%. In control group the percentage of the recovered animals was the lowest with 16.7%.

Table 1: Efficiency of treatment protocols for digital dermatitis.

Groups	Treatment	№ of the cows	% of recovered cows	% of non recovered cows	Significance P
G1	gel + bandaging	13	86.4	13.6	<0.01
G2	gel	13	46.1	53.9	<0.05
CG	bandaging	12	16.7	83.3	>0.05

Discussion

Measures implemented to control DD in dairy farms are related to management of risk factors, using regular footbaths and individual treatment of affected animals. Treatment is one of the ways to control DD in dairy herds it is because M2 lesions are proven to be highly contagious. Various treatment schedules often applied empirical mainly rely on antibiotics (Hernandez et al., 1999; Laven, 2006). Classical treatment relies on oxytetracycline in the form of a topical spray with different types of dressing (Berry et al., 1996; Hernandez et al., 1999; Manske et al., 2002). In most studies of topical antibiotic treatments effectiveness is between 60 and 70%. Non-antibiotic products such as formaldehyde, copper sulphate, peroxide-based products, peracetic acid, zinc sulphate, etc., have also been reported as effective (Britt and McClure, 1998; Read and Walker, 1998; Hernandez et al., 1999; Laven and Hunt, 2002; Stevančević et al., 2009).

Results of this study indicate that the topical application of the gel containing copper and zinc chelates with a bandage is an excellent option for the treatment of DD which is consistent with studies by other authors (Holzhauer et al., 2011). A study of a similar product Hoof fit spray (called Repiderma®) of the same company in seven herds showed an average healing rate of 89.15% (Versteegen, 2014). It is assumed that the composition of Hoof fit gel is the same as Hoof fit spray which is why the results of both trials are similar. Treatment with the same product without bandaging was moderately effective. In this case risk factors as housing system, type of floor and environmental hygiene were not investigated. Deficiencies in environmental hygiene and the possible impact of moisture, feces and urine of the effect of healing support necessity of using a bandage. In this trial, we confirmed that bandaging hoofs after surgical debridement, without the use of topical antimicrobial agent is insufficient to cure DD lesions.

Conclusion

Copper and zinc have an antimicrobial effect and assist in the healing of the lesion. The use of Intra Hoof fit gel under bandage is a good option to treat digital dermatitis and alternative to combat antibiotic resistance.

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CLINICAL AND HEMATOLOGICAL STUDIES IN SHEEP WITH SUBCLINICAL AND CLINICAL KETOSIS

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ABSTRACT

The investigation was performed on 136 ewes, 106 (Lacaune) and 30 (Mouton Charollais). The ewes were divided in three groups: pregnant; recently lambed and lactating. Sheep of the three groups, we performed a chemical blood test to determine the level of β-hydroxybutyrate. Clinical examination was performed on all animals by routine methods of clinical diagnostics. In haematological studies were monitored parameters of red and white blood count.

The investigation of the sheep from the three groups with subclinical ketosis (SCK) showed that clinical parameters varied within the reference ranges. The studied red and white blood cell indices in Lacaune sheep with SCK and clinical ketosis (CK) indicated erythropenia, oligochromemia, reduced haematocrit, leukocytosis and lymphocytosis. The meat breed Mouton Charollais did not exhibit any changes in studied haematological parameters. The sheep from the dairy breed Lacaune were affected with SCK and CK during the pregnancy, parturition and lactation while those from the meat breed Mouton Charollais did not suffer.

Key words: ketosis, β-hydroxybutyrate, clinical and hematological parameters, ewes.

Introduction

In sheep, ketosis is observed during the last 6-4 weeks of pregnancy (pregnancy toxaemia) and after lambing (Van Saun, 2000). The condition is caused by the negative energy balance (NEB), occurring from increased demand for glucose of developing fetuses (Van Saun, 2000; Schlumbohm and Harmeyer, 2008). Some predisposing factors are the number of body weight of fetuses, body condition of the dam, number of lactations, age and breed, feeding, stressors etc. (Hefnawy et al., 2011).

Subclinical ketosis (SCK) is a pathological state associated to increased systemic levels of ketone bodies but without the ketosis-specific clinical signs (Duffield et al., 1997). From health and economic point of view, the SCK leads to lower milk yield (McArt et al., 2013), reproductive disorders, prolonged duration and severity of mastitis (Suriyasathaporn et al., 2000) and/or clinical ketosis (LeBlanc et al., 2005).

Clinical ketosis (CK) is manifested with loss of appetite, dehydration, depression, decreased milk secretion. Affected sheep stay away from the herd, exhibit purposeless movements, and often press their heads against the feeder or wall. They show weak chewing muscles, seizures and tremor of the head and neck, opisthotonus, grinding with teeth, amaurosis, ataxia, sternal recumbency and loss of the wool along the entire length of the back, liquid faeces, coma and death (Henze et al., 1998; Van Saun, 2000). Kabakci et al. (2003) and Balikci et al. (2009) established that sheep carrying twins, toxicosis was manifested with neurological signs (convulsions and vision disturbances) as compared to sheep pregnant with a single foetus which did not show such signs. A scent of acetone was perceived in exhaled air in all sheep with pregnancy toxaemia, and in some sheep with CK – bradypnea (8 min^{-1}), abdominal breathing, hypothermia (36^0C) and tachycardia (120 min^{-1}). Barakat et al. (2007) and Balikci et al. (2009) did not establish changes in body temperature, respiratory and heart rates in sheep with SCK and CK. In previous studies of ours

(Binev et al., 2014; Marutsova et al., 2015) in goats and cows with SCK, significant alterations in their clinical status were not observed, and in cows with CK – gastrointestinal signs.

Hematological and blood biochemical analysis results are inconsistent and not always reliable as markers of metabolic and clinical status disturbances in sheep with CK and SCK. Barakat et al. (2007) and Gupta et al. (2008) found out erythropenia in goats and sheep with ketosis, while Iriadam (2007) did not observe any substantial changes in hemoglobin content in lactating goats. Iriadam (2007) and Abba et al. (2015) reported leukocytosis accompanied with lymphopenia, eosinopenia and monocytopenia in goats with SCK during the first three weeks of the lactation.

The main ketone bodies in blood are BHBA, acetoacetic acid (AcAc) and acetone. A number of researchers (Herdt, 2000; Sordillo and Raphael, 2013) outline BHBA as a parameter for evaluation of NEB and lipolysis extent in dairy animals and a primary numerical parameter of ketosis, as its concentrations define the states of SCK and CK.

Various threshold blood BHBA levels in sheep are commented in the literature. Lacetera et al. (2002); Balikci et al. (2009) and Anoushepour et al. (2014) accept BHBA concentrations up to 0.8 mmol/l as normal; those from 0.8 to 1.6 mmol/l – indicative for SCK and those over 1.6 mmol/l – for CK. In this study, we used the same threshold values.

The aim of the present study was to perform a comparative evaluation of the changes in clinical parameters, hematological indices and blood BHBA in two sheep breeds (one dairy and one meat-type) with SCK and CK.

Materials and methods

Animals. A total of 136 ewes (2nd and 3rd lactation), 106 from the dairy breed Lacaune with 200 l annual lactational yield, average weight 60–80 kg, and 30 from the meat breed Mouton Charollais weighing 70–100 kg were included in the study.

Experimental design. The sheep from studied sheep farms were divided into three groups according to their physiological condition: group I – pregnant sheep (between pre-partum days 15 and 0); group II – recently lambed (from postpartum days 0 to 15) and group III – lactating (from postpartum days 30 to 45). Blood chemical analysis of BHBA concentrations was performed in all sheep in order to classify them as control (C, BHBA <0.8 mmol/l), affected with SCK (BHBA from 0.8 to 1.6 mmol/l) and CK (BHBA >1.6 mmol/l). Target groups of sheep from both breeds were reared under equal conditions and fed the same ration.

Group I from the dairy breed Lacaune included 45 animals – 14 healthy (control), 8 with SCK and 23 with CK. The second group (n=30) comprised 8 healthy controls, 10 with SCK and 12 with CK. The third group consisted of 31 sheep (8 healthy, 11 with SCK and 12 with CK).

The three groups of the meat breed Mouton Charollais included 10 animals each. All of them did not exhbit blood BHBA concentrations indicative for either SCK or CK, e.g. they were healthy.

Clinical investigation. All sheep were submitted to examination of the rectal body temperature, heart rate, respiratory and rumen contraction rates using routine clinical diagnostic procedures.

Blood samples and analyses. Blood samples were collected through puncture of the jugular vein using sterile 21G needles and vacutainers with K_2EDTA (3 ml, Biomed, Bulgaria). Samples were obtained in the morning before feeding and were stored and transported at 4^0C. Analysis was performed within 24 hours after sampling. Blood BHBA concentrations were determined in situ using a portable Xpress-I system (Nova Biomedical, UK). The following indices were determined:

RBC (10^{12}/l), HGB (g/l), HCT (%), MCV (fl), MCH (pg), MCHC (g/l), RDW (%), RDWa (fl), WBC (10^9/l), PLT (10^9/l) and MPV (fl). The parameters of the Differential Blood Count – LYM (10^9/l), MON (10^9/l) and GRA (10^9/l). Hematological investigations were analyzed on an automated analyser Exigo EOS Vet (Boule Medical AB, Sweden).

Statistical analysis. Statistical analysis was done with Statistica 6.0, StatSoft, Inc. (USA, 1993) and ANOVA test. Results were presented as mean (x) ± standard deviation (SD). The level of statistically significance was $p < 0.05$.

Results

The results from the clinical investigations in the three groups of Lacaune sheep are shown in Table 1. The studied clinical parameters in control and SCK sheep from group I, II and III were within the reference ranges. The clinical exam of pregnant sheep (group I) with CK revealed reduced appetite, enhanced thirst and depression. In some sheep (21.7%), locomotor disturbances of progressing nature followed by lying down, tremor of the head and neck, and bruxism were present. As the disease progressed, ataxia, sternal recumbency, abortions, coma and death were observed. Sheep from the second and third group affected with CK exhibited polypnea, tachycardia, rumen atony, hind limb locomotor weakness, weakness of chewing muscles, salivation, seizures with clonic head movements, liquid faeces and dehydration with reduced skin elasticity.

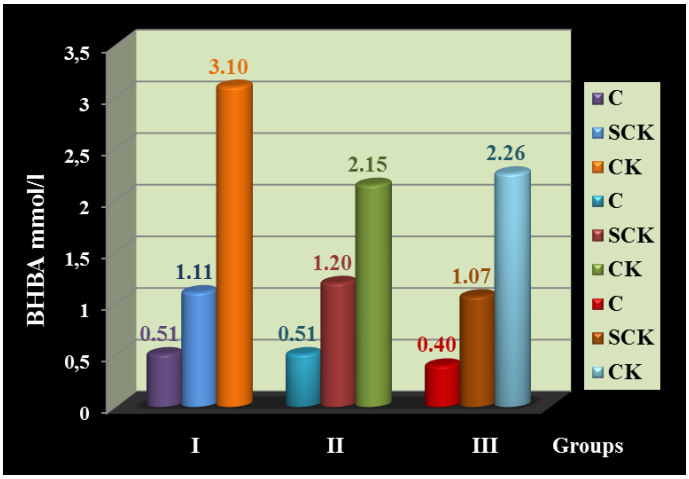
Table 1: Changes in clinical parameters of Lacaune ewes from first, second and third group with subclinical and clinical ketosis (average mean ± standard deviation).

Parameters / Groups	Study period (days)								
	Group 1 (15–0 days prepartum)			Group 2 (0–15 days postpartum)			Group 3 (30–45 days postpartum)		
	C	SCK	CK	C	SCK	CK	C	SCK	CK
Temperature (°C)	39.6±0.5	38.7±0.8	38.5±0.3	39.3±0.7	38.8±0.5	38.6±0.8	39.5±1.0	39.2±1.2	39.0±0.9
Heart rate (min^{-1})	76.5±0.8	78.8±0.6	81.2±1.2	74.8±0.7	76.4±0.5	79.5±0.8	75.8±0.6	77.1±0.8	79.3±0.8
Respiratory rate (min^{-1})	19.7±0.7	25.8±0.6	28.2±0.9	22.6±0.5	24.8±0.7	27.4±0.5	21.3±0.5	28.4±0.6	30.0±0.4
Rumen contractions (min^{-5})	11.6±0.6	10.4±0.3	9.2±0.2	12.6±0.7	9.8±0.5	9.2±0.4	12.2±0.6	9.54±0.3	8.0±0.5

Legend: $^a p<0.05$; $^b p<0.01$; $^c p<0.001$; 1-vs. control groups; C – control group; SCK – with subclinical ketosis; CK – with clinical ketosis

The physical examination of Mouton Charollais sheep from the three groups (pregnant, recently lambed and lactating) did not show any deviations from norms.

Blood BHBA analysis in the three control groups of Lacaune sheep were within the reference range (Fig. 1). Sheep from the first group with SCK had statistically significantly higher BHBA concentrations than controls: 1.11±0.24 mmol/l ($p<0.001$); those from group II: 1.20±0.28 mmol/l, ($p<0.001$), and from group III – 1.07±0.24 mmol/l ($p<0.001$) (Fig. 1). Sheep from groups I, II and III with CK had BHBA levels in blood substantially higher than both controls and SCK – 3.10±0.60 mmol/l ($p<0.001$), 2.15±0.63 mmol/l ($p<0.001$) and 2.26±0.23 mmol/l ($p<0.001$) respectively (Fig. 1).



(C-control group; SCK-with subclinical ketosis; CK-with clinical ketosis)

Figure 1: Changes in blood β-hydroxybutyrate (BHBA) levels in Lacaune ewesfrom first, second and third group with subcl inical and clinical ketosis.

Mouton Charollais sheep did not exhibit blood BHBA higher than 0.8 mmol/l, i.e. no SCK and CK was present (0.44±0.08 mmol/l in group I, 0.40±0.17 mmol/l in group II and 0.18±0.08 mmol/l in group III).

The haematological analysis of red and white blood cell picture in the three control Lacaune groups showed that all parameters were within the respective physiological ranges (Table 2).

In SCK sheep from the first group, erythrocyte counts, hemoglobin and hematocrit decreased insignificantly vs controls. Leukocyte and lymphocyte counts increased considerably and attained 11.13±2.3x109/l ($p<0.05$) and 4.85±1.0x109/l ($p<0.05$). In sheep with SCK from groups II and III, significant changes consisting in erythropenia, oligochromemia, reduced hematocrit, leukocytosis and lymphocytosis were observed. The other red blood parameters were within the reference limits (Table 2). Hematological analysis data in Lacaune sheep with CK from the three groups showed more pronounced changes, even more significant vs controls – erythropenia, oligochromemia, reduced hematocrit, leukocytosis (Table 2).

There were no statistically significant differences than reference values in red and white blood cell parameters in any of studied Mouton Charollais sheep.

Table 2: Changes in the hematological parameters in Lacaune ewes from first, second and third group with subclinical and clinical ketosis (average mean ± standard deviation).

Parameters / Groups	Study period (days)								
	Group 1 (15–0 days prepartum)			Group 2 (0–15 days postpartum)			Group 3 (30–45 days postpartum)		
	C	SCK	CK	C	SCK	CK	C	SCK	CK
RBC (x10^{12}/l)	8.83±2.1	7.88±0.5	6.39±2.3 ^{1b}	11.69±3.4	8.48±3.0 ^{1c}	8.03±0.4 ^{1c}	9.44±3.4	7.73±1.7 ^{1b}	6.96±1.1 ^{1c}
HGB (g/l)	113.00±8.9	98.66±5.5	87.66±8.9 ^{1b}	149.00±8.7	92.00±8.5 ^{1b}	88.00±4.1 ^{1c}	116.80±5.3	86.16±4.9 ^{1b}	80.00±5.5 ^{1b}
HCT (%)	30.10±4.3	25.51±1.6	20.50±3.2 ^{1a}	40.66±3.9	30.66±3.0 ^{1a}	23.00±1.4 ^{1c}	31.66±2.1	20.65±3.5 ^{1b}	19.66±1.7 ^{1b}
MCV (fl)	33.95±2.9	33.70±1.9	35.16±0.4	35.06±1.9	35.36±0.9	34.65±3.1	33.32±2.4	31.80±2.8	33.86±1.5
MCH (pg)	12.76±0.8	12.56±0.5	12.86±0.1	12.70±0.5	12.88±0.4	12.85±0.07	12.30±0.8	11.98±0.7	12.63±0.2
MCHC (g/l)	377.0±15.2	373.33±12.2	366.66±3.5	364.5±12.0	363.16±8.6	351.5±3.5	369.8±4.5	372.75±0.9	373.33±10.6
RDW (%)	21.55±1.4	20.81±0.8	20.03±0.1	20.90±0.2	20.88±0.5	20.65±0.07	21.54±0.9	21.42±1.0	21.90±1.0
RDWa (fl)	20.03±1.4	19.30±0.8	20.00±0.4	20.40±1.6	20.80±0.7	21.65±0.4	19.46±1.2	19.45±1.4	19.90±0.8
WBC (x10^{9}/l)	8.88±1.1	11.13±2.3 ^{1a}	12.70±3.3 ^{1b}	8.13±1.9	12.50±2.8 ^{1b}	13.30±1.7 ^{1c}	7.46±1.4	10.05±1.6 ^{1b}	13.33±1.3 ^{1c}
LYM (x10^{9}/l)	3.90±0.5	4.85±1.0 ^{1a}	4.63±0.4 ^{1a}	3.80±0.1	5.77±0.9 ^{1b}	6.60±0.2 ^{1c}	4.06±0.8	5.97±0.6 ^{1b}	6.25±0.6 ^{1c}
MON (x10^{9}/l)	0.63±0.1	0.83±0.1	0.86±0.2	0.60±0.1	0.68±0.2	0.55±0.07	0.66±0.1	0.60±0.2	0.90±0.2
GRA (x10^{9}/l)	4.35±0.6	5.45±1.3	6.20±0.6	3.70±1.0	5.07±0.2	5.00±0.2	2.74±0.7	3.41±1.0	4.53±1.3
PLT (x10^{9}/l)	197.30±18.2	173.80±13.3	254.66±26.3	291.30±22.5	394.50±19.2	312.00±28	335.40±28.2	274.00±18.6	489.00±32.8
MPV (fl)	5.51±0.4	5.53±0.4	5.36±0.4	5.25±0.07	5.05±0.3	5.20±0.1	5.14±0.3	5.20±0.5	5.13±0.1

Legend: $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$; 1-vs. control groups; C – control group; SCK – with subclinical ketosis; CK – with clinical ketosis

Discussion

The observed field cases of ketosis in high-yielding dairy sheep conformed the reports of many authors (Van Saun, 2000; Schlumbohm and Harmeyer, 2008), that this condition was provoked by overfeeding with concentrate feed and deficiency of easily digestible carbohydrates in diets especially during the late gestation and early lactation, which are accompanied by substantial changes in the maternal organism.

With regard to its clinical manifestation, ketosis is defined as subclinical and clinical by numerous authors (Herdt, 2000; Gordon et al., 2013); we also agree with their opinion. Dairy sheep breeds (Lacaune) could be affected with SCK and CK during all physiological states (pregnancy, lambing, lactation). The sheep with SCK did not show any deviation in general clinical parameters except for rumen movements, whose strength and frequency decreased at a various extent and were close to the lower limits secondary to alkalization of rumen and duodenal content by increased ammonia and biogenic amines concentrations as reported elsewhere (Kabakci et al., 2003; Balikci et al., 2009). The established changes in the general clinical status in sheep with CK were localized in the alimentary, respiratory and nervous systems. Enhanced heart and respiratory rates in sheep points at functional damage of the cardiovascular and respiratory systems from one part, and could be due to rumen metabolites subsequently to CK on the other. Respiratory and heart failure reflect on the color of visible mucous coats that from pale rose-red become diffusely red and before the lethal outcome, have an icteric tint. The rumen movements in the course of development CK decreased both in strength and frequency compared to control groups. These results are in line with other reports (Kabakci et al., 2003; Balikci et al., 2009).

A main chemical blood parameter used as an early marker for SCK and CK in ruminants is blood BHBA concentration (Lacetera et al., 2002). This was confirmed by our studies as well. We found out that the sheep from the dairy breed Lacaune suffered from SCK and CK during the pregnancy, lambing and lactation when blood BHBA was about 1.10–1.20 mmol/l (in SCK) and 2.15–3.10 mmol/l (in CK). Sheep from the meat breed Mouton Charollais were not affected with both forms of ketosis as indicated by the lack of individual blood BHBA concentrations over 0.8 mmol/l. High BHBA levels in blood are a compensatory mechanism in response to occurring carbohydrate deficiency and Krebs cycle inhibition (Ingvartsen, 2006). In cases of excessive lipolysis accompanied by production of large acetyl CoA amounts, the tricarboxylic acids cycle is not capable to convert entirely fatty acids. Consequently, acetyl CoA is metabolized to acetoacetate, which is reduced to BHBA through BHBA dehydrogenase or is spontaneously decarboxylated to acetone (Roche et al., 2013). The increased BHBA concentration in blood reveals the incomplete oxidation of non-esterified fatty acids (NEFA) in the tricarboxylic acid cycle at the time of NEB (Grummer, 1993; Doepel et al., 2002).

The established erythropenia, resp. oligochromemia and reduced hematocrit in sheep with SCK and CK could be attributed to the body NEB during pregnancy, lambing and intensive lactation. The negative energy balance activates the function of adrenal glands with resulting increase in catecholamines, cortisol and endorphins levels (Antonov, 2000; Găvan et al., 2010). These suggestions are in agreement with data reported in sheep (Gupta et al., 2008), goats (Abba et al., 2015) and cows (Belić et al., 2010). The leukocytosis and lymphocytosis in the three groups of sheep with SCK and CK could be associated to the presence of acute and chronic inflammations (mastitis, endometritis, etc.) during the postpartum period (Găvan et al., 2010). The enhanced lipolysis,

ketogenesis and hypoglycemia could contribute to erythropenia, leukocytosis with lymphocytosis (Belić et al., 2011), or these could be a sequel to stress manifested with increased glucocorticosteroids (cortisol) (Burton et al., 2005; Abba et al., 2015). Our data were therefore comparable to results reported by others (Hoeben et al., 1999).

Conclusion

In conclusion, the values of studied clinical parameters in the three groups of Lacaune sheep with SCK were within the reference ranges, while sheep affected with CK exhibited deviations from the general and specific clinical status. The primary blood biochemical marker of the form of ketosis in sheep is BHBA. Its amount in blood defined pregnant and recently lambed sheep as being affected with either SCK or CK. The sheep from the dairy Lacaune breed suffered from SCK and CK during the pregnancy, lambing and lactation, while those from the meat breed Mouton Charollais were healthy (BHBA <0.8 mmol/l). The studied red blood cell parameters in Lacaune sheep with SCK and CK showed erythropenia, oligochromemia, reduced hematocrit, leukocytosis and lymphocytosis.

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LEVELS OF LEAD IN TISSUES OF MALLARDS (*Anas platyrhynchos,* L) EXPERMENTALLY EXPOSED TO SHOT PELLETS

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ABSTRACT

The paper presents for determination of lead in the liver, kidney, breast muscles and humerus of mallards (*Anas platyrhynchos*, L), treated orally with lead shot pellets. The results obtained show significant elevation tissue concentrations of lead in relation to the control values. The highest values were established in the humerus, followed by the kidneys, liver and breast muscles.

Key words: mallards, lead, hunting pellets, tissues, toxicological analysis.

Introduction

Waterfowl are exposed to the toxic effects from lead due to the ingestion of lead pellets from the bottom of the natural reservoirs in areas with intense hunting activities. The lead pellets used in hunting activities contaminate wetlands worldwide as their density in many places in the upper 20 cm of sediment is over 100 pellets /m2 (Mateo, 2009). Lead acts at a molecular level and may lead to a number of toxic effects which have been well described in waterfowl (Beyer et al., 1988; Pain et al., 2009).

According to Sanderson and Bellrose (1986), the ingestion of 1 or 2 pellets can be fatal, accompanied by morphological and functional changes in the birds and a loss of 30–50% of their normal body weight. Lead concentrations are highest after direct absorption into the bloodstream, after which the lead accumulates in the kidneys and liver for days or months, and if the process becomes chronic, it is then deposited in the bones with the possibility of a lifetime exposure (De Francisco et al., 2003). The monitoring of the poisoning in waterfowl is difficult due to their inaccessible habitat, mobility and, last but not least, the fact that the poisoned birds become an easy prey for predators and vultures. Making a definitive diagnosis of the poisoning requires specialized laboratory analyzes.

The purpose of the current study is the quantification of lead in liver, kidney, breast muscles and humerus of mallards, experimentally treated with lead pellets in various doses.

Material and methods

Sixteen clinically healthy mallards (Anas platyrhynchos) aged between 9 and 12 months were evenly divided (n = 4) in four groups and housed in separate aviaries. After a 7-day adaptation period the birds were treated orally once with lead pellets #3 (medium weight 0.267 g), as follows: I group –

3 lead pellets; II group – 2 lead pellets; III group – 1 lead pellet. The ducks from the IV group were used as a negative control.[1]

The survey was conducted between 23.02.2016-28.03.2016, in the Laboratory of Ecology and technical tests "Akvateratest" Sofia. The samples were analyzed by the methods of inductively coupled plasma spectrometry mentioned above. The spectrometers Varian Vista-MPX CCD Simultaneous ICP OES, Varian Australia, and Plasma Quant MS S-NR 105000-AQ032, Analytik Jena A G, Germany were used.

The analysis and statistical processing of the data were performed by the computer program SPSS 19.0. The data is expressed as mean plus standard error. In this study the assessment is made with guaranteed probability 0.95 (significance level $\alpha = 0,05$), where $p < 0,05$ was adopted as the lowest level of statistical reliability.

Results

During the experiment, the severity of clinical signs and pathological lesions correlated with the levels of lead in soft tissues and bones, and were proportional to the dosage of the applied lead pellets in the mallards tested.

One of the earliest clinical signs to appear was the reduced food intake. Either the appetite of the affected bird dropped completely or the food consumption decreased to a level below the minimum nutritional requirements which lead to a progressive loss of weight. The characteristic bright greenish diarrheic feces occur as early as the seventh day after the mallards were dosed with factory lead pellets #3. A few days before their death a high degree of cachexia, photophobias, vocal changes, loss of appetite and completely altered motor function with paralysis of the limbs were registered, along with body weight loss between 25 and 50% in the experimental mallards.

Following the death of the mallards, the main macroscopic findings included cachexia, loss of body fat, bright green coloration in the area of the cloaca, slightly enlarged, yellowish colored liver and enlarged and filled with content gallbladder. The mucosa of the muscular stomach was greenish-gray with the presence of ulcerative lesions where the lead pellets could be found. In the experimental animals of I and II group the weight of the lead pellets had dropped averagely by 25% from the initially measured. The most typically expressed pathomorphological changes are detected in the liver and kidneys where acidophilus intranuclear inclusions in hepatocytes and proximal renal tubular cells are observed.

From the toxicological analysis is observed repeatedly increased content of lead in the liver, kidney and the humerus in experimentally treated mallard ducks, compared with the control group (Table 1).

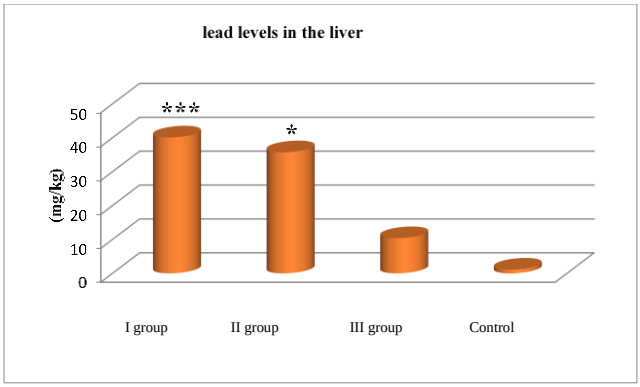
[1] This study was approved by the Commission for animal welfare at the Faculty of Veterinary Medicine, University of Forestry - Sofia (Permit Number: № 80, valid until 2018 for educating students and conducting scientific research in veterinary medicine) and is conducted in compliance with EU and the national legislation.

Table 1: Lead content in biological material from the liver, kidney, breast muscle and humerus in mallards after experimental application of lead ammunition.

Material / Group	Liver mg/kg	Kidney mg/kg	Breast muscle mg/kg	Humerus mg/kg
I n = 4	40.100 ± 1.597***	65.203 ± 7.067***	2.0575 ± 0.301**	86.695 ± 10.801***
II n = 4	35.725 ± 12.874*	53.607 ± 8.741**	1.450 ± 0.144*	68.907 ± 11.657**
III n = 4	10.375 ± 6.201	7.862 ± 2.780	0.650 ± 0.150	59.475 ± 13.169**
IV Control n = 4	1.097 ± 0.184	3.200 ± 0.286	0.500 ± 0.032	1.000 ± 0.161

Statistically significant difference compared to the control group:
**p < 0.05; **p < 0.01; *** p < 0.001*

There were statistically significant differences respectively, p <0,001, for the content of lead in the liver of the mallards from the first experimental group (three pcs. of lead pellets # 3) compared to the control group, and p <0,05 between the second test group (two pcs. of lead pellets # 3) compared to the control group. The results of the comparative analysis of lead levels in the liver, between the mallards in the third test group and the control group, did not show statistically significant differences, but graphically and quantitatively reflected significant difference of tenfold elevated level in the test subjects treated with one lead pellet # 3 (Fig. 1).



* *Statistically significant difference compared to the control group: p <0,05.*
*** *Statistically significant difference compared to the control group: p <0,001.*

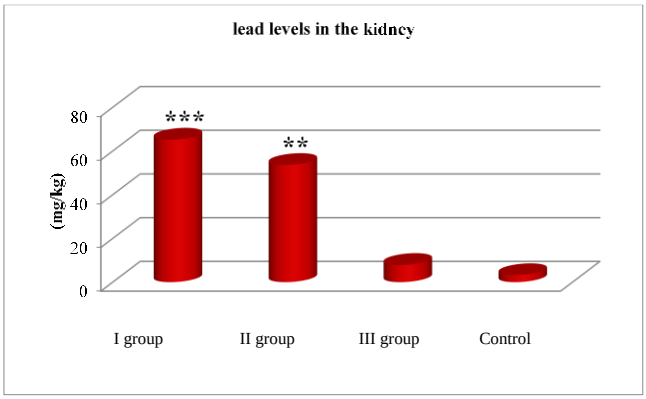
Figure 1: Lead concentrations in the liver of mallards

Regarding the content of lead in the kidney, the comparative quantitative analysis was similar to the data reported in the liver.

Again, we found absolute statistical reliability between the individuals treated with the highest dose in the first test group and the control group (Fig. 2).

In the mallards from II group the result confirmed that the administered dose of two lead pellets # 3 with a total mass of over 0,5 g resulted in an increase of the lead levels in the kidney over twenty times compared to the reported values in the control group (Fig. 2).

In the least dosed experimental mallards from the third test group, there are no statistically significant differences in comparison to the control group (Fig. 2).



*** Statistically significant difference compared to the control group: where p <0,01.*
**** Statistically significant difference compared to the control group: where p <0,001.*

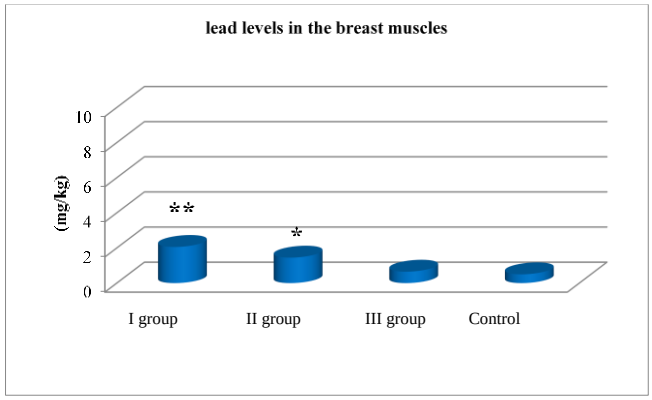
Figure 2: Lead concentrations in the kidney of mallards

Unlike the highly elevated levels in the liver and kidneys, the lead concentration in the breast muscles showed low concentrations of lead, however only the control group only meets the regulatory standards of 0.1–0.5 mg/kg (Fig. 3).

The obtained results for the lead concentration in the breast muscle of the mallards from the first test group were four times higher than those of the control group, corresponding to a statistically significant difference of $p < 0,05$ (Fig. 3).

In the ducks from the second test group the levels of lead in the breast muscle were increased three times compared to the control group, and a statistically significant difference of $p < 0,05$ (Fig. 3).

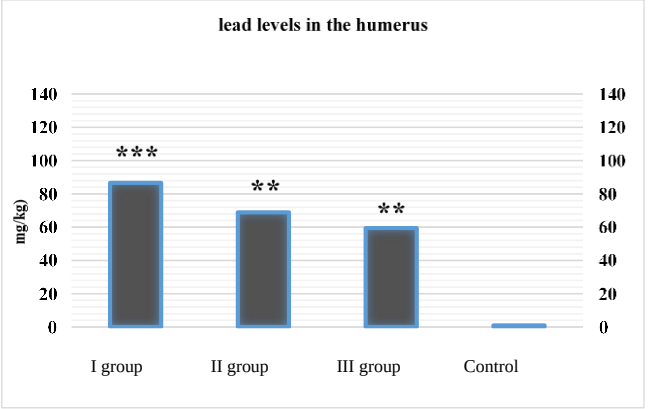
Regarding the lead concentration in the breast muscles of the least exposed mallards in the third test group (one lead pellet # 3) compared to the control group, slightly elevated levels of 0.65 ± 0.15 mg/kg were reported (Fig. 3).



** Statistically significant difference compared to the control group: where p <0,05.*
*** Statistically significant difference compared to the control group: where p <0,01.*

Figure 3: Lead concentrations in the breast muscles of mallards

The analysis of the humerus of the experimental mallards, clearly showed statistically significant differences in all three experimental groups in terms of lead concentration, compared to the individuals in the control group (Fig. 4).



*** Statistically significant difference compared to the control group: where p <0,01.*
**** Statistically significant difference compared to the control group: where p <0,001*

Figure 4: Lead concentration in the humerus of mallards

The most experimentally exposed to lead individuals from the first group show lead concentration in the humerus of 86.695 ± 10.801 mg/kg, i.e. between 50–120 times more than those from the control group, which after a statistical processing of the data is expressed as an absolute statistical reliability ($p < 0,001$).

In the mallards in the second test group (treated with two lead pellets # 3) average levels of 68.907 ± 11.657 mg/kg ($p < 0,01$) in the humerus or about 40–100 times higher than In those in the control group were reported (Fig. 4).

Unlike the other quantitative analyzes of the rest of the target tissues, in which in the third test group there were relatively low concentrations, the lead concentration in the humerus is very high, with the presence of a wide individual range and an average of 59.475 ± 13.169 mg/kg ($p < 0,01$), compared to the low concentrations reported in the control group (Fig. 4).

Discussion

The obtained values by us above 40–50 mg/kg for lead content in the liver and between 60–70 mg/kg in the kidneys in individuals from the first and second experimental groups, indicate signs of acute poisoning, accompanied by serious tissue damages. The higher content of lead in the kidneys compared to the liver described by other authors as well (Di Giulio and Scanlon, 1984; Jeng et al., 1997; Szymczyk and Zalewski, 2003; Hutařová et al., 2015), could mean that the kidneys are damaged irreversibly and cannot excrete the cumulating lead. Unlike the third test group whose lead content is in the range of less than 7 mg/kg for the kidneys and 10 mg/kg for the liver, whereas such levels are characteristic of a subclinical effects with possible cumulative effect and chronification. In some of the individuals it a partial recovery could be possible because the liver and kidneys, along with the blood flow and cardiac tissue are so called "fast-exchanging systems" and a small amount of lead could be absorbed and excreted (Friend, 1987; Szymczyk and Zalewski, 2003).

The lead content in the breast muscles is a rather secondary indicator for the lead concentration in case of an oral exposure unlike the liver and kidneys (Jordan and Bellrose 1951; Mateo et al., 2001; Binkowski et al., 2013 b). In our study, the lead concentration is low compared to the other target organs. Nevertheless, the obtained elevated levels in the first (2.05 ± 0.301 mg/kg) and second (1.4500 ± 0.144 mg / kg) experimental groups contrasted greatly with of the individuals from the control group, whose levels did not exceed 0.5 mg/kg. The least treated (with one lead pellet) individuals of the third test group showed low lead concentrations in the breast muscles of 0.65 ± 0.150 mg/kg, without statistical significance compared to the control group, which is consistent with the results published by other authors (Hutařová et al., 2015).

The results obtained from analyzing the levels of lead in the humerus are indicative of a severe clinical form of poisoning with a fatal outcome or with the possibility of long-term (lifetime) exposure and chronification of the process (Kalisińska et al., 2003; Pain et al., 2009; Hutařová et al., 2015). The high lead concentration measured in the bones of all three experimental groups contrasted sharply with the low levels of the control group, and was in accordance with the severity of the administered dose (Del Bono and Braca, 1974; Kamil et al., 2012). The bone tissue is the last link in which the lead is deposited after active transport by the hematopoietic and excretory systems. As a result, there was a strict correlation between the concentrations of lead in the bones, and those in the liver and kidney, acting as tissues with the greatest importance for the diagnosis of lead poisoning (Clausen et al., 1982; Fisher et al., 2006).

The main reasons for such greatly increased lead concentration in the bones of mallards, according to Mateo et al. (2001), are the elevated needs of calcium and mineral components required for the activation of bone metabolism, especially in the case of restricted and grown for experimental purposes younger subjects.

Conclusiones

The oral exposure to lead shot pellets # 3, leads to significant changes in certain haematological and biochemical parameters, manifested with anemia and metabolic changes. The observed toxicological effects are reflecting on the hematopoietic, excretory and musculoskeletal systems, generating repeatedly elevated levels in the soft tissues and in the bones.

Acknowledgements

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TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2017, vol. 2, No 2(3): 52–55

INFLUENCE OF CALCAREA CARBONICA 30C ON WEIGHT AND THICKNESS OF EGG SHELL AND INDEX OF EGG SHAPE IN DOMESTIC OSTRICHES (STRUTHIO CAMELUS DOMESTICUS)

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ABSTRACT

Influence of Calcarea carbonica 30c on weight and thickness of egg shell and index of egg shape in domestic ostriches (Struthio camelus domesticus) during the first and second egg-productivity years was investigated. Only according to the index of egg shape remedy has done some work; weight as well as thickness of egg shell did not increase statistical significance between treated and untreated animals, and values of coefficient of variation of the index of egg shape was lower in comparison with the control group.

Key words: ostriches, homeopathy, egg shell, egg index.

Introduction

The experiments with Calcarea carbonica had been done by Hanuman and he used for this purpose the powder of crushed middle layer of the oyster shell. The rich chemical composition of the substance makes it a drug with a wide variety of indications applicable at all stages of life: it contains mainly $CaCO_3$, but also a lot of various substances such as: Si, Mn-salts, Fe, heavy metals, Mg-salts, and a plurality of amino acids. This composition explains the powerful effects on the metabolism, especially the metabolism of P and Ca, lymph and bone tissues, skin and mucous membranes. It gets worse during cold weather, full moon and new moon. There are desires for disgusting food and sweets. Calcarea carbonica is a drug with a deep action indicated for carrying out the treatment in subjects with both psoric and with sycotic diseases in dominance still a psoric reactive type. (Shefdevil et all., 2000). Calcarea carbonica is a huge disease with extensive symptoms. The state Calcarea is rarely added in the form of a new layer, but more often is a primary, "primary cell" interference (Vitulkas, 2001). As with most non-toxic substances in normal doses, Hahnemann carried pathogenesis by using dilution 30c. Therefore, the resulting symptoms are not just primary subtoxic or toxic effects but clinical symptoms, which are an expression of the general type of reaction of the individual. Nowadays, it is also preferable to use this dilution. (Demark et all., 1998). This is the maximum potency that is recommended for human beings (Alefirov, 2002). In farm birds Calcarea carbonica was used much more limited in the past (The Poultry Doctor. 1999) and at significantly greater number of states today (Madrewar, 2003).

Aim

In the present study, we aimed to test the influence of homeopathic remedy Calcarea carbonica 30c on weight and thickness of egg shell and index of egg shape in domestic ostriches (Struthio camelus domesticus) during the first and second egg-productivity years.

Material and methods

The study was conducted on a farm in the municipality of Kyustendil in the period from April to September 2014th and 2015thr. The animals (ostriches – bearing of the same origin and of the same age entering into the first oviparous year in the spring of 2014, 12 individuals) were divided into two groups of six birds: test treated with Calcarea carbonica 30c with water drinking once per every three days for the months of April, May and June and a control group. The measurement of the small and large diameter (to the nearest 0.5 mm) of the egg was performed in all laid eggs over the study period as the egg index was calculated by dividing the multiplications hundred small diameter to the large diameter of the egg (to the nearest 0.01). The measurement of the mass (to the nearest 0.1 g) and the thickness (to the nearest 0.1 mm) of the eggshell was made only of those eggs which were classified as fertilized at the first candling (sixth day of incubation) as the thickness of the egg was measured through the opening through which we eliminated the contents of the egg (the differences in the thickness between different points in the ostrich egg is considered insignificant (Turevich, 2000)). The variation-statistical processing of the results was carried out with the program Statgraphics Statgraphics Plus Version 7.0 (Statistical Graphics Corporation (and Manugistics, Inc.), 1993).

Results

The summerd results of the carried out measurements had been presented in Table 1. Only regarding on the index of the egg it was found a significant effect; in regard to the mass and thickness of the eggshell it was not observed a statistically significant difference between the treated animals and the untreated ones.

Table 1: Effect of applying of Calcarea carbonica 30c on weight of egg, weight of egg shell, thickness of egg shell and index of the egg shape

Traits (Average ± Standard deviation)	Groups		Significance level
	Control (n = 6)	Treated (n = 6)	
First egg-productivity year			
Egg weight (g)	1497.5 ± 319.55	1518.5 ± 317.95	0.5739
Eggshell weight (g)	270.1 ± 30.80	272.9 ± 30.99	0.3901
Eggshell thickness (mm)	1.98 ± 0.24	1.99 ± 0.23	0.1670
Egg index	85.0 ± 5.22	83.8 ± 3.13	0.0513
Second egg-productivity year			
Egg weight (g)	1510.1 ± 309.03	1.520 ± 295.50	0.5209
Eggshell weight (g)	273.1 ± 29.00	275.1 ± 27.86	0.1099
Eggshell thickness (mm)	2.00 ± 0.19	1.99 ± 0.20	0.2007
Egg index	84.9 ± 4.92	83.1 ± 3.01	0.0480

The comparison between the variation coefficients for the individual groups is an interesting topic. In the treated birds it is observed statistically significant (at P = 0.0408) lower degree of variation in the index of the form of the eggs. The comparison of the coefficient of correlation between the mass of the shell and the mass of the egg in the various groups is another interesting thing – in this case there is practically no difference. (at P = 0.9611).

Discussion

As it is seen from the results of the measurements it is difficult to be defined some explicit trends. The large number of factors that influence on the quality of the eggshell (Delchev and Mitkov, 1984) suggests that homeopathic medicines, with their balancing effect on the body, have the potential to optimize the underlying signs. The results show that when the relevant drug is chosen we shouldn ‘t pays so much attention on the superficial comparison of the starting substance and the desired effect. It is worthwhile to be paid attention on the fact that the predominant side of Calcarea carbonica in the human being is the right one (headache of the most varied nature, localized in the upper region on the right, tuberculosis with predominant lesions in the upper right lung, etc.) (Alefirov, 2002)), and the lateralisation in the anatomy of the reproductive system in birds is so highlighted and obvious as for any other group of organs (Gigov, 1985).

In the treated animals, we have a stronger degree of "clustering" with respect to the index of the shape of the eggs obtained from the treated group around values that may be supposed to have been optimal prior to the expansion of the artificial incubation. Ultimately, the most important function of the shell is the preservation of the egg shape (Kartashev and Shilov, 1982), while the deviations from the normal form lead to a deterioration of other qualities of the eggs (Mitkov, 1984) and from this standpoint it is understandable that the homeopathic preparations activate the vital force of the organism and lead to such effects.

The fact that we have no certain reasons to think that the homeopathic preparation which was used has any effect may be associated with the possible undesirable effects of external factors which obviously can largely neutralize to a great extend the contained information inside when it is added to the total drinking water. It is relatively easy for a person to comply with a number of conditions when using homeopathic products in humans, which under actual production conditions of the livestock farm cannot or at least very difficult could be avoided (getting fodder and litter in the drinking water, sunlight, high temperature, etc.). This, of course, does not mean that in the process of working with animals, we shouldn‘t makes efforts to ensure the information stored in the homeopathic medicine access to the body.

Conclusion

Only in relation to the egg index it was received statistically significant difference to a very small extent. Also, with regard to the form index, it was observed a smaller degree of variation which could be interpreted as "striving" to lay eggs that were optimal during the natural (or close to) breeding of the species. The homeopathic remedies, balancing the individual's vital energy, lead to the expression in the phenotype of such signs that have been suppressed during the rise of industrial livestock. When prescribing homeopathic medicines, we should not glide over the surface comparison of the starting substance and the desired effect. The present results were obtained in female ostriches during the first two ovine years, the subject of future research will be how Calcarea carbonica affects older maturing as well as growing animals.

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INFLUENCE OF MELATONIN TREATMENT ON PUBERTY ONSET IN BUFFALO HEIFERS FROM BULGARIAN MURRAH BREED

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ABSTRACT

Current study aimed to investigate the influence of melatonin treatment on puberty onset in buffalo heifers from Bulgarian Murrah breed. The experiment was carried out with eleven clinically healthy pre-pubertal Bulgarian Murrah buffalo heifers alloted in two groups – control (non treated, n=6) and experimental (melatonin-treated, n=5). According to used plan, the treatment was done three time by subcutaneous melatonin implant, containing 18 mg melatonin. Seven days after the last melatonin treatment, progesterone levels were measured by ELISA method and used as an indicator for a presence of cyclic ovarian activity. Data were processed using of a computer statistical program. The average age (12.7±1.1 months and 13.2±0.9 months), body weights (184±22 kg and 232±33 kg) and progesterone levels (0.94±0.37 ng/ml and 1.08±0.16 ng/ml) among the groups did not differ considerably. However, the minimal and the maximal progesterone values in the experimental group (0.96 ng/ml до 1.36 ng/ml) were indicative for a presence of cyclic ovarian activity in 100% of the animals versus 80% in the control group. The concluded analysis shows that melatonin implants application is connected with a trend for earlier induction of cyclic ovarian activity and hastening of a puberty onset in Bulgarian Murrah buffalo heifers.

Key words: buffalo, melatonin, puberty, sexual cycle.

INTRODUCTION

The time for a puberty onset differs and is influenced by various factors – climatic and geographical region, breed, season of birth, feeding etc. (Peeva et al., 1993; Borghese et al., 1996; Campanile et al., 2001; Singh et al. 2010; Roy et al., 2016; Planski et al., 2017). Most of the studies have been connected with an influence of the age and the body weight on the expression of cyclic ovarian activity as its determination is done by rectal palpation of the ovaries, progesterone (P_4) concentration measurement or ovarian ultrasonography (Campanile et al., 2001; Terzano et al., 2007; Roy et al., 2016; Planski et al., 2017). The obtained data among the investigation differ and sometimes are discrepant, which is a precondition for searching of others factors than abovementioned, responsible for a puberty initiation.

Melatonin is biological active substance included in the sequence of processes leading to the onset of puberty in ruminants. It is N-acetyl-5-methoxytryptamine synthesized by the pineal gland during the dark phase of the photoperiod which transfer an information to synchronize cell physiology with the dark and the light part of the day-night cycle. Besides, melatonin stimulates gene expression of antioxidative enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase (Reiter, 1993; Rodriguez et al., 2004; Baychev, 2008; Kanchev et al., 2010). Recently, melatonin implants have been successfully applied for stimulation of ovarian cyclicity in beef heifers and anestral buffaloes (Ghuman et al., 2010; Singh et al., 2010; Ramadan et

al., 2014). In spite of all, the precise mechanisms, the treatment plan and the melatonin dose for a puberty initiation in different buffalo breeds remain unclear.

Current study aimed to investigate the influence of melatonin treatment on puberty onset in buffalo heifers from Bulgarian Murrah breed.

Material and methods

The experiment was carried out with eleven clinically healthy pre-pubertal Bulgarian Murrah buffalo heifers, aged between 9-12 months, weighing from 160 to 220 kg and cultivated of free-penned regimen in experimental farm of Agricultural Institute, Shumen, Bulgaria.

The nutrition plan included giving of all buffalo milk until the first month after calving, followed by milk replacement unit the third month. From third to eight months the animals received alfa-alfa hay at libitum and 1.2 kg concentarate for growing-up buffaloes daily. After that the ration included vetch-barley hay and 1.8 kg concentarate daily. During experimental period all animals had unlimited approach to water. The experiment was performed between September and January.

At the begin of experiment, the animals were alloted proportionally in two groups according to their body weight - control (non treated, n=6) and experimental (melatonin-treated, n=5). The treatment was done three time (at days 0.45 and 110) by subcutaneous melatonin implants, containing 18 mg melatonin (Melovine, Ceva Animal Heath, France). The time of first melatonin treatment was accepted for day 0. Buffalo heifers with body weight <180 kg. were implanted with 1, 2 and 3 melatonin implants per animal through abovementioned periods whereas those weighing >200 kg were treated with 2–2–3 implants per animal.

Blood samples for progesterone analysis were obtained from v. jugularis of previously restrained animals, seven days after the last melatonin treatment. The blood was centrifuged at 3000 rpm for 15 min and separated sera were stored in sterile tubes at -18 ^{0}C until analysis. Hormonal concentrations were assays with a commercial Progesterone ELISA Kit (Monobind Inc, Lake Forest USA) with analytical sensitivity of 0.105 ng/ml and coefficients of variation <10%. Progesterone levels of individual samples were measured in triplicate and mean arithmetic value of the three measurements was retained as final value.

After data processing, average age and body weight, minimal, maximal and average progesterone concentrations and a percentage of animal with cyclic ovarian activity in both groups were calculated. Progesterone levels >0.71±0.24 ng/ml were accepted as an indicator for cyclic ovarian activity in this buffalo breed (Planski et al., 2017).

Data were analysed using statistical software Statistica version 7.0 (Stat-Soft 1984-2000 Inc., Tulsa, OK, USA) using of the non parametric method for a comparison of mean values and proportions, based on Student t-test. The level of significance was set at $P<0.05$.

Results and discussion

The obtained data are showed in a table 1. The values about average age and body weight of the buffalo heifers in the control (12.7±1.1 month; 184±22 kg) and experimental (13.2±0.9 month; 232±33 kg) groups did not differ significantly (P=0.43 и P=0.48). These results showed that in a case the aforementioned parameters have no considerable influence on the investigated reproductive findings (P_4 and cyclic ovarian activity) and are indicative for equalization of both groups. According to some authors (Peeva et al., 1993; Terzano et al., 2007) the age and the body weight

have a pivot role for a puberty initiation in buffalo heifers. In the contrary, other studies (Parmeggiani et al., 1992; Singh et al., 2010) reported that significant parts of animals remained acyclic in spite of gaining of the typical age and body weight for the investigated buffalo breed. This is in agreement with our hypothesis that other mechanisms (melatonin production for example) are responsible for activating of the hypotalamus-pituitary-ovarian cascade, leading to early puberty onset. It is also in correspondence with the results of different investigations in beef heifers (Tortonese and Inskeep, 1992; Jaeger et al., 1998) and anestral buffalo heifers (Singh et al., 2010), reporting for stimulation of ovarian activity after melatonin treatment.

Table 1: Age, body weight, progesterone concentration and animals with a presence of cyclic ovarian activity in the control and the experimental group.

Group	Control (n=6)			Experimental (n=5)		
Parameters	**Min.**	**Max.**	**Mean±SD**	**Min.**	**Max.**	**Mean±SD**
Age (months)	11.4	14.4	12.7±1.1	12	14.2	13.2±0.9
Body weight (kg)	170	220	184±22	180	290	232±33
P_4 (ng/ml)	0.24	1.23	0.94±0.37	0.96	1.36	1.08±0.16
	%			%		
Cyclic ovarian activity	80			100		

The present study aimed to investigate the influence of melatonin treatment on puberty onset in buffalo heifers from Bulgarian Murrah breed by using of a progesterone concentration as an indicator for cyclic ovarian activity. The obtained progesterone values (0.94±0.37 ng/ml и 1.08±0.16 ng/ml) for non treated and melatonin-treated buffalo heifers, respectively, did not differ considerably (P=0.45). However, the minimal and the maximal concentrations of P_4 within the groups showed high variation in control group (from 0.24 ng/ml to 1.23 ng/ml), whereas in treated group it was low (from 0.96 ng/ml to1.36 ng/ml) and all animals had a progesterone levels over accepted reference value for ovarian activity in Bulgarian Murrah breed. Furthermore, 20% non treated heifers had hormonal level below the reference. On this base could be accepted that melatonin implants administration is connected with a tendency for earlier initiation of cyclic ovarian activity (80% vs. 100%). It is in agreement with information by other authors (Borghese et al., 1994; Jaeger et al., 1998; Ghuman et al., 2007, 2010; Singh et al., 2010), studied effect of exogenous applied melatonin for acceleration of a puberty in beef heifers or induction of ovarian cyclicity in anestral buffalo heifers. The absence of significant differences between the values of the investigated parameters could due to low number of animals. On the other hand, the phase of estrous cycle for samples collecting can also influence progesterone results. If a part of animals is in the start or the end of estrus during blood collection, the hormonal level could also be lower and determining lack of statistical significance. In addition, there are data for shorter luteal phase, lower size of corpus luteum and decreased progesterone during the first estrus cycle after melatonin treatment than in normal estrus in anestral buffalo heifers (Ramadan et al., 2014). In our opinion, the treated of us animals are being with ovarian cyclicity, but through of previously showed reasons some of them are had decreased progesterone production.

Regardless of abovementioned, all progesterone values in melatonin-treated buffalo heifers were above the reference for the investigated breed and it could be accepted as an argument for a positive impact of the melatonin treatment on the puberty onset. In respect of that, Singh et al. (2010) reported successful induction of ovarian activity in buffaloes by melatonin implants administration.

Ramadan et al. (2014) determined the second estrous cycle after melatonin application is chracterized with greater size of the ovulatory follicle, the corpus luteum and progesterone level, compared to the same parameters in the second estrus cycle in lack of treatment. Probably at the begin, the longer melatonin exposition results in temporary drop in GnRH releasing and because of that the first corpus luteum has a short half-life with low progesterone production. After that, GnRH production and releasing are increase and it led to ovarian activity induction. However, proving of this hypothesis is need from additional investigations and will be a subject of future study.

In conclusion, the present study shows that melatonin implants administration is connected with a trend for earlier induction of cyclic ovarian activity and hastening of a puberty in Bulgarian Murrah buffalo heifers. Numerous questions about effect of melatonin on the reproductive performance in buffalo heifers remain debatable and detailed investigations with a large number of animals can clarify this area.

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COMPARATIVE ECHOCARDIOGRAPHIC STUDIES OF DOGS WITH MIXOMATOUS DEGENERATION OF THE MITRAL VALVE, DEPENDENT ON THE PRESENCE OR ABSENCE OF PULMONARY EDEMA, CONSECUTIVELY INDEXED BY THE LINEAR AND WEIGHT IDEALIZING AORTIC SIZE

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ABSTRACT

Echocardiographic dimensions of 20 dogs with myxomatous degeneration of the mitral valve were compared in this paper. The patients were decided into two groups depending on the presence or absence of pulmonary edema. ERIs were obtained after consecutive indexing of Aom and Aow. In the indexing of Aom we received significant differences for aLA***, aLVODd** and aΔA**. In the indexing of Aow we received significant differences for more ERIs (wLA***, wLVODd**, wLVODs*, wΔA** and wWAd*).

Key words: dogs, mitral, mixomatous, degeneration, echocardiographic.

Introduction

Many authors consider the mitral valve disease (MVD) the most common heart disease in small dog breeds (5, 6, 7). This disease is also the most common cause of heart failure in this species (4).

Echocardiography is the primary method in the diagnosis of cardiac disorders. The problem with this method of testing is the different size of the animals in the dog population and the different size of the cardiac structures. In this connection, in 2003 a new method for quantitative echocardiographic interpretation based on the calculation of the ratios, where each unprocessed measurement in M-mode is divided by the size of the aortic root (Ao) was introduced. "Aortic based" indices are calculated using the aortic root (Aom) as a standard for length. Furthermore, the authors offer "weight based" indices, representing an idealized assessment of the aortic size (Aow), where Aow = KxW1/3. The use of these indexes bypasses the unwanted statistical characteristics typical for the linear regression of the echocardiographic measurements compared to the body weight and, to a lesser extent, to the body surface (2). Compared to the regressions, the ratios led to a significant improvement of the predictable range for any M-mode measurements in dogs, particularly with reducing the body size.

The same authors in 2005, carried out a comparative study on M-mode echocardiographic indices (ERIs) in dogs with mitral regurgitation due to chronic valvular disease (CVD / MR), about the ability of these ratios to discriminate and quantitatively connect echocardiographic changes. They determined that one M-Mode ERI index (WΔA), designed to express by weight the normalized short-axised impact area, is sensitive (90%) and specific (98%) in establishing a difference between normal and CVD / MR dogs. Predicting the left-sided heart failure within the group of CVD / MR is more problematic, but the ERI expressed size of the left atrium (WLA) showed 76% sensitivity and 81% specificity in this regard. Their study of echocardiographic models of pathology associated with CVD / MR determined as ERI, are sensitive and specific for CVD / MR and LSHF. Based on

the results they claim that ERI normalizes the echocardiographic data regarding body size in dogs and are suitable for quantitative description and analysis (1).

There is considerable debate on the existence of a linear relationship between echocardiographic measurements and body size. In M-mode and 2D images, the size of the LA is often indexed to the aortic diameter at the level of the aortic cusps because of the lightness of the image and the assumption that in the cases of the most common heart diseases the aortic size does not change significantly, which determines this ratio as a relatively independent internal index which is a better reflector of the body frame (skeleton) compared to the body weight (3). The aortic diameter is less likely to change over time in comparison to the body weight, which makes the ratios based on it, probably, more accurate at tracing the development of heart disease in the individual animals over an extended period of time.

Purpose of the study

To make a comparison between the weight indexed and linearly indexed M-mode echocardiographic indices (ERIs) in dogs with mitral regurgitation due to chronic valvular disease (CVD / MR), regarding the ability of these ratios to identify and quantitatively bind echocardiographic changes with cardiac decompensation.

Material and methods

To achieve the objective the dogs with leftward apical systolic murmur had X-rays and ultrasound done. Based on the results obtained, they were divided into two groups. First group - dogs with MVD without pulmonary edema, and the second group – MVD dogs with pulmonary edema.

The ultrasonographic examination was performed with the apparatus My Lab 70 vet XV (the most modern veterinary Doppler apparatus of the Italian company Esaote – one of the leaders in the design and manufacture of ultrasonic systems and software for veterinary medicine in Europe). The patients were examined with specialized cardiac phasometric transducers PA023E (at a frequency of 4-11MHz) and PA122E (with a frequency of 3–7 MHz) suitable for small dog breed cardiac patients in a right-sided parasternal position. The patients were examined in the right-sided parasternal sections - longitudinal (long axis) and transverse (short axis).

The chest radiographs were made with a direct digital radiography (DR X-ray system) in LLR (left lateral), and VD (ventro-dorsal) projections.

The patients were divided into two groups depending on the presence or absence of a radiographically established pulmonary effusion.

M-mode echocardiographic indices (ERIs) of the dogs were obtained from the raw echocardiographic measurements. They were calculated by the formulas of Brown D.J. et al., (2003), in which in order to allow the comparison of patients with different sizes, they are indexed first by the aortic size (AOm), and then in terms of the weight idealized aortic size (AOw).

The statistical processing was carried out with the program StatMost.

Results

Standardized, statistically summarized, linearly indexed by Aom data from Table 1 of dogs with MVD without pulmonary edema were compared to dogs with MVD with pulmonary edema. In the table the mean values and the standard deviation of the studied ratios are shown. Then the

same was done in regard to the echocardiographic M-mode measurements, but indexed to the weight idealized aortic size (Table 2), and, finally, the values from the two different types of indexing were compared.

Table 1: Statistics – linearly based (Aom) derivatives

Indicator	Without pulmonary edema n = 9		With pulmonary edema n = 11		Accuracy
	mean	SD	mean	SD	
aLA	1.4209	0.3100	2.1054	0.3554	0.0003***
aLVODd	2.9112	0.4602	3.4815	0.3038	0.0037**
aWTd	1.0024	0.1505	0.9993	0.2003	0.9691
aLVODs	2.6216	0.4323	2.8437	0.2823	0.1829
aWTs	1.6529	0.5313	1.5488	0.2415	0.5673
aΔA	2.8678	1.0590	4.5446	1.4493	0.0097**
aWAd	5.0997	1.2979	5.8778	0.8374	0.1220
aWAs	5.6990	1.6060	6.3771	1.2489	0.3017

a – Aorta-Based; aLA – Index of left atrial dimension; aLVODd – Index of left ventricular outer dimension, diastole; aWTd – Index of combined septal and left ventricular wall thickness, diastole; aLVODs - Index of left ventricular outer dimension, systole aWTs - Index of combined septal and left ventricular wall thickness, systole; aΔA – Index of change in left ventricular internal area (ie, short-axis stroke area); aWAd – Index of left ventricular short-axis myocardial wall area, diastole; aWAs - Index of left ventricular short-axis myocardial wall area, systole (Brown D.J. et al., 2003).

Table 2: Statistics – weight based (Aow) derivatives

Indicator	Without pulmonary edema n = 9		With pulmonary edema n = 11		Accuracy
	mean	SD	mean	SD	
wLA	1.4273	0.2555	2.1237	0.2715	1.511E-005***
wLVODd	3.0237	0.3169	3.5308	0.2952	0.0016**
wWTd	1.0132	0.1399	1.0114	0.1971	0.9813
wLVODs	2.6284	0.2655	2.9327	0.2855	0.0250*
wWTs	1.5087	0.2224	1.5575	0.2212	0.6304
wΔA	2.8306	0.7971	4.7611	1.4701	0.0024**
wWAd	5.1100	0.9789	6.0173	0.6106	0.0206*
wWAs	5.6812	1.1607	6.5494	1.1332	0.1090

w – Weight-Based; wLA – Index of left atrial dimension; wLVODd – Index of left ventricular outer dimension, diastole; wWTd – Index of combined septal and left ventricular wall thickness, diastole; wLVODs – Index of left ventricular outer dimension, systole wWTs - Index of combined septal and left ventricular wall thickness, systole; wΔA – Index of change in left ventricular internal area (ie, short-axis stroke area); wWAd - Index of left ventricular short-axis myocardial wall area, diastole; wWAs - Index of left ventricular short-axis myocardial wall area, systole (Brown D.J. et al., 2003).

The mean value of the linearly indexed size of the left atrium (aLA) in the group without edema is (1.4209 ± 0.3100) which is statistically significantly (0.0003 ***) lower than the mean value in the group with edema (2.1054 ± 0.3554). When comparing the same dimentions but indexed by the weight idealized aortic size, very close mean values were obtained (1.4273 – in the group without edema and 2.1237 – group with edema), where the fact that the values of standard deviation are lower makes impression (0.2555 – in group without edema and 0.2715 – in the group with edema). Also, a higher accuracy (as an absolute value) between the group without edema and the group with edema indexed with this type of indexing (1.511E-005 ***) were obtained. The mean value of the diastolic linearly indexed left ventricular outer dimension (aLVODd) in the group without edema is (2.9112 ± 0.4602) which is statistically significantly (** 0.0037) lower than its mean value in the group with edema (3.4815 ± 0.3038). The mean value of the same dimensions, but weight indexed

(wLVODd) in the group without edema is (3.0237 ± 0.3169) which is statistically significantly (** 0.0016) lower than the mean value in the group with edema (3.5308 ± 0.2952). As far as this indicator is concerned a higher accuracy was obtained, but only as an absolute value with the weight indexed dimensions in comparison to the linearly indexed dimensions. The mean value of the linearly indexed sum of the wall thicknesses of the septum and the left ventricle (aWTd) in the group without edema is (1.0024 ± 0.1505) which is statistically insignificantly (0.9691) higher compared to the mean value in the group with edema (0.9993 ± 0.2003). Similar values and lack of statistical significance (0.9691) were also obtained after the indexing of this dimension with the weight index.

The mean value of the systolic linearly indexed left ventricular outer dimension (aLVODs) in the group without edema is (2.6216 ± 0.4323) which is statistically insignificantly (0.1829) lower than the mean value in the group with edema (2.8437 ± 0.2823). The mean value of the same dimension, but weight indexed (wLVODs) in the group without edema is (2.6284 ± 0.2655) lower than the mean value in the group with edema (2.9327 ± 0.2855). For this indicator, in contrast with the linear indexing, there was a statistically significant difference (0.0250 *) when indexing the size by using the weight idealized aorta.

The mean systolic linearly indexed sum of the wall thicknesses of the septum and left ventricle (aWTs) in the group without edema is (1.6529 ± 0.5313) which is statistically insignificantly (0.5673) higher than the mean value in the group with edema (1.5488 ± 0.2415). Similar values and lack of statistical significance (0.6304) were obtained when indexing this dimension with the weight index as well. The mean value of linearly indexed short-axised impact area (aΔA) in the group without edema is (2.8678 ± 1.0590) which is statistically significantly (** 0.0097) lower in comparison to the mean value of the group with edema (4.5446 ± 1.4493). The mean value of the same size, but weight indexed was (2.8306 ± 0.7971) which is statistically significantly (** 0.0024) lower in comparison to the mean value of the group with edema (4.7611 ± 1.4701). For both types of indexing of the short-axised impact area (aΔA) results with similar values, standard deviation and statistical significance were obtained.

The mean value of the diastolic linearly indexed left ventricular myocardial short-axised wall area (aWAd) in the group without edema is (5.0997 ± 1.2979) which is statistically insignificantly (0.1220) lower than the mean value of the group with edema (5.8778 ± 0.8374). The mean value of the same dimension, but weight indexed (wWAd), in the group without edema is (5.1100 ± 0.9789) which is lower than the mean value in the group with edema (6.0173 ± 0.6106). For this indicator, in contrast with the linear indexing, there was a statistically significant difference (0.0206 *) when indexing the size with the weight idealized aorta.

The mean value of the systolic linearly indexed left ventricular short-axised myocardial wall area (aWAs) in the group without edema is (5.6990 ± 1.6060) which is statistically insignificantly (0.3017) lower than the mean value of the group with edema (6.3771 ± 1.2489). Similar values and lack of statistical significance (0.6304) were obtained with the indexing of this dimension with the weight idealized aorta.

Discussion

After concluding the results from both methods for indexing the M-mode echocardiographic measurements, it was found that the ratios measured based on the weight idealized aortic size are obtained with higher reliability in comparison to the results obtained with the indexing of the same dimensions with the linear aortic size. I suppose that the differences obtained in the two ways of

compiling the ratios are due to the weight loss in patients, which is associated with the progression of the cardiac decompensation. Under these conditions the indexing of the raw echocardiographic dimensions and the lower body weight leads to higher values of the ratios and register of a higher reliability. The question that arises is – which is the more correct indexation? When developing a clean left-sided heart failure the isolated development of pulmonary edema cannot significantly affect the body weight. On the other hand, the real weight loss is another sign of the advancing heart disease and as such it should not be excluded when reporting the progression of the process.

Conclusions

1. Upon the indexing of the raw M-mode echocardiographic measurements with the weight idealized measured aortic size, the results obtained are with a higher reliability in comparison with the indexing of the same dimensions with the linear aortic size

2. Based on the reasoning in the discussion of the results, I believe that as far as the mixomatous valvular disease is concerned, the indexing of M-mode echocardiographic measurements with weight aortic size is more suitable in comparison to the indexing with the linear aortic size, where in the case of this indexing the weight loss with progression of the heart failure is also noted.

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HISTOLOGICAL STRUCTURE OF THE GREY WOLF (*CANIS LUPUS*) STOMACH

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ABSTRACT

The aim of the current study was to investigate the microscopic anatomy structure of the grey wolf stomach wall and to compare it with another carnivorous as dog, fox, jackal, cat and tiger, respectively which were surveyed previously. The stomach of 2 male grey wolfs obtained after unplanned hunting, were investigated microscopically. The observation and morphometry was done by using „Olympus“ microscopic computer system.

The mucous membrane of the grey wolf stomach wall contains the typical tubular glands in different anatomical regions of the stomach and a chief, parietal and mucous exocrinocytic cells are presented. But no signs of the layer stratum compactum belt-like collagen formation was observed in wolf stomach mucosa. In layer tela submucosa well developed network of myotypical arterial and venous blood vessels are observed. The muscle layer of the wall is proportionally developed from three-positional situated smooth muscle cells bundles and an autonomic intramural myenteric nervous plexus is found between. The most outer layer serosa is morphologically presented and it enveloped the organ.

Dismissing the layer stratum compactum in grey wolf stomachs is similar fact as another canine animal species like dog, fox and jackal but it differs in cat and tiger where it was demonstrated. This fact supports the hypothesis that the mucosal layer stratum compactum may not to be presented in animals belonging to Canidae family carnivores but usually exists in Felidae animals.

Key words: carnivorous, stomach, histology, stratum compactum.

Introduction

The carnivorous animals have a simple stomach with a classic four layered structure. In histological literature the authors as Bacha W. Jr. and L. M. Wood, 1990, Krystev H. and S. Vitanov, 1993, are described the presence of specific lamina subglandularis into the most inner mucous layer of carnivorous stomach wall. As a wild animal it feeds on meat together with bones, but also supplemented the diet with fruits and vegetables which is supposition for specific structure of the stomach. The information about its designation and composition shows a certain amount of discrepancies. Its presence and double-layered structure in lamina propria mucosae underneath the bases of the stomach glands has also been noted by Smollich A., 1972, who however named it as stratum subglandulare. According to him, as well as Stinson A. W. and M. Lois Calhoun, 1993, the first layer which is situated closer to the bases of the glands and it is designated as stratum granulosum due to the fact that it is rich of connective tissue cells and their nuclei give impression for granulation. The other deeper layer which is positioned just to the lamina muscularis mucosae and has denser collagen structure is designated as stratum compactum. According to other authors only a single-layer dense fibrous acellular membrane, which protects the stomach wall from perforations in carnivorous stomach propria exists. In Nomina Histologica nomenclature, 1992, as Frappier B.L., 2006, it is noted that only one additional layer of the stomach mucosa in carnivores exists and it is called as stratum compactum.

The major aim of the current study was to examine the histological structure of the grey wolf stomach wall and the next one was to compare results with another stomachs from carnivorous

animals as dog, fox, jackal belonging to Family *Canidae*, and also cat and tiger from Family *Felidae*, respectively which were surveyed previously in another studies.

Materials and methods

The stomachs of 2 male grey wolfs obtained after unplanned hunting in Balkan range, were examined microscopically. The samples taken for histological investigation were fractionally fixed in 10% buffered formalin after the gastrotomy. Histological slides for microscopic investigation were conventionally prepared. The tissue samples were embedded in paraffin wax and sections 7 μm thick were stained with hematoxylin and eosin. An observation and morphometry of prepared samples by using „Olympus“ (Olympus Co Ltd., Tokyo, Japan) microscopic computer system was done.

All investigated methods are done in accordance with the Bulgarian low for protection and human attitude with animals and were under observation of institutional ethics committee of FVM.

Results and discussion

As we expected the wall of wolf's stomach showed a typical layered structure (Fig. 1.). The fourth main layers, as glandular mucosa followed by tela submucosa, then musculature and after that serosa were histologically presented. In stomach glands the excretory epithelial cells as mucous cells – mucocytes cervicales, chief cells – exocrinocytes principales, and parietal cells – exocrinocytes parietals, were shown. Cervical mucocytes included vacuoles and their cytoplasm was transparent, chief cells which included pepsinogen were stained basophilic and parietal cells included respectively chlorides were eosinophilic. Between glandular tubules an individual smooth muscle cells were observed. They theoretically belong to the most inner layer of mucosal muscle laminae. No sign of the layer stratum compactum as belt-like collagen formation was observed in the wolf stomach mucosa just under the tubular glandular bottoms and before mucosal muscular lamina. Furthermore, stratum compactum was not established, neither in small intestine or in large intestine mucosal layer, in our additional research of the wolf intestinal duct.

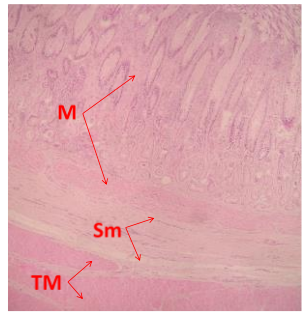

Figure 1: Wolf stomach wall. M – mucosa, Sm – submucosa, TM – tunica muscularis.

Adequately to these facts in another investigated stomachs belonging to animals of Family Canidae the mucosal layer stratum compactum also dismisses, as for example in fox, jackal and dog species Zahariev, P. et all, 2010, 2014. This distinctive feature is only comment by Bacha W. J. and Wood L. M., 1990, who reports that the cat stomach has layer stratum compactum, but the later one may be absent in dog's stomach. In comparison to this characteristic, in another investigated

stomachs of animals belonging to Family *Felidae*, the mucosal layer stratum compactum was presented, as for example in domestic and wild cat and tiger. The final one mucosal lamina muscularis is well-developed as three layer organization and thick smooth musculature bundles interrupted by thin connective tissue streaks were established. The existence of smooth muscle cells between the glandular tubules conforms the data of Frappier, B. L., 2006, who also present three layered structure of this lamina in another animal's stomaches. In submucosal layer a branched network of arterial and venous blood vessels was observed. These vascular frames were formed by vessels of muscular type of construction and triple-layered vessel wall was clear demonstrated. The smallest arterioles, venules and capillaries as well as lymphatic vessels were also visible. The muscle layer – tunica muscularis, of the wall is proportionally developed from three-positional situated bundles of smooth muscle cells which are better presented in cardiac and pyloric regions. An autonomic intramural myenteric nervous plexus containing multipolar neurons was found between muscular fascicles. The most outer denser serous layer was morphologically presented and it's composition of fibrous layer and superficial mesothelium, consisting of squamous epitheliocytes, was observed.

Conclution

Dismissing of the layer stratum compactum in grey wolf stomachs is a similar fact as in another canine animal species like dog, fox and jackal but it differs in cats and tiger where it was demonstrated. This fact supports the hypothesis that the mucosal layer stratum compactum probably is not presented in all animals belonging to Family Canidae but usually permanently exists in animals of Family Felidae, nevertheless that these both families belong to the Order *Carnivora* animals.

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TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2017, vol. 2, No 2(3): 69–74

GENITAL MYCOSIS IN MAIL DOGS

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ABSTRACT

As a result of microscopic and microbiological tests on semen from two dogs with signs of infertility necrospermia was established and fungi of the genus Fusarium were isolated. When develop in food and feed they emit toxins that cause mycotoxicosis in animals and birds after oral intake. Zearalenone, one of mycotoxins of these mushrooms is known as the cause of the atrophy of the gonads, spermatogenesis disorders, and infertility. This, however, is the first release for the isolation of fungi from the seminal fluid and setting them as the cause of genital mycosis.

Key words: infertility, Fusarium sp., genital mycosis, dogs.

Introduction

It is a well-known fact that for the successful application of various reproductive techniques, the good reproductive health of the breeding animals is of particular importance. In this connection, the quality of the ejaculates is crucial to obtaining optimum results in animal reproduction. There are, however, a number of cases in the practice, in which the quality of the semen does not meet the requirements for an optimal reproduction process. Possible causes of this are abnormal temperature regulation of testicles, trauma, haematocele, hydrocele, inflammatory process in testicles or epididimis, prolonged systemic disease, obesity (increased scrotal fat), prostatitis, brucellosis, medications, autoantibodies to sperm and others (Ticer, 1965; Johnston, 2000).

In this regard, the aim of the present study was to determine the cause of infertility in male dogs, a Middle Asian shepherd breed.

Materials and Methods

Animals under study. The research was carried out on materials from two male dogs aged 7 and 8 years of Middle Asian shepherd breed. They are grown together under the same living and eating conditions, in the yard of a house with farm animals. Throughout all period of the study, the dogs were in good general condition, without signs of infectious or non-infectious pathology. However, in attempts to fertilize female dogs infertility was established.

Clinical materials. Seminal fluid samples from both dogs were examined. They were obtained in sterile vials twice with an interval of one month. Seed fluid analysis was done on a computer sperm analyzer Nikon Eclypse 200.

Microscopic studies of native materials and after staining using the classical methods of Gram and Romanowsky-Giemsa were performed under an immersion at magnification of 1200 x.

Nutrient media. Selective nutrient media were used for isolation and quantification of the microorganisms (Antisel – Sharlau Chemie S. A., Spain): agar of Mueller Hinton, Eosin Methylene Blue agar for *E. coli* and the Gram-negative aerobic bacteria, Cetrimide agar for bacteria of the genus *Pseudomonas*, Chapman Stone agar for those of the genus *Staphylococcus*, Sabouraud agar

for fungi, selective agar for enterococci, differentiation liquid media (for indole determination, nitrate degradation, methyl red and Voges-Proskauer reactions) selective agar for *Clostridium perfringens* (Merck, UK), blood agar and broth of Tarocchi for obligate anaerobes (BUL BIO NCIPD Ltd. - Bulgaria).

For isolation of microorganisms, cultures from the samples were made in the elective and selective nutrient media for bacteria of different groups, as well as for fungi. They were cultured at 37° C and 28° C for 24 to 72 hours under aerobic conditions.

The taxonomic identification of the isolated bacteria was performed by microscopic examination of their morphology, reading the cultural features and biochemical properties using differentiating liquid media and additional tests for oxidase, hydrogen sulphide and others with reagents from Antisel (Sharlau Chemie S. A., Spain). The isolation and identification of the bacteria has been carried out in accordance with the Bergey International Identifier (Holt et al., 1994), and of the fungi was made according to Murray et al. (2003).

Determination of the sensitivity of isolated bacteria to antimicrobial means was carried out by the classic agar-gel diffusion method of Bauer et al. (1966). Standard antibiotic discs (BULBIO – NCIPD Ltd. – Sofia) and such prepared by us were used after inoculation of bacterial suspension in exponential growth phase with a concentration of 2.10^6 cells/ml on blood agar. Incubation was performed at 37°C for 24 hours. The results were interpreted in a three-tier system of Bauer et al. (1966) after measuring the diameters of inhibitory zones in mm.

Results

Microscopic studies. The results of the spermiogram of the materials from the studied dogs are presented in Table 1 and Fig. 1.

Table 1: CASA analysis of semen of dogs (Nikon Eclypse 200)

progression	Total	Percentage (%)	CONCENTRATION millions per ml	CONCENTRATION in total ejaculated
Static	101	99,0%	113,2	226,3
Non-progressive motile	1	1,0%	1,1	2,2
Progressive motile	0	0,0%	0,0	0,0
	102	100,0%	114,3	228,6
			(≥ 20 mill/ml)	(≥ 40 mill/total)

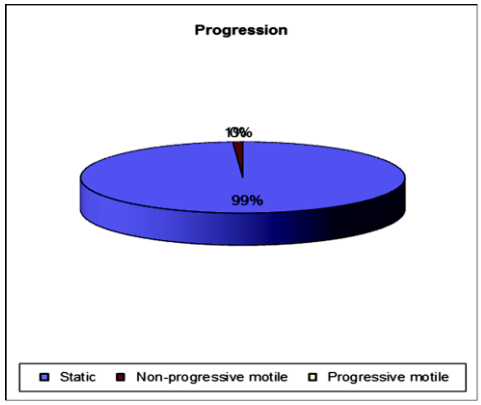

Figure 1: CASA analysis of semen of dogs (Nikon Eclypse 200)

As evidenced by the data of semen analysis, 99% of the sperm were non motile and only 1% showed signs of movement, in that not progressive but passive, ie. they were also not alive. Overall, no significant changes in sperm cells morphology were observed.

In the microscopic studies of the materials, conidia of fungi with morphology of representatives of the genus *Fusarium* were observed. They were large (about 8 μm) and pear-shaped. In examining the samples taken initially, bacteria were not found in the microscopic preparations. However, in seminal fluid samples obtained from both dogs after a one month period, except fungal conidia with morphology of *Fusarium* genus, Gram-negative rod-shaped bacteria were also observed.

***Cultural studies*.** The results of the cultural studies of both initially taken seminal samples are presented in Figure 2. Colonies of fungi with morphology characteristic of the genus *Fusarium* are seen. They developed after cultivation for 10 days and were large, mossy, oval or irregular in shape, with white to pink color. Data from colony and conidia morphology as well as growth indicators give reason to refer these to the species *Fusarium sporotrichella*. From these samples there were no isolated bacteria on the used elective and selective nutrient media.

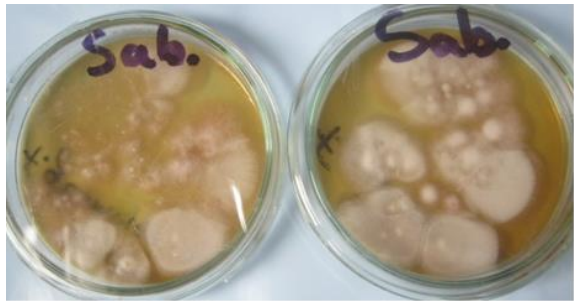

Figure 2: Colonies of fungi of genus *Fusarium* on Saburough agar 10 days after application of semen samples of two dogs.

In the culture studies of semen samples obtained from the two patients after a one-month period, fungi of the same genus were isolated and determined by the morphology of conidia and colonies such as *Fusarium sporotrichella* (Figure 3a). From the same samples, Gram-negative facultative anaerobic bacteria were isolated, whose colonies can be seen in Figure 3b. According to the culture and biochemical indicators, they were defined as *Enterobacter agglomerans*. At the same time and Gram-negative obligate anaerobic bacteria with morphology characteristic of the *Dichelobacter* genus were isolated from both samples.

a b

Figure 3: Colonies of fungi of genus *Fusarium* on Saburough agar 9 days after application of semen samples of one of the dogs (a) and colonies of *Enterobacter agglomerans* on Mueller-Hinton agar of the same sample.

Sensitivity to antimicrobial means. The results of the *in vitro* tests to determine the sensitivity of the isolated *Enterobacter agglomerans* to antimicrobials from different groups are presented in Table 2.

Table 2: Sensitivity of the isolated bacteria Enterobacter agglomerans to antimicrobial means in vitro

Antimicrobial mean	Disc content (μg)	Sensitivity of the strains	
		P 1	P 2
Thiamphenicol	30	R	R
Tetracycline	30	R	R
Lincomycin	15	R	R
Oxacillin	1	R	R
Amoxycillin+Clavulanic acid	10	R	R
Penicillin	10	R	R
Cefuroxime	30	S	S
Cefotaxime	30	S	S
Novobiocin	30	S	S
Gentamicin	10	S	S
Amikacin	10	S	S
Enrofloxacin	5	S	S
Ciprofloxacin	5	S	S
Sulfamethoxazole+Trimethoprim	23,75/1,25	S	S

The data in the table shows the multiple resistance to antibiotics of the two isolated strains of *E. agglomerans.* It covers some broad spectrum products such as tetracyclines and amphenicols as well as those from the penicillin group, including amoxicillin in combination with clavulanic acid (β-lactamase inhibitor). The strains were sensitive to aminoglycosides, quinolones and potentiated sulfonamides.

Treatment. It was performed with metronidazole in combination with quinolone (enrofloxacin orally at a dose of 10 mg / kg with food every 12 or 24 h.

After administration of this therapy for three weeks a good effect was achieved. This is evident from the results of the spermogram of the materials from the examined dogs after the treatment, which are presented in Table 3 and Fig. 4. Data show that 24% of the sperm were mobile after completion of therapy.

Table 3: CASA analysis of semen of dogs after the applied antimicrobial therapy (Nikon Eclypse 200)

	Total	Percentage	CONCENTRATION	
progression		(%)	millions per ml	in total ejaculated
Static	3174	74,1%	389,2	389,2
Non-progressive motile	1036	24,2%	127,0	127,0
Progressive motile	76	1,8%	9,3	9,3
	4286	100,0%	525,5	525,5

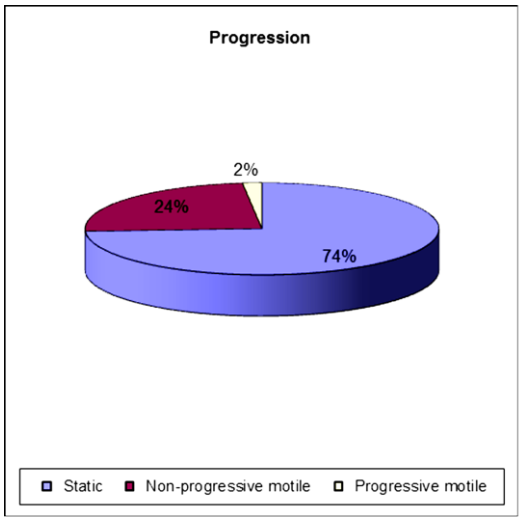

Figure 4: CASA analysis of semen of dogs after the administered treatment with antimicrobial means (Nikon Eclypse 200).

Discussion

The data from the performed studies show mycosis of the genital system of the studied patients and a following secondary bacterial infection. It is mixed with the participation of conditionally pathogenic Gram-negative aerobic and anaerobic bacteria. Most likely, the secondary infection was provoked by the fungi of the genus *Fusarium*, found in the seminal fluid. In animals and birds, the mycotoxins, and predominantly zearalenone, emitted by these fungi, after oral ingestion in the body, cause gonadal atrophy in both sexes with signs of excitability, false oestrus and infertility (Popova, 2016). However, there is insufficient research on their role as causative agents of mycoses. The isolation of *Fusarium sporotrichella* from such material is an indication for such a diagnosis. There are no literary data for mycoses of the genital system. This is the first report for isolation such fungi from semen, and twice, of two patients living under the same zoo-hygienic conditions and nutrition. Predisposing factors are intake of food with spores of molds and mycotoxins, as well as living in a damp premises and poor hygiene. The dogs studied were grown under similar conditions.

The use of quinolone was chosen in accordance with the antibiotic test results for *Enterobacter agglomerans*. Because the two isolated strains exhibited sensitivity to quinolones or aminoglycosides, but these are not active against obligate anaerobes, this therapy was supplemented with metronidazole directed against the isolated strict anaerobes of the genus *Dichelobacter*, as well as against the fungus *F. sporotrichella*. The established in vitro multiple resistance of the isolated *E. agglomerans* to antibiotics makes an impression, particularly to those of the penicillin group and to some broad-spectrum such as tetracycline and amphenicols. Such resistance is increasingly common among clinical isolates today. Even amoxicillin in combination with β-lactamase inhibitor (clavulanic acid) did not show effect *in vitro*. The uniform results of the antibiotic tests are an indicator of the role of uniform conditions of breeding and treatment of animals, as their close coexistence being a prerequisite for the exchange of microorganisms, including resistant antibiotics.

Good results from applied combination therapy over three weeks have shown that infertility caused by a combined genital infection involving fungi and bacteria can be treated successfully.

However, the prophylaxis it is of great importance, in which the role of providing good zoo-hygienic conditions and quality food has a leading significance.

Conclusions

Genital mycosis was established in dogs with signs of infertility. Fungi of the species *Fusarium sporotrichella* were isolated from the seminal fluid of the patients. Dogs breeding and feeding conditions may be a factor in the development of such a fungal mycosis resulting in infertility.

The development of fungi in the genital tract turns out to be a prerequisite for the development of a combined bacterial infection with the participation of conditionally pathogenic bacteria – a facultative anaerobe (*Enterobacter agglomerans*) and a strict anaerobe of the genus *Dishelobacter*.

Combined therapy for three weeks with metronidazole and enrofloxacin had a very good result – 24% mobile sperm.

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RADIOLOGICAL STUDIES OF SECONDARY COMPLICATED SINUSITIS IN A RACING MARE – CASE REPORT

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ABSTRACT

The purpose of this research reflects the development process of secondary sinusitis in horses regarding the topographic-anatomical preconditions for unilateral involvement of the all six sinuses complex. The medical anamnesis and diagnostic imaging tests conducted previously in a veterinary clinic in Germany were used. The head of the mare was examined by X-ray radiography and computed tomography (CT) methods after its death. The following procedure included a treatment of the skull and the established osteolytic alterations have been compared by us with those obtained from the X-ray images and CT scans, as well as the applied CT slices and 3D reconstructions of the alive patient. This prominent clinical case reveals an opportunity for an interpretation of the expansion and complications of sinusitis in horses with an emphasis on the anatomical characteristics of the sinuses, visualized by diagnostic imaging methods. Through this study we hope to contribute to the timely diagnosis and treatment of the paranasal sinuses inflammation in horses.

Key words: paranasal sinus system, secondary sinusitis, computed tomography, horse.

Introduction

The equine paranasal sinuses are an intricate area of interests. The horse head had six pairs of sinuses, three paranasal; the frontal, maxillary and sphenopalatine sinuses and three nasal; dorsal, middle and ventral conchal sinuses and all of these spaces communicate with each other and the nasal passage either directly or indirectly. Different sinus compartments communicate with each other, grossly creating a rostral and more caudal complex (Vlaminck, 2013). The rostral complex consists of the ventral conchal sinus which communicates with the rostral maxillary sinus over the infraorbital canal through the conchomaxillary opening. The caudal complex consists of the caudal maxillary sinus which broadly communicates with the conchofrontal sinus through the frontomaxillary opening. Over the infraorbital canal, the caudal maxillary sinus also communicates with the more medially located sphenopalatine sinus. Caudal maxillary sinus communicates with middle conchal sinus. Rostral and caudal maxillary sinuses communicate with the nose through separate narrow nasomaxillary openings into the middle meatus. This close communication with the nose renders these sinuses vulnerable for development of infectious problems.

The large size and complex anatomy of the sinuses can allow a pathologic process to be present for weeks or months before any external signs, such as facial swelling or nasal discharge were noticed by the owner or veterinarian. This can negatively affect the prognosis (Waguespack, 2011).

Disease processes that can develop in the sinuses include: ethmoid hematomas, cysts, neoplasia, and bacterial and fungal infections.

Clinical signs of any type of sinusitis usually include unilateral purulent nasal discharge, ipsilateral mandibular lymph node enlargement, and epiphora. Less common signs include facial swelling, exophthalmos, abnormal respiratory noises, head shaking, and exercise intolerance (Lane 1993; Tremaine & Dixon, 2001a).

Diseases involving the head are frequently encountered in horses and require imaging studies for further work-up (Tucker and Farrell, 2001). There is apparently no breed, age or gender predisposition to sinusitis.

Historically, radiography has been the primary imaging technique for assessing the skull, nasal cavity, dental structures and paranasal sinuses (Wyn-Jones 1985a,b; Tremaine and Dixon 2001a). However, the anatomic complexity and superimposition of the osseous, dental and soft tissue structures complicate radiographic interpretation (Tucker and Farrell 2001). Cross-sectional imaging modalities, such as computed tomography (CT) and magnetic resonance imaging (MRI), are therefore particularly useful for this area (Kraft and Gavin 2001; Solano and Brawer 2004).

The purpose of this research reflects the development process of secondary sinusitis in horses regarding the topographic-anatomical preconditions for unilateral involvement of the all six sinuses complex. Through this study we hope to contribute to the timely diagnosis and treatment of the paranasal sinuses inflammation in horses.

Materials and Methods

Animals

The object of this study was a head of racing mare obtained from a veterinary clinic in Gessertshausen, Germany. The mare was eight years old. The medical anamnesis and diagnostic imaging tests conducted previously in a veterinary clinic in Germany were used. The head of the mare was examined by X-ray radiography and computed tomography (CT) methods after its death. The following procedure included a treatment of the skull and the established osteolytic alterations have been compared by us with those obtained from the X-ray images and CT scans, as well as the applied CT slices and 3D reconstructions of the alive patient.

Computed tomography study

The head of racing mare were placed in lateral and dorso-ventral recumbency on the CT table. CT was performed along the transversal planes from the muzzle to the atlas in 1.5 mm thick helical CT slices at 10 mm intervals, by a Picker® CT PQ 5000 scanner. The CT scan images were analyzed with computer software DIKOM-VIEWER and were subsequently compared with radiographic images and pictures of the processed skull and are compared right with left side too.

The CT study and 3D reconstructions provided from German clinic were used.

Radiography

The head of mare wes captured by X-ray machine – Eickemeyer® Vet, model E 7239X in standard orthogonal projections – dorsal-ventral and lateral images. The available dissection results were clarified, supplemented and compared right with left side.

Bone cleaning technique

From the head a faded bone sample was prepared. The scull was cleaned by boiling and soaked for overnight in peroxide solution. The results from CT and Radiography was compared with osteology results.

Results and Discussion

This case observes an eight-year-old mare, which had developed secondary sinusitis after an apical dental disease of the upper right premolar. The inflammation of the teeth roots progressed

into the right rostral maxillary sinus. The development of this sinusitis resulted in cavity formation and accumulation of purulent exudate. This cavity has led to hyperventilation which alleviates the breathing and as a result, the mare was stopped from racing. Due to the difficult drainage of the sinus, the exudate has been inspissated. The cavity started to enlarge and led to face asymmetry with clear distinguishable swelling of the right maxilla (Fig. 1), which is one of the clinical signs of sinus diseases, especially in an apical infection of the first three premolars, according to a large number of authors (Waguespack, 2011; Khairuddin, 2016; Tremaine and Freeman). On the CT scans and 3D imagining is established asymmetrical inflection of external lamina of the maxilla dorsally of the right infraorbital foramen (Fig. 1). On the CT scans is visible an asymmetry caudally of *apertura nasi ossea* and *incisura nasoincisiva* (Fig. 1), signs that the above-mentioned authors do not describe.

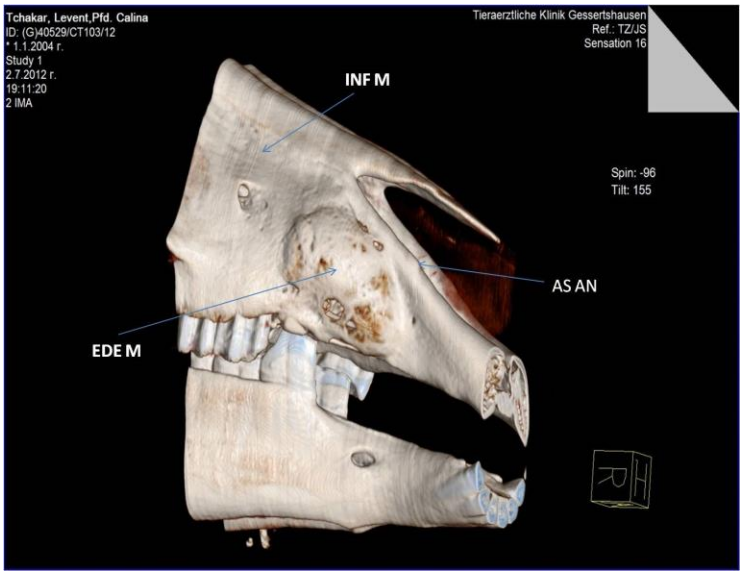

Figure 1: 3D reconstructions of head of the mare (Veterinary clinic in Gessertshausen) – INF M – inflexion of the external lamina of maxilla; EDE M – facial swelling of the maxilla; AS AN – asymmetry caudally of apertura nasi ossea.

The adjacent dorsal and ventral nasal concha were affected with the development of the inflammation process. Osteolytic changes had occurred in the bones that had affected the maxilla and its palatine process, as well as the ventral and dorsal nasal concha (Figs 1; 2). Similar changes are described from Schumacher & Crossland, 1994; Waguespack, 2011. The ventral conchal sinus is relatively isolated and communicates only with the rostral maxillary sinus. This is a predisposition for accumulation and inspissation of exudate (Waguespack, 2011), which is fully confirmed by this clinical case (Fig. 2).

We observed deviation of the cartilage nasal septum and the vomer in the right direction under the inflamed tissue pressure and osteolytic changes in right maxillary sinus (fig.2), as Tremaine and Freeman describe.

Osteolytic changes between right infraorbital canal and molar's alveoli are observed on CT scans and X-ray images. Canal's bone proliferation indicates a possible inflammatory process that

we identified when comparing it to the left side of the mare's head (Fig. 3). There is no data in the literature regarding this changes.

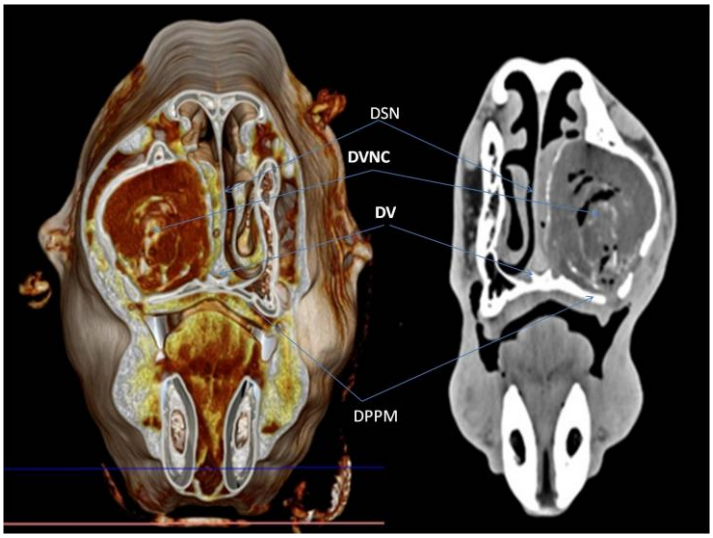

Figure 2: 3D reconstructions (left) and CT(right) through swelling region (Veterinary clinic in Gessertshausen) – DSN – deviation of the nasal septum cartilage; DVNC – destroyed ventral nasal concha with inflammatory and osteolytic mass; DV – deformation of the vomer; DPPM – deformation of the palatine process of the maxilla.

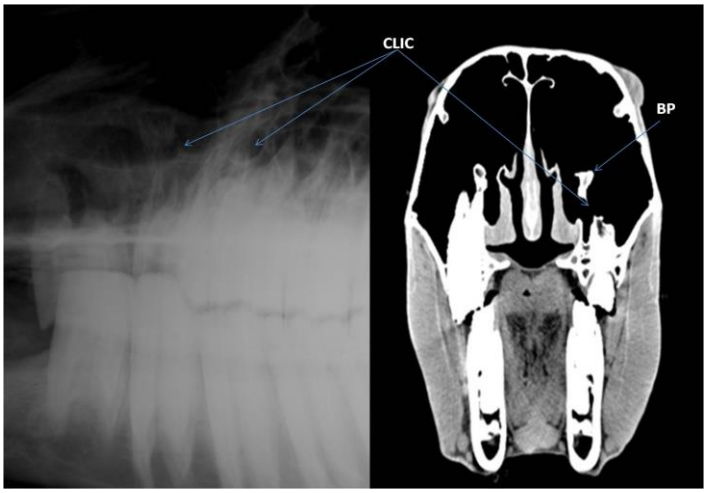

Figure 3: Lateral radiography of the head (left) compared with CT (right; Veterinary clinic in Gessertshausen) through first molar – CLIC – cavity after osteolytisis lower infraorbital canal; BP – bone proliferation of the right infraorbital canal.

In horse, compared to donkey, the septum between the rostral and caudal maxillary sinus is complete, as is described from El-Gendy and Alsafy, 2010. On the CT scans the destruction of *septum sinuum maxillarium* is evident, compared with the left sinus (fig. 4), in contrast to that observed by Tremaine and Freeman.

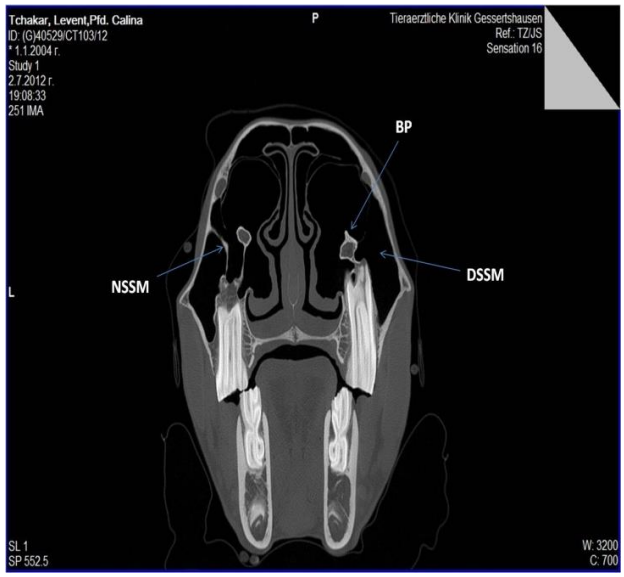

Figure 4: CT before orbit of head of the mare (Veterinary clinic in Gessertshausen) – BP – bone proliferation around right infraorbital canal; NSSM – normal septum sinuum maxillarium; DSSM – destroyed septum sinuum maxillarium.

Khairuddin (2016) observe a painless facial swelling formation of the frontal bone, due to inflammation of the frontal sinus. This is confirmed by our research as well, where the process was complicated and affect the conchofrontal sinus because of its communication with caudal maxillary sinus. We observed an inflection of *lamina externa* of the right frontal bone and deviation *of septum sinuum fronatalium* on the level of supraorbital foramen due to the osteolytic changes (Fig. 5).

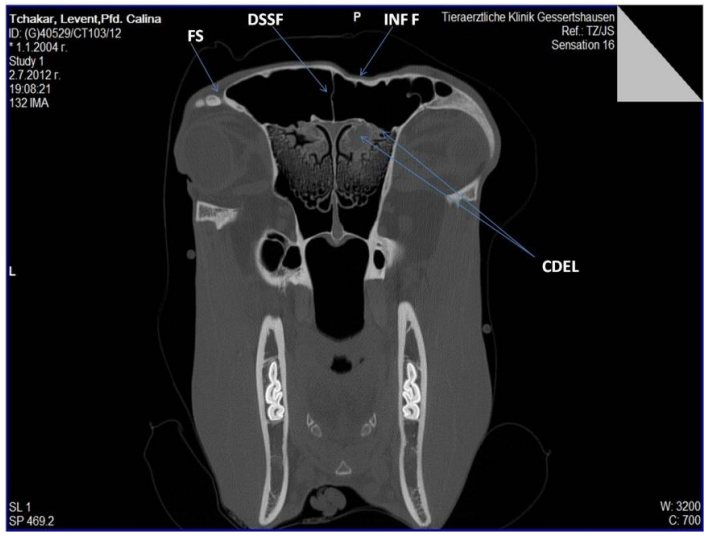

Figure 5: CT through the level of supraorbital foramen (Veterinary clinic in Gessertshausen) – FS – foramen supraorbitale; INF F – inflexion of the external lamina of frontal bone; DSSF – deviation of the septum sinuum fronatalium; CDEL – edema and asymmetry of dorsal part of the ethmoidal labyrinth.

We observed an asymmetrical location of *septum sinuum sphenoidalium* in the sphenopalatine sinus (Fig. 6), which is not very rare with healthy horses and donkeys (El-Gendy, 2010). Due to this often normal position of the septum, we cannot affirm the inflammatory changes in it. This was proven with the ventral part of the ethmoidal labyrinth, which was not affected.

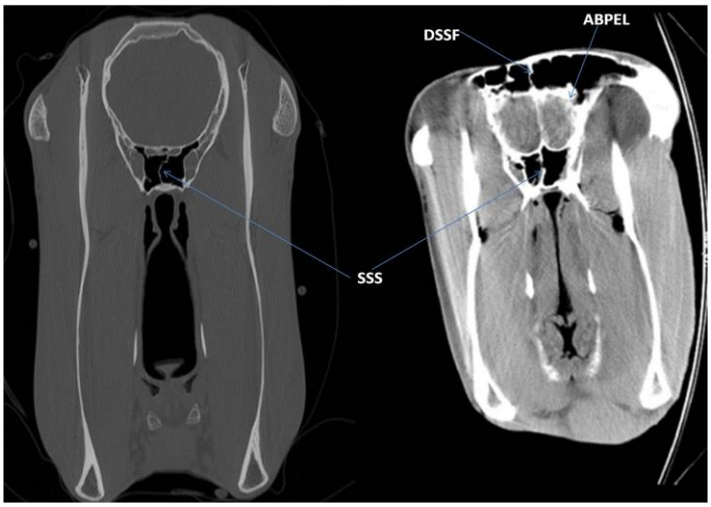

Figure 6: CT through the level of the orbit (left (Veterinary clinic in Gessertshausen) and right) – SSS – asymmetry of the septum sinuum sphenoidalium; DSSF – deviation of the septum sinuum fronatalium; ABPEL – asymmetry and bone proliferation of the cribriform plate.

We did not establish affected middle nasal concha from the radiological examination. Only the dorsal part of the ethmoidal labyrinth, which is located at the base of the frontal sinus, is influenced. It is evident that a bone proliferation and swelling had occurred, compared to the left half of the head (Figs. 5, 6). This is also seen in the rostral part of the telencephalon. Not very often, purulent meningoencephalitis and neurological signs can occur after severe chronic sinusitis with an erosion of the cribriform plate. Similar changes are described by Waguespack (2011).

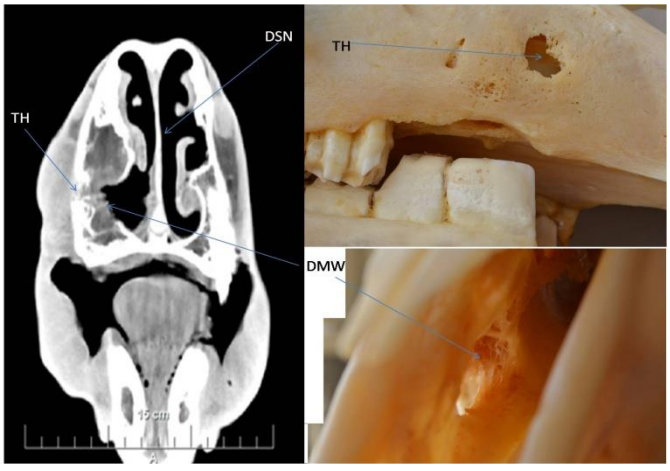

Figure 7: CT through the level of the trepanation hole (left) compared with native pictures of the skull (right) – TH – trepanation hole; DSN – deviation of the nasal septum cartilage; DMW – destroyed of medial wall of the maxilla.

The second and third right molars were removed for healing purposes. The removing of the teeth was made through a trephination opening above the second premolar (Fig. 7), which subsequently was filled with granulation tissue. So formed openings were used for lavage and drainage of the sinus, as some authors recommend (Schumacher & Crossland, 1994; Nickels, 2006; Dixon et al., 2012).

The cavity formed by the inflammation is medially destroyed, which led to wide communication with nasal cavity (Fig. 7). Similar pathologic changes are not mentioned from other authors. This osteolysis is identified on present radiological examinations and confirmed on the mare's skull. The great expansion and development of the chronical sinusitis are confirmed with comparing our examinations with those from the German veterinary clinic.

From our studies, we established that the changes are distinguishable and differentiable on CT scans, compared to x-ray images. On the radiographic views, there was a severe overlaying of the images, which confirmed Manso-Díaz, (2015) findings.

Conclusions

The specific morphology and communications of the horse paranasal sinus system predisposes sinusitis to spread and become chronic. All sinus compartments can be involved in sinus disease. The large size and complex anatomy can negatively affect the prognosis of horse sinusitis. They can exists for weeks or mounts before any signs will be noticed by the owner or veterinarian. CT is an imaging technique with high diagnostic value for evaluation the equine head, yielding additional information over multiple radiographic views, which may alter the outcome of the case. Computed tomography and skull radiography are of great value in diagnosing the presence and causes of equine sinus disease. X-ray examination alone is insufficient for accurate and timely diagnosis of sinuses diseases in the horses.

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НЕМСКИ КАСТРАЦИОНЕН ЦЕНТЪР

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