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COMPARATIVE ANALYSIS OF COMPLIANCE OF GENETIC POTENTIAL OF THE RACE FOR MILK PRODUCTION BREED OF GOATS ALPINA IN MUNCIPALITY OF STRUGA IN THE REPUBLIC OF MACEDONIA

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ABSTRACT

Alpine race belongs to the most dairy breeds of goats, whose race genetic potential for milk production amounts to 900 liters per lactation, which lasts about 280 days.

The purpose of this research is to carry out a comparative analysis of the implementation of the race genetic potential for milk production in the Alpine breed reared in the municipality of Struga R. Macedonia. The survey was conducted in the municipality of Struga on a sample of 20 individual goat breeders of this race.

The smallest variation of the variation width (VŠ = (Max-Min) in the sample ranges from 495 liters or 55 %, and the biggest variation of the variation width is 644 liters or 71.5 %.

The average deviation of the progress genetic potential for producing milk of goats of the Alpine breed in Municipality of Struga with extensive local technology compared to race genetic potential for milk production (which is 900 liters per lactation) variation of the variation width VŠ=(Max–Min) is 571 liters or 63.4 %.

From the results, the sample of 20 goat breeders, which is chosen based on: the use of similar technology for accommodate goats, assistive technology equipment and tools, micro-climatic conditions, selection and improvement of the quality of the resulting offspring and nutrition, we can conclude that all breeders have derogation in fulfilling the genetic potential for milk production of goats of the Alpine breed in Struga in the ratio of the race genetic potential for milk production.

Key words: goat, alpine race, genetic potential, milk.

Introduction

The production of milk and dairy products from goats are increasingly found application in nutrition at all ages of people, because of the great economic importance, which stems from many respected nutritional, dietary and medicinal properties of the products. Goat milk contains easy digestibility due to the small and fine fatty acids (linolenic and arachidonic acid), which enzymes of the digestive tract of man quickly and easily decompose (unlike cow's milk), casein is easily digestible and clot that forms in human stomach is much softer compared to milk from other animals. Also, goat milk contains calcium, phosphorus, smaller amounts of iron, lesser amounts of folate that is necessary for synthesis of hemoglobin, less vitamin C, E and B12 which are in the correct ratio and that goats hardly become infected by RNA viruses have good antiviral activity, it is recommended in the diet of children and sick persons (Popovski K, Stefanovska J., 2002).

Goat milk is suitable for manufacture of many kinds of quality cheese and yogurt, which from year to year the interest is growing. Diversity of cheeses is very large, as they usually are produced locally from farmers themselves. Therefore, cheeses are typical of the area and the region from which originate (Sutton J. D. & Mowlem A. 1991; Hetherington L. & Matthews G. J. 1999).

Alpine is a French breed of goats reared in the Alpine, created with the participation of the Swiss breeds. The animals are of medium size, strong and developed bone system and expressed the depth of the body. The head is without horns with slightly indented profile, the forehead is broad and has upright ears. Hairy coat is short and often with yellow-brown or black-brown with black legs and black dorsal line. Often you can meet and black goats. The average live weight of the goats is 60–70 kg, while at the male goats is 80–100 kg. Fertility of this breed ranges from 180–190 %.

The breed genetic potential for production of milk of this breed is up to 900 liters per lactation. In this breed of goat selection emphasis is on increasing the level of proteins, therefore characterized by higher levels of protein, because the milk of this breed are used to produce various types of cheeses (Franic I. 1993).

In The Republic of Macedonia, this race is so organized imported there are and reproduction centers aimed at improving the domestic goat population. In Struga and elsewhere in our country, there are a small number of professional, highly specialized goat farms. Breeding goats is usually combined with existing herds of sheep and the number of goats ranges from about 2–20 throats.

Materials and Methods

In the survey was conducted comparative analysis of the results on a sample of 20 individual breeders of goats in order to determine how to meet racial genetic potential for milk production from goats of the Alpine breed, which is grown in the municipality of Struga. The results was obtained from purchase center for milk Struga IMB Bitola.

For the statistical analysis of the data we used variable width VŠ = (Max – Min) in the sample. Results are presented in tables and graphics.

Results and Discussion

The survey was conducted with a comparative analysis of the results on a sample of 20 individual goat breeders to determine how to meet the racial genetic potential for milk production from goats of the Alpine breed, which is grown in the municipality of Struga.

The results are presented in Table 1 and Chart 1.

Table 1: Comparative analysis of the results obtained for the racial genetic potential for milk production from goats of the Alpine breed, which is grown in the municipality of Struga (a representative sample of 20 breeders).

Breeders	Number of heads	Quantity of milk production per farm in lactation (litres)	Average obtained by head litres per lactation	Genetic potential by head litres per lactation	Deviation from genetic potential	
					litres	%
1	22	7 810	355	900	545	60.5
2	19	6 555	345	900	555	61.6
3	17	6 885	405	900	495	55.0
4	12	4 140	345	900	555	61.6
5	11	3 872	352	900	548	60.8
6	10	3 390	339	900	561	62.3
7	9	3 105	345	900	555	61.6
8	9	2 871	319	900	581	64.5
9	8	2 816	352	900	548	60.8
10	8	2 400	300	900	600	66.6
11	8	2 408	301	900	599	66.5
12	7	2 338	334	900	566	62.8
13	7	2 086	298	900	602	66.9
14	7	2 478	354	900	546	60.6
15	6	2 250	375	900	525	58.3

Breeders	Number of heads	Quantity of milk production per farm in lactation (litres)	Average obtained by head litres per lactation	Genetic potential by head litres per lactation	Deviation from genetic potential	
					litres	%
16	4	1 180	295	900	605	67.2
17	3	765	255	900	645	71.6
18	3	882	294	900	606	67.3
19	2	600	300	900	600	66.6
20	2	608	304	900	645	71.6
Total:	**74**	**59 439**	**341.6**	**900**	**558.4**	**62.0**

VŠ = (VŠ = (Max – Min) = 900 – 405 = 495l or 55 %; VŠ = Max – Min) = 900 – 255 = 645 l or 71.6 %;
VŠ = (Max – Min) = 900 – 341.6 = 558.4 or 62 %

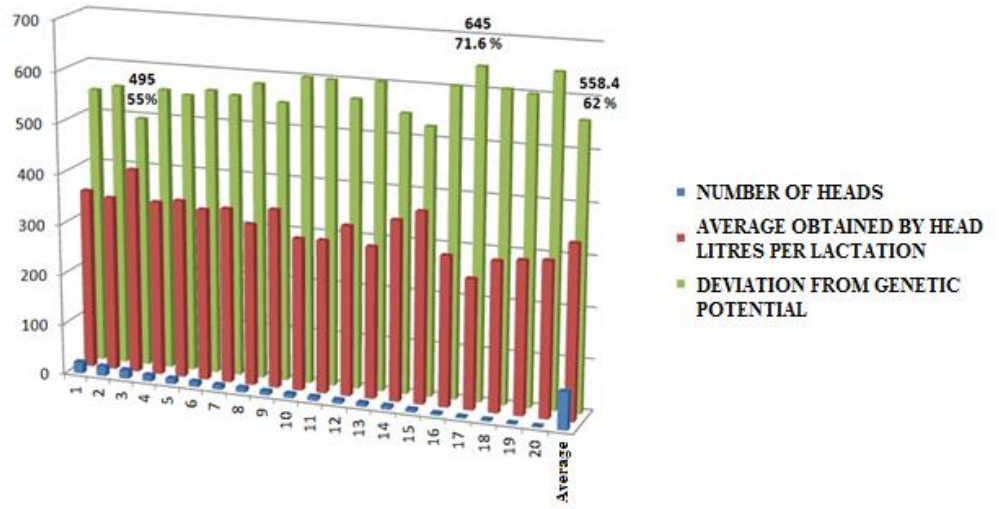

Chart 1: Comparative analysis of the results obtained for the racial genetic potential for milk production from goats of the Alpine breed, which is grown in the municipality of Struga (sample of 20 breeders).

The results, a sample of 20 goat breeders, which is chosen based on: the use of similar technology to accommodate goats, assistive technology equipment and tools, micro-climatic conditions, selection and improvement of the quality of the resulting progeny and diet can be concluded that in all breeders have derogation in fulfilling the genetic potential for milk production of goats of the Alpine breed, bred in Struga in the ratio of racial genetic potential for milk production.

The smallest variation, of the variational width VŠ = (Max – Min) is the sample of 495 liters or 55 %, while the largest variation of the variational width amounts to 644 liters or 71.5 %.

The average deviation for the entire sample of the achieved genetic potential for the production of milk goats of the Alpine breed, bred in the municipality of Struga with local extensive technology compared to racial genetic potential of the milk production (which is 900 liters per lactation), varying varacionata width VŠ = (Max – Min) is 558.4 liters or 62 %.

Similar results were obtained in studies conducted by the Ministry of Agriculture of the Republic of Macedonia, and published in the Strategy of improving and monitoring the quality of milk 2013–2020, the share of the population dairy goats which are covered in the survey, that is under the control of productive and reproductive traits. It was found a significant reduction in usability of genetic capacity of of heads as a result of the low level of primary education of the farmers, educating farmers and the secondary use of contemporary technologies in goat production (breeding, accommodation, care, selection, improving the quality of the resulting progeny and nutrition of heads

among the majority of the farms). Also in this study, the stated reasons are limiting factors in terms of improving the milk yield of heads, and improve the genetic capacity of heads.

Conclusions

From the obtained results of a sample of 20 goat breeders, we can conclude that all have derogation in fulfilling racial genetic potential for milk production of the goats of the Alpine breed (which is 900 liters per lactation per head), grown in the municipality of Struga.

The smallest variation, of the variational width VŠ = (Max-Min) is the sample of 495 liters or 55%, while the largest variation of the variational width amounts to 644 liters or 71.5 %.

The average deviation for the entire sample of the achieved genetic potential for the production of milk goats of the Alpine breed, bred in the municipality of Struga with local extensive technology compared to racial genetic potential of the milk production (which is 900 liters per lactation), varying varacionata width VŠ = (Max-Min) is 558.4 liters or 62 %.

Farmers, breeders of goats from dairy breed Alpina grown in Struga, do not achieve the racial genetic potential for milk production, as a result of the low level of primary education of the farmers, educating farmers and the secondary use of contemporary technologies in goat production (breeding, accommodation, care, selection, improving the quality of the resulting progeny and nutrition of heads among the majority of the farms). Also in this study, the stated reasons are limiting factors in terms of improving the milk yield of heads, and improve the genetic capacity of heads.

The research we concluded that the National Extension Agency of the Republic of Macedonia, responsible for implementing the advisory component in their advisory programs, should plan to put greater emphasis on the development of goat breeding in the municipality of Struga and wider in our country, because the goat as a branch of animal husbandry, has great significance for the development of rural areas, rural tourism in the production of quality products.

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THE ROLE OF DG SANTE IN THE ADOPTION OF VETERINARY LEGISLATION AND FOOD SAFETY

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ABSTRACT

The law-making process in the EU is a complex and sometimes very long process. Depending on the nature, scope of application and the type of the draft act, this process is a multistep one. It is implemented by the three institutions in the European Union (EU)- the Council, the European Commission (EC) and the European Parliament (EP), working in coordination. In operational line the law-making procedures carried out by DG SANTE within the margin of the internal consultation procedure (intra-SANCO consultation) and the inter-service consultation level (inter-service consultation) are discussed.

The work of the Standing Committee on Plants, Animals, Food and Feed (SCPAFF) is described in details. In addition, a mechanism of operation and interaction between the Council, the European Parliament and the Commission and three types of procedures for the adoption of primary legislation: the consultation procedure (consultation) cooperation (cooperation) and a co-decision or the ordinary legislative procedure (co-decision) has been analyzed.

Key words: European Union, primary and secondary legislation, food safety, legislative procedure.

Introduction

The EU law making process is a complex and sometimes very long process. It depends on the nature, scope of application and the type of draft acts and this process is a multistep one. The process is implemented by the three institutions of the European Union (EU) - the Council, the European Commission (EC) and the European Parliament (EP), working in coordination with each other. The EU institutions have competence in the field of law. It is not universal, but limited within the defined and permitted by the Member - States (MS) legislative competence.

Concerning the legislative competencies in the veterinary field (including animal health and welfare and food safety and quality) the EC is being supported by DG SANTE. In some cases, the views of stakeholders' organizations are taken also into account when the proposals for amendment of veterinary legislation are being prepared by experts. Examples how the social and public choices reflect the policy making in the veterinary field are given by Matt (2014), McInerney (2004), Garner (1993) [7, 8, 5].

Material and methods

For the purpose of the study we made a content analysis [9] of official documents from the EU legislation, concerning the procedures for the exercise of implementing powers conferred on the Commission and procedures in matters of food safety.

Results and discussion

European Union law consists of primary legislation - Treaties and secondary legislation - regulations, directives and decisions adopted by the Union institutions on the basis of the Treaties.

In the association with the Lisbon Treaty [6] only the Commission has the right to submit legislative proposals – draft acts (right of initiative), except where the treaties provide otherwise. It may also change or modify any its own proposal (Article 293, paragraph 2) [12].

The primary legislation is constantly relevant and covers treaties establishing the European Community and the European Union, the accession treaties of the new Member States and other treaties and protocols, bringing changes or complementary norms of Treaties. By its nature primary law is comparable to the constitutional law at national level. It regulates the fundamental principles and essential characteristics of the European Communities and, later on, of the European Union, the legislative procedures, the Union institutions and their competence. The adoption of the treaties themselves is a subject of the direct negotiations between the governments of the Member States, after which they are ratified in accordance with national procedures used in relation to international contracts (usually by national parliaments or by referendum).

The secondary legislation is the second major source of Union law. It involves unilateral acts and agreements. Unilateral acts can be divided into two categories:

- mentioned in Art. 288 of TFEU [12]: regulation, directives, opinions and recommendations;
- not mentioned in Art. 288, so called non typical secondary legislation that includes the communications, white and greens books et cet.

The secondary legislation introduces the primary legislation where necessary and is therefore known as the implementing legislation. Most legislation adopted by Council and Parliament provide a legislative basis for the introduction of further legislation (secondary or supplementary legislation), which is prepared and adopted by the Commission. With its adoption, it certifies that the measures taken correspond to the particular circumstances or refer to the technical details and that their updating can be done as quickly as possible. The vast majority of EU legislation or secondary implementing legislation is made by the Commission.

The main mission of DG SANTE is to make Europe a healthier, safer place, where citizens can be confident that their interests are protected. A zero-risk society may not be possible but it is doing as much as it can to reduce and manage risks for the EU citizens.

DG SANTE is organized into seven directorates (Fig. 1) and has approximately 850 employees, 600 of whom are based in Brussels, 75 in Luxembourg and the other 175 in Ireland (Grange, County Meath).

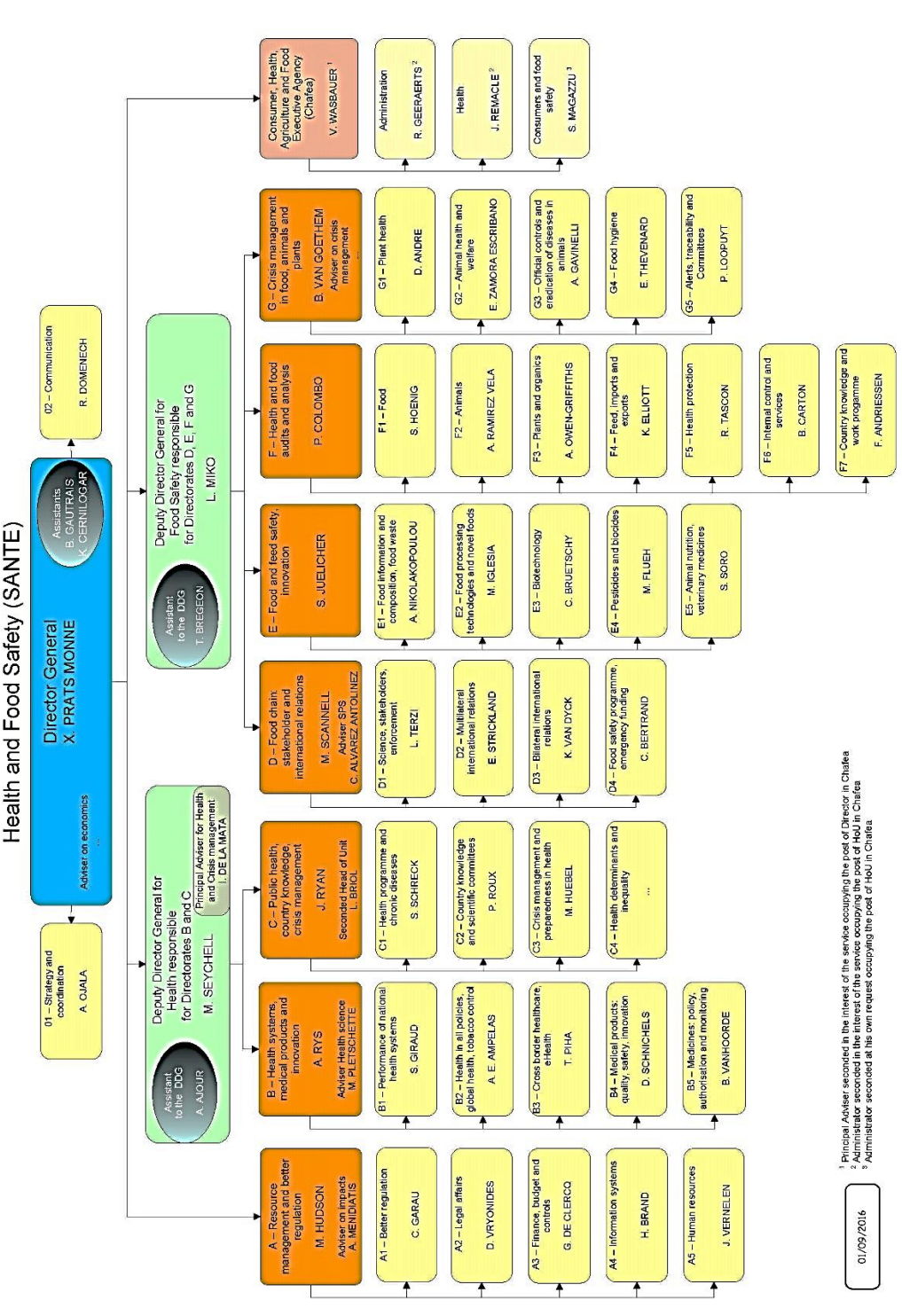

Figure 1: Structure and organization of DG SANTE.
(Source: http://ec.europa.eu/dgs/health_food-safety/chart.pdf)

Directorate D „Veterinary Affairs and International Relations" consists of seven units that are relevant to veterinary legislation and food safety:

Unit 1 – Animal feed;

Unit 2 – Animal Health;

Unit 3 – Animal welfare;

Unit 4 – Food systems for rapid communication and training;

Unit 5 – Food Chain and spending in the veterinary field;

Unit 6 – Multilateral international relations;

Unit 7 – Bilateral international relations.

The main tasks of the directorate include:

- ensuring a high level of health protection and animal welfare, including the drafting of the legislation on animal by-products, and zootechnical standards;
- ensuring the free movement of feed / food and feed / food materials, rules on labeling requirements for hygiene, as well as registration and control of producers and traders of feed and food for animals;
- to oversee the application of the requirements for animal health and food safety in bilateral international agreements with third countries to which the EU is a party;
- to contribute to the rights and obligations of EU health and food safety included in multilateral international agreements, especially WTO and international standard-setting bodies - the OIE and Codex Alimentarius;
- Management of the Standing Committee on plants, animals, food and feed (PAFF Committee).

The proposals to amend the veterinary legislation in DG SANTE are prepared by the experts in the relevant departments of the Directorate "D". If necessary, underservice groups of officials from other DGs, which are relevant to a given problem (DG Trade, DG Agriculture, etc.) are created. In some cases the views of stakeholders' organizations (producers, NGOs) and the international organizations of the relevant committees and "ad hoc" panels, which include selected veterinary experts from some MS. With the creation of the European Medicines Agency (EMA) [4], the European Food Safety Authority (EFSA) [3] and the European Centre for Disease Prevention and Disease Control (ECDC) [2], recommendations of a scientific or technical nature given are also requested from these organizations. All proposals for legislation initially follow so called „road map" and must be accompanied by an impact assessment and a detailed financial statement.

The next step in the legislative process is intra-SANTE consultation procedure, where the draft document is sent to all other departments of the Directorate for comments and observations. After gathering of the all reflections, the document is sent for inter-service consultation the other relevant Directorates General (mostly these are DG Agriculture, DG Trade) and the Commission's Legal Service.

The Commission is often empowered to implement EU legislation with the assistance of committees. It is done through the comitology process. The term „comitology"refers to the way in which the Commission exercises its implementing powers conferred on it by the EU legislation by the support of the working groups called committees. The latest are composed of representatives from the EU Member States and chaired by the Commission staff.

Broadly speaking, before being able to perform a legal act of the EU, the Commission must consult the detailed implementing measures (known as "implementing acts") offered by the committee. The Committee provides opinion on the Commission's proposed measures. This opinion may

be mandatory or optional for the Commission depending on the procedure referred to in the legal instrument applicable. A detailed description of the functioning of comitology is defined in the Regulation (EC) № 182/2011 [11] that lays down the rules and general principles concerning mechanisms for control by Member States on the exercise of implementing powers of the Commission. It defines two types of procedures. The choice of procedure for each committee is indicated by the EU legislator in the basic instrument depending on the type of implementing powers.

According to Article 3 of Regulation (EC) № 182/2011 [11], PAFF Committee is a regulatory committee which assists the European Commission in developing measures for food safety and veterinary issues. The Committee shall consist of representatives of the 28 Member States and observers from member countries of the EEA (Iceland, Liechtenstein and Norway) and Switzerland. The Committee is chaired by a representative of the European Commission (level department heads in the „D"). Its power covers the entire food chain including animal health and plant, animal welfare, animal feed and animal feed, food safety and animal products. Thereto the following sections are created:

- General food law;
- Biological safety of the food chain;
- Toxicological safety of the food chain;
- Controls and requirements on imports;
- Animal nutrition;
- Genetically modified food and feed and environmental risk;
- Health and welfare, plant protection products, plant health;
- Seedlings and ornamental plants seeds in agriculture;
- Horticulture and forestry, for variety testing;
- Wine.

Three sections of the committee - animal health and welfare, biological safety of the food chain and controls and requirements on imports cover a wide range of food safety and other veterinary issues so that decisions of the Commission are relevant for the implementation of veterinary measures. The Commission provides for the opinion of the committee all proposals of the secondary legislation. The Committee works in the following way:

The Chairman brings the draft of the implementing act, which is to be adopted by the Commission (it is included in the invitation and agenda for each meeting of the Section Committee). Except in duly justified cases, the Chairman shall convene a meeting not earlier than 14 days after the date of submission of the draft implementing act and the draft agenda of the committee. Within this period, the members of the committee have the opportunity to consider the proposed agenda and draft acts to express their views or to make a proposal for changes. Usually, the procedure is performed electronically. Until the committee delivers an opinion, any committee member may suggest amendments and the chair may present to all modified versions of the draft implementing act. In duly justified cases, the Chairman may obtain the committee's opinion by written procedure. Usually this is done in exceptional cases of implementing acts immediately applicable. The Chairman sends to the committee's members the draft implementing act and set a deadline for an opinion according to the urgency of the matter. For each committee member who does not oppose the draft implementing act or not explicitly abstain from voting before this deadline shall be deemed to have given his tacit agreement to the draft implementing act.

PAFF Committee adopts implementing legislation for two procedures: the examination and consultation procedure

The examination procedure applies in particular to adopt implementing acts of a general nature and other implementing acts, mainly related to programs with a significant impact on food safety, the common agricultural policy and the common fisheries, environment, security and safety or protection of health or safety of humans, animals or plants and common commercial policy. When the procedure applies, the committee shall deliver its opinion by the majority provided for in Article 16, paragraphs 4 and 5 of the Treaty on functioning of the European Union ([12]) and, where applicable, Article 238 paragraph 3 of the TFEU ([12]), for acts should be adopted on a proposal from the Commission. The votes of the representatives of the Member States within the committee shall be weighted in the manner set out in those articles.

The current system for reporting the votes in the SCFCAH is given in Table 1.

Table 1: Member States Votes in the SCFCAH.

Member States	**Votes**
France, Germany, Italy, United Kingdom	**29**
Poland, Spain	**27**
Romania	**14**
The Netherlands	**13**
Belgium, Czech Republic, Greece, Hungary, Portugal,	**12**
Austria, Sweden, Bulgaria	**10**
Denmark, Finland, Ireland, Lithuania, Slovak Republic, Croatia	**7**
Cyprus, Estonia, Latvia, Luxembourg, Slovenia	**4**
Malta	**3**

Qualified majority: 352 votes expressing the votes of 28 Member States

When the committee gives a favorable opinion (in favor), the Commission shall adopt implementing act. If the committee gives an unfavorable opinion (not in favor), the Commission does not adopt the draft implementing act. Where an implementing act is deemed necessary, the Chairman may either submit to the same committee an amended version of the draft within two months after giving an adverse opinion or within one month of such delivery to submit a draft implementing act in appeal committee for further discussion.

When there is no opinion (failure to deliver an opinion), the Commission may adopt the draft implementing act. If it does not accept the project implementation, the President may submit an amended version thereof. However, when it relates to the protection of health or safety of humans, animals or plants, the basic act provides that the draft implementing act cannot be adopted if it is not delivered, or a simple majority of committee members opposed the draft, then the Commission shall not adopt the implementing act.

The consultation procedure applies as a rule for the adoption of implementing acts not falling within the scope of the examination procedure. However, in duly justified cases, it can also be applied for the adoption of implementing acts in other procedures. Through it, the committee shall, where necessary, deliver its opinion by voting. If the committee votes, the opinion shall be adopted through the simple majority of its members. The Commission shall decide on the draft, which is to be adopted, taking into account the full extension of the conclusions of the discussions within the committee and of the opinion.

The PAFF Committee may adopt implementing acts in exceptional cases and implementing acts immediately applicable. Furthermore, the regulatory procedure with scrutiny provided for in Article 5a of Decision 1999/468/EC ([1]) on comitology still apply in relation to all the main legal

instruments in which reference is made to this procedure. If the Commission is prevented from applying its proposed implementing measure (especially when the committee voted against it under the examination procedure), it may refer the case to the Appeal committee.

The Appeal Committee is composed of representatives of the Member States, chaired by the Commission's representative and has the same voting rules as other committees. It is not a permanent body, but rather a procedural tool that allows the Member States to hold a second discussion with representatives of the higher level. If the Appeal Committee votes against the Commission's proposed measure, the Commission must comply with this decision. When the Appeal Committee gives a favorable opinion, the Commission shall adopt the draft implementing act.

In the event that no opinion is delivered by the Appeals Committee, the Commission may adopt the draft implementing act.

Within the work of the PAFF Committee a Member State may delegate another right to represent it at meetings of the Committee, but a country may represent only one other Member State.

More information on the Standing Committee on animal health and the food chain, including the most recent reports and opinions can be found at:

http://ec.europa.eu/food/committees/regulatory/index_en.htm

Once the final text is finalized and the text is translated into the official languages of the EU, the draft act is published in the Official Journal of the European Union.

As for veterinary legislation to be approved within the context of Article 152 (public health) and Article 251 (procedures) of TFEU ([12]), the legislative procedure used is a co-decision procedure (co-decision). By applying its many legislative acts are entitled as "Regulation or Directive of the European Parliament and the Council", rather than just regulations and directives. It should be emphasized also that a large amount of primary legislation on food safety is adopted by this procedure, such as Regulation (EC) 178/2002/EC ([10]).

Summary of key legislation on food safety is available at the following link: http://europa.eu/legislation_summaries/food_safety/index_bg.htm.

In conclusion it can be said that the work in the EU law making structures is well organized with a view to achieving maximum effect process and making consensus decisions.

Conclusion

In conclusion it can be said that the work in the EU law making structures is well organized with a view to achieving maximum effective process and making consensus decisions. Finally, the most important point remains the implementation of legislation. Although there is a technically and legally appropriate legislative basis, which is correctly drafted and designed for long term, its effective and satisfactory implementation depends on several important factors. Among them we could point out the competencies and capacities of national Food Safety Agencies for execution of their enforcement duties, and the involvement of all stakeholders in the whole process of decision-making in order to be interested in the implementation of the adopted measures.

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TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2016, vol. 1, No 1(1): 15–20

HISTOLOGIC FEATURES OF THE DOG'S MAMMARY GLANDS BLOOD SUPPLY

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ABSTRACT

The purpose of this study was to establish and characterize the kinds and types of blood vessels microcirculation from parenchyma of the dog's mammary glands.

The incoming blood vessels in mammary complexes and their branches in inter- and perilobular connective tissue septs, as so the following smaller blood vessels in glandular parenchyma were investigated. These vessels presented on histological slides from bitches' mammary gland in diestrous cycle were obtained and prepared by conventional histological method and was observed. The samples were fixed in solution 10 % neutral formaldehyde, then cut by microtome at 7 μm and stained with Hematoxylin and Eosin.

Myotypical arteries and arterioles in specific position as rosette were established in mammary teats and postarteriolar capillaries as so venules and small myotypical veins in inter- and perilobular connective tissue septs were observed. Capillaries decrease their number in correlation of glandular parenchyma involution.

The established blood vessels morphologic features in bitches' mammary glands are important for glandular functional characteristic in norm and as so frequently happened pathological conditions, especially in mastitis and neoplasms.

Key words: dog, microcirculation, blood vessels, mammary gland, histology.

Introduction

Mammary gland (MG) is the study subject by many authors, both because of important economic significance of its secretion and therefore frequently observed pathological changes associated with functional impairment. In this regard, the basic morphology knowledge of the lymph and blood vessels is necessary, and also in connection of the metabolic processes provided, especially on microcirculatory level.

Diagnosis and treatment against mammary gland diseases are serious problem in clinical pathology. There are events, e.g., where after a false pregnancy accompanied with lactation, it may pass without treatment. But the problem is serious in connection with cases of mastitis and mammary oncologic disorders.

Neoplasms of the dog's MG are common in this species and have higher percentage for further development in comparison with other domestic mammals. Dogs 25–35 % of all tumors, and 24 % cats, and mainly get ill adult animals (8–11 years).The tumors grow slowly, about two-thirds of them are localized mostly in the last or penultimate mammary complex (MC). In comparative aspect, tumors are more common in the dog, while in the cat they are more malignant (85 %). Average about 50 % of mammary tumors in dogs are clinically malignant neoplasias in which metastasize through blood and lymph way.

Object and purpose of the research is blood circulation to the MG in the dog, because the bloodstream and lymphatic drainage have contemporary relevance for both anatomical structure and for surgical interventions and manipulations for treatment in disease processes.

Materials and Methods

For the purpose of the study was used fresh tissue material from a female dog euthanized in private veterinary clinic for reasons other than the tasks of our research. The animal was in later dioestrus or anoestrus, showing different stages of MG involution significantly advanced in breast MC, while caudal MC were still larger size, which is characteristic for delayed involution. The material was taken from different MC from sections with incoming blood vessels and other parenchyma of the MG. Samples with cuboidal form and dimensions of the walls of about 1 cm, were preserved in 10% buffered formalin and subsequently treated conventionally to their inclusion in paraffin blocks. Sections were made with a microtome in a thickness of 7 μm, and stained with hematoxylin and eosin, and subsequently prepared permanent histological slides.

The slots were observed with a light microscope Olympus CH 21FS1 (China), and morphometry was performed by eyepiece-micrometer. Foto documentation was processed by camera Olympus C-5050Z (Japan).

Results and Discussion

The investigated dog according to the criteria of Chandra, S. A. et al. (2010) was determined in cycle of stage IV from latest diestrus or anoestrus, which was conformed by histological findings and macroscopic state of the mammary glands. In this condition we decided to trace the blood circulation but we could not find enough details about microvascularisation of the dog's mammary gland in the literature.

Svilikova (according to Kovatchev, (1976)), experimentally studied the microvascular bad of canine mammary gland in comparative aspect that was seen in women. She observed dendritic branches of the large gland vessels into interlobular septes connective tissue and she founded that terminal outside branches cover densily every lobule. Some vessels, mostly blood capillaries, enter the lobule like a loop which cover the glandular alveoli. It was stated that there is a correlation between vascularization, age and functional state of the gland expressed by strong development of vasculare meshwork in actively lactating MG, which fact has been conformed also in a sheep [1].

Vascularization of the excreting system of the mammary gland is poorly researched, especially in the wall of the lactiferous ducts and channels. For them, there is not much information as well as for productive animals and for the woman. The blood circulation to the mammary gland's fascia is slightly investigated but it is important morphological and functional part of the gland.

Furstenberg, Riederer and Rubeli according to Kovachev, G. (1976) found in the cow papilla wall a specific vasculary-musculare layer which is richer with blood vessels, mainly longitudinal veins, and they have identified this vascular area as corpus cavernosum.

In our previous studies by contrast radiography was found that dog's papilla shows several mainly longitudinal myotypic arteries and arterioles.

Kovatchev (1976), wrote that Kaeppeli, describing the microscopic structure of the papilla in sheep indicated that in the middle layer of it's wall between the lining mucosa of the milk cystern and skin at longitudinal direction several large blood vessels has passed. The veins are more numerous than the arteries and are equipped with valves. Such a picture described by other authors [2], we have confirmed in several sections of teats in the dog. In a papilla, counting about 8–12 transversely cuted arterial vessels which are ring-like placed around the terminal lactiferous ducts. The vessels are located at the position between the skin and the excretory system of the gland. Valves of the

veins in the dog in this area were not observed (Fig. 1). Into dog mammary complexes several channels systems usually from 6 to 12 were observed in our sections.

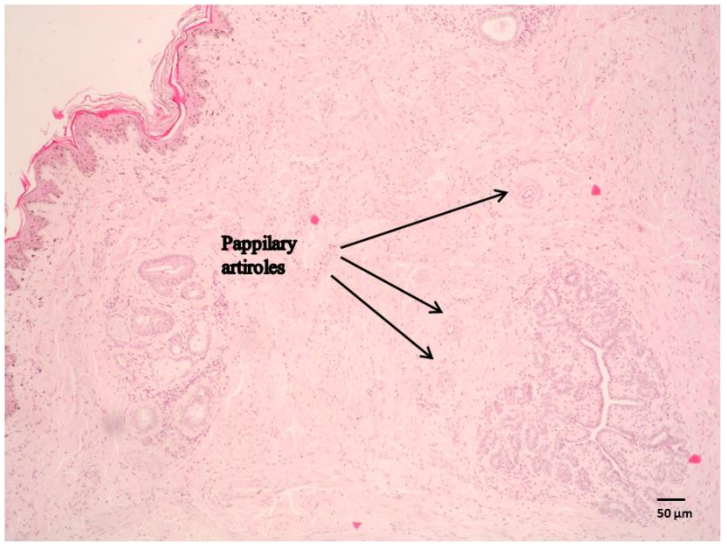

Figure 1: Transversal (cros) section of some pappillar arterioles. H&E. X40.

According to literature information (4) and our macro- and microscopic observations the involution of the gland is occured first in cranial MC and at least on the lenth of the abdominal and iliac MC. In glandular lobes, there are single alveoles with eosinophilic excretion in it (Fig. 2).

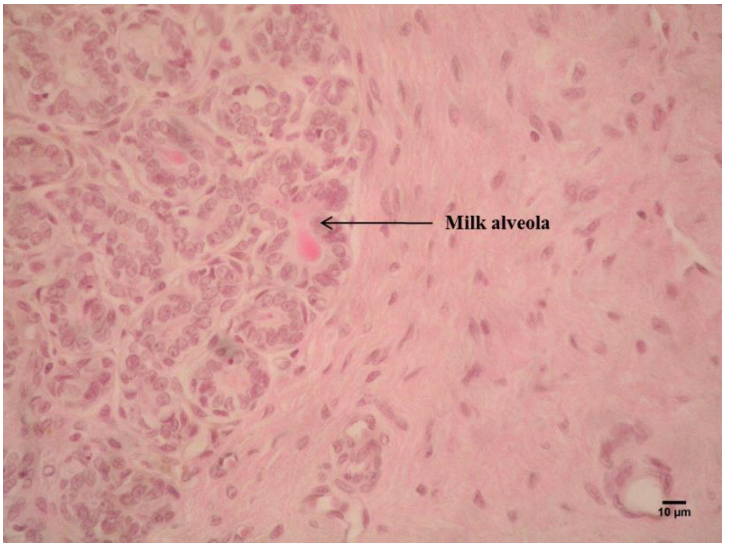

Figure 2: A milk alveola from inguinal mammary gland with an eosinophilic secret in the lumen. H&E. X400.

On histological slides of thoracic MC was observed highly developed arteriolo-venular and capillary circulation, and lymphocapillary meshwork around slightly developed parenchyma, against which prevails well-developed ductal system. On most preparations especially the thoracic

MC prevails connective tissue that replaces lobes of glandular parenchyma. This illustrates the morphological process of involution of the MG, which is in the advanced stage of abdominal and iliac MC.

It was counted that one lob of the gland is average 11–28 cross cut capillaries (average of random lobs or ductal systems of different sections and parts of the gland). This gives a partial quantification of blood flow to the mammary gland.

A. thoracica lateralis can be defined as arteria myotypica, in which media is occured in trace single elastic fibers among smooth muscle cells. There is not observed valves in the corresponding vein.

Rr. perforantes and rr. mammarii of a. thoracica interna are covered by a capsule or rather sleeve of connective tissue together with satellite venous vessels and nerve fibers. In the media of these vessels it was noticed hardly a single elastic fibers. In our view (Fig. 3) these are transition forms from mixtotypical (rr. perforantes) to the typical muscle type arteries (rr. mammarii). Some of the relevant medium veins have valves.

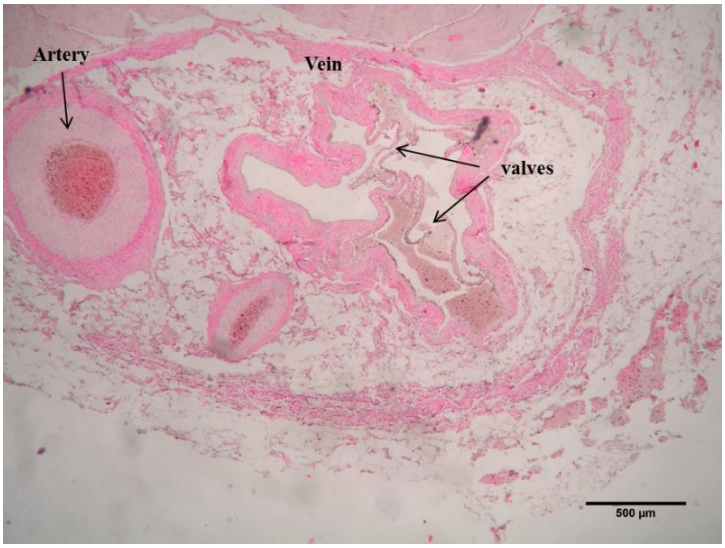

Figure 3: Rr. perforantes near to second thoracic mammary gland surrounded with connective tissue capsule. A vein with a valves is visible on the right of the artery. H&E. X40.

A cut which consists skin of the gland, parenchyma and underlying muscle in the second thoracic MC was observed relatively large muscle type artery and medium-sized vein (with a few smooth muscle layers). These vessels were derived probably from direct branch of a. thoracica lateralis for the glandular complex. The location of these vessels from the lateral side of the gland gives us reason to believe that there are not a branches from a. thoracica interna, which as perforating must first pass through the muscles (Fig. 4).

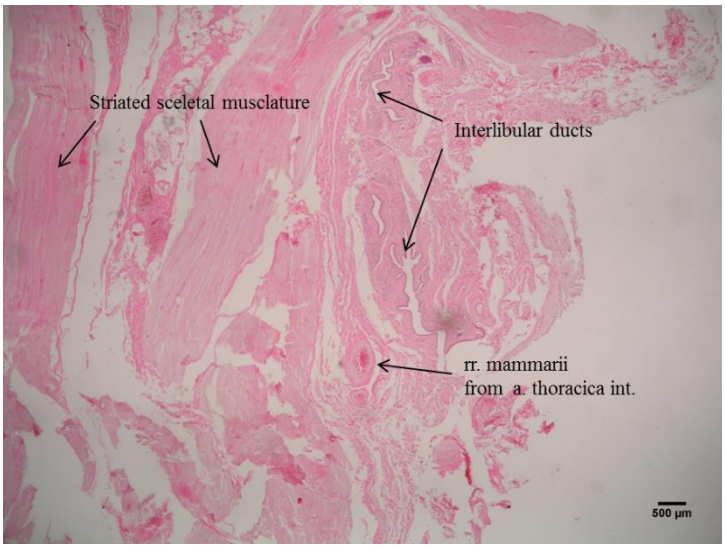

Figure 4: Slice from dog's thoracic mammary gland. On the right are interlobular lactiferous ducts, on the left are bundles from thoracic musculature. H&E. X40.

A. pudenda externa is more of some mixed type arteries. It owns as many elastic fibers so many smooth muscle fibers. The internal elastic membrane is clearly visible on the border with tunica intima, after which follows a broad middle layer of smooth muscle cells and elastic fibers. It is notable that the elastic fibers are concentreted peripherally from the smooth muscle layer, followed by the middle layer passes in a thin layer adventitia in which the capillaries were observed by the composition of the vasa vasorum. Several outgoing from gland parenchyma postcapillary venules were observed (Fig. 5).

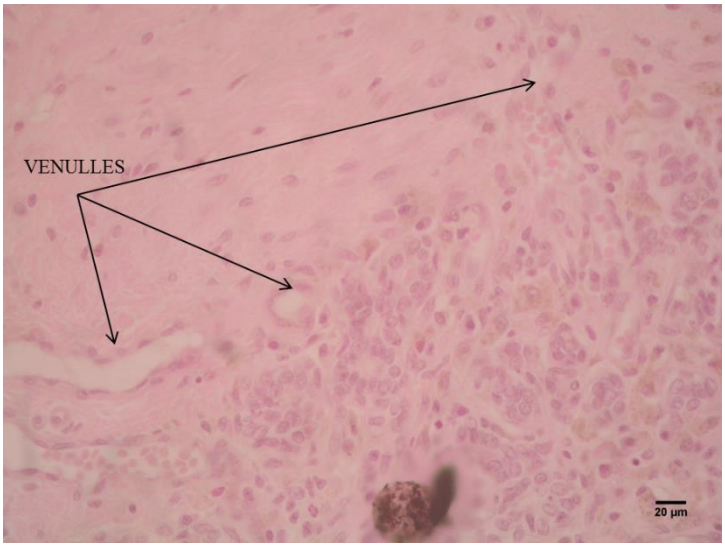

Figure 5: Outgoing postcapillary venulles from a mammary lobule of inguinal dog's mammary gland. H&E. X400.

Conclusions

1. The Incoming arteries to mammary complexes are the type arteria myotipica, or arteria mixtotypica. The arteries in perylobular connective tissue are arteries of muscular type or arterioles. In arteries there are relatively more elastic fibers, beacause of its proximity to the heart.
2. Thoracic mammary complexes undergo faster involution of the parenchyma compared with abdominal and inguinal.
3. In lower parts of the mammary gland and its papilla is mostly seen longitudinal blood vessels as arterial prevail over venous.
4. In glandular lobs was seen well-developed blood and lymphatic vessels system, which on average quantify 11-28 transversely cut capillares.

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HEPATIC PRENEOPLASIA INDUCED BY N-NITROSODIMETHYLAMINE AND N-NITROSODIETHYLAMINE IN JAPANESE QUAIL EMBRYOS

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ABSTRACT

Toxic and carcinogenic effects induced *in ovo* by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos were studied by histopathological methods. The obtained results indicate that both compounds induce preneoplastic hepatic alterations. The spectrum of macroscopic and microscopic lesions identified in carcinogen-treated embryos has been presented. The significance of avian embryos as an inexpensive and reliable model system for studies on hepatocarcinogenesis has been briefly discussed.

Key words: hepatocarcinogenesis, preneoplasia, avian embryos, japanese quail, N-nitrosodimethylamine, N-nitrosodiethylamine.

Inrtroduction

Neoplastic diseases are a serious health problem with a great importance for both veterinary and human medicine. Experimentswith with laboratory rodents are still the main approach used in the scientific investigations on the factors and mechanisms responsible for the initiation and progression of cancer.

In recent years, issues related to the ethical aspects of biomedical research and the welfare of experimental animals have been gaining an increasing significance. There is a growing interest and a desire for implementation of more reliable, rapid and cost-effective alternative methods to supplement and/or replace animal experiments (Knight et al., 2006; Benigni et al., 2013; Anadón et al., 2014; Marone et al., 2014).

Avian embryos are a model system attracting the attention of experimental oncologists as an alternative to laboratory animals, which provides a multitude of possibilities for exploration of various processes related to carcinogenesis such as genotoxicity, mutagenicity, metastasis, angiogenesis, etc. as well as for assessment of carcinogenic/antineoplastic activity of various environmental factors (Enzmann et al., 1997; Wolf et al., 2008; Enzmann et al., 2013; El Hasasna et al., 2016).

Here, we present results from a study of the ability of the known carcinogenic compounds N-nitrosodimethilamine and N-nitrosodiethilamine to induce preneoplasia in the Japanese quail embryonal liver.

Materials and methods

Avian embryos. Fertilized Japanese quail (Coturnix japonica) eggs were obtained from pathogen-free flocks bred in a certified Bulgarian farm.

Chemical carcinogens and in ovo treatment. N-nitrosodimethilamine (NDMA; CAS № 62-75-9; Sigma-Aldrich) and N-nitrosodiethilamine (NDEA; CAS № 55-18-5; Sigma-Aldrich) were dissolved in sterile double distilled water and applied as a single dose of 100µg/egg with an injection volume of 100µL. The eggs were incubated at 37.8 ± 0.5 °C and 70 ± 10 % relative humidity in an automatic rotating incubator. Carcinogens were applied into the egg albumen during the first hours of incubation. Control eggs were inoculated with an equal volume of the vehicle. Four days before hatching, the incubation was terminated and the embryos were weighed and examined for gross lesions.

Histopathology. The livers of the control and of the treated embryos were dissected, weighed and immediately fixed in 10 % buffered formalin. The tissue samples were routinely dehydrated, paraffin embedded, sectioned at 5µm and stained with hematoxylin and eosin (H&E). Histopathological lesions were identified and documented with microscope Leica DM 5000 B.

Statistical analysis. The statistical significance of the differences between the control and the treatment groups was evaluated by GraphPad Prism software package, using one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test. Values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

Results and discussion

Gross pathology revealed a significant reduction of body mass (Tabl. 1) and well-demarcated hepatic lesions in carcinogen-treated embryos. In both treatment groups, the livers were with diffuse reddish-green coloration, occupying in most cases 2/3 of hepatic parenchyma. In addition, multiple petechial hemorrhages were found in some experimental embryos. Gross pathological lesions were observed in 85.7 % and 63.30 % of the embryos treated with NDEA and NDMA, respectively (Fig. 1). The alterations of the embryo weight, the absolute and relative liver weight, induced by NDEA and NDMA, were examined as important indicators for the toxic and carcinogenic potential of the tested compounds (Tabl. 1).

Table 1: Effects of the *in ovo* treatment with N-nitrosodimethylamine and N-nitrosodiethylamine on the embryo and liver weigh.

Experimental groups	Number of embryos	Embryo weight (g)	Liver weight (g)	Relative liver weight (%)
NDEA	14	5.28±0.12***	0.14±0.01	2.54±0.21
NDMA	11	4.86±0.09***	0.15±0.02	2.97±0.30
Control	12	5.76±0.10	0.12±0.01	2.01±0.18

The *in ovo* treatment with both chemical compounds induced a statistically significant reduction of the embryo weight. The absolute and the relative liver weight were increased as compared to the control; however, the established differences were not statistically significant (Table 1).

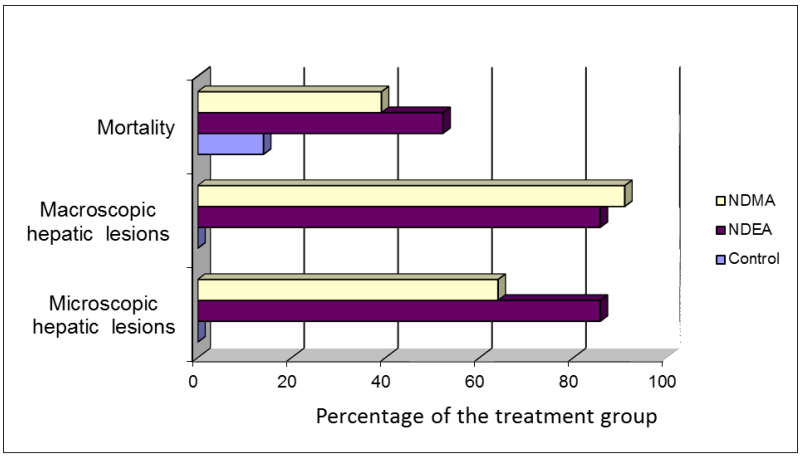

Figure 1: Mortality and hepatic lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos

Histopathological examination of the liver sections from NDMA- and NDEA-treated embryos showed the presence of foci of altered hepatocytes (FAHs) with a clear cell and basophilic phenotype (Fig. 2a). The application of both hepatocarcinogens induced spongiosis (Fig. 2b) of liver parenchyma and obstruction of bile ductules by bile plugs (Fig. 2c). In addition, megalocytes and a clearly pronounced hyperplasia of cholangiocytes (Fig. 2d) were found in some carcinogen-treated embryos.

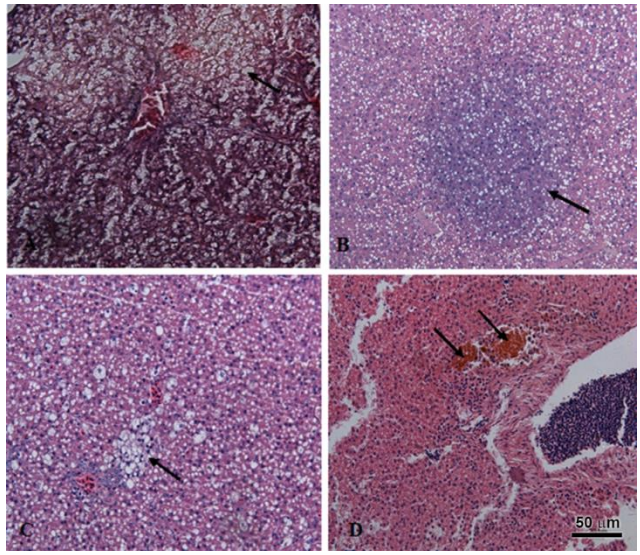

Figure 2: Light microscopy of liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos

A – Basophilic focus of altered hepatocytes; B – Spongiosis hepatis; C – Bile thrombi; D – Hyperplasia of cholangiocytes; a-,b- in ovo treatment with 100 µg NDMA/ egg; c-,d- in ovo treatment with 100 µg NDEA/ egg; H&E staining; bar = 50 µm.

Our results are in accordance with previous findings (Enzmann et al., 1997), which demonstrate that the application of NDEA induce preneoplastic lesions in Japanese quail embryonal liver. In addition, we were able to demonstrate formation of bile thrombi and marked hyperplasia of cholangiocytes in the livers of NDEA-treated embryos. Identical focal hepatic alterations were identified after the *in ovo* application of NDMA, which confirms the previously published data indicating that the FAHs show similar cellular phenotype irrespective of the carcinogenic agents by which they was induced (Bannasch et al., 2003, Su and Bannasch, 2003).

Foci of altered hepatocytes 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). These preneoplastic lesions have been widely used as endponds in carcinogenicity testing as well as in studies on the molecular mechanisms of early neoplasia (Bannasch et al., 2003; Pitot et al., 2007; Tsuda et al., 2010; Enzmann et al., 2013).

Conclusion

The results of the present study indicate that the hepatocarcinogens NDEA and NDMA initiate carcinogenesis in embryonal Japanese quail liver. The fact that preneoplasic hepatic lesions develop within just 14 days highlights the significance of avian embryos as a valuable model system that could contribute for the reduction of animals used in experimental oncology.

Acknowledgement

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CYTOLOGICAL CHARACTERISTICS OF ENDOMETRITIS IN DAIRY CATTLE

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ABSTRACT

In the last decades, related to increased milk yield, the reproductive performance has rapidly decreased in dairycows, especially in the Holstein breed. Although milk yield is negatively associated with reproductive-performance, there are other additional factors which affect the fertility in dairy cattle, such as animal health-condition, management and balanced rations. Additionally, physiologic dysfunctions, such as uterine infections, areelements which are responsible for decreased reproductive performance and fertility in dairy cattle. The objective of this study was to obtain a clear view over normal cell clusters in cow`s vagina and uterus, so this information will be useful for comparison in future examination related to rapid cytology diagnosis. Neutrophils are the first and most significant inflammatorycell involved in endometritis, but are also foremost duringnormal uterine involution. The inflammatory cell response in cases of subclinical endometritis is widely believed to bequantifiably more severe than that associated with normalinvolution yet milder than clinical endometritis. Such cytological diagnostic approach is useful for both – normal and infected vagina/uterus with or without presence of discharge. Vaginoscopy is a rapid and simple technique for thediagnosis of purulent vaginal discharge. Clear mucus isnormal, whereas purulent and foul-smellingdischarge are indicative of disease. Other ways of detecting uterine dischargehave been studied, including the gloved handand theMetricheck device (Simcrotech, Hamilton, New Zealand).

The results show clear relation between cytological positive diagnosis and affected condition of the reproductive function.

Key words: subclincal endometritis, cytological diagnosis, dairy cattle

Introduction

In the last decades, related to increased milk yield, the reproductive performance has rapidly decreased in dairy cows, especially in the Holstein breed. Although milk yield is negatively associated with reproductive performance, there are other additional factors which affect the fertility in dairy cattle, such as animal health condition, management and balanced rations. Additionally, physiologic dysfunctions, such as uterine infections, are elements which are responsible for decreased reproductive performance and fertility in dairy cattle (Chebel et al. 2007).

The objective of this study was to obtain a clear view over normal cell clusters in cow`s vagina, so this information will be used for comparison in future examination related to rapid cytology diagnosis.

The cytological criteria for the diagnosis of subclinical endometritis continue to be refined, with the postpartum interval for sampling being a key variable (Chapwanaya et al. 2008). Assessments of the severity of inflammation are made by determining the percentage of polymorphonuclear (PMN) cells per 100 cells (PMNs plus endometrial cells) at 400x magnification by method of Barlund et al. They reported that a threshold of more than 8 % PMNs was the lowest proportion of PMNs significantly affecting pregnancy status at 150 days postpartum. Despite the specificity of this threshold at 89.9 %, the sensitivity was poor at 12.9 %, indicating that there are many reasons for nonpregnancy apart from cytological evidence of endometritis.

Material and methods

- The specimens were been obtained in two groups of dairy cattle in the town of Troyan in Central North Bulgaria. First group was Mountbeliarde breed with 13 representatives. Second group of 14 Jerseys. Days in milk from 44 to 346. The average BCS in both groups was 2,5 – 3,5 (Mean 2,78±1,41). Age 3 – 8 years. Data for breeding and calving history was taken.

The examination protocol was equal to both groups including the following:

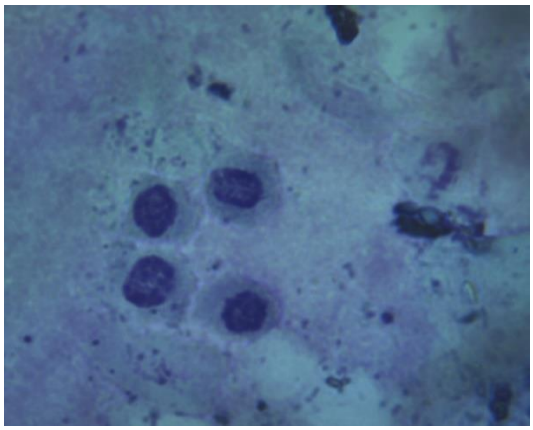
- Fixing in metal chute
- Wipe up the external genitalia
- Metricheck sampling and vaginal discharge scoring (Mc Dougall et al 2007)
- Smear preparation on a microscopE slide
- SMEAR Dry off on room temperature
- Staining a Diff Quick dye
- Microscope examination under magnification 40x and 400x with immersion

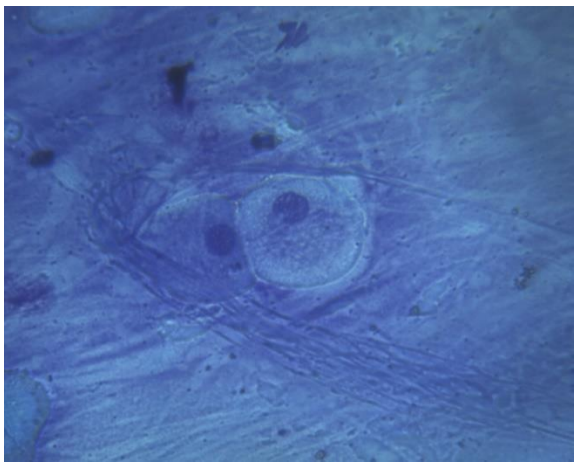
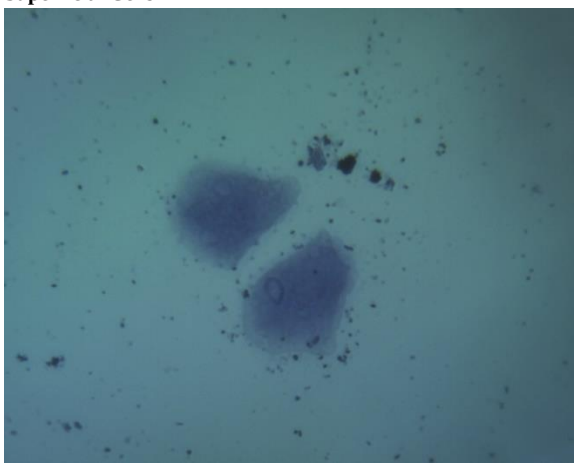
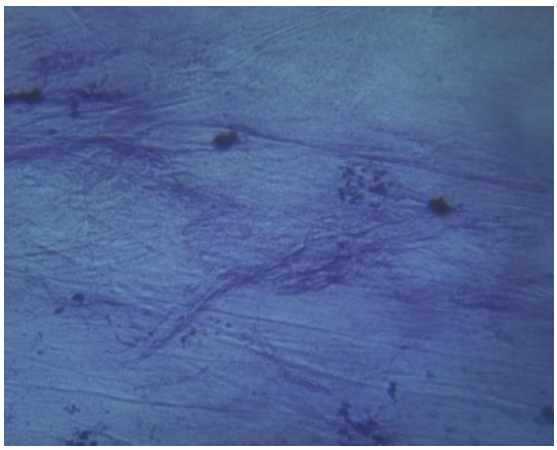
One clinician took all the specimens. Moreover, different 2 pathologist checked all the smears without knowing history of the animals.

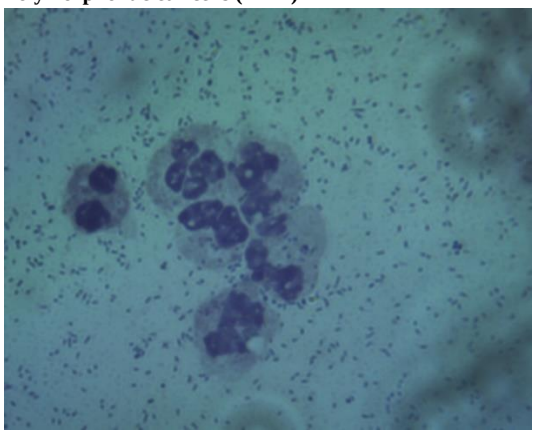
Results

Firstly a microscopic findings are presented. The results show the cells of different type in the smears. The different clusters are clearly distinct with specific features summarized in the table 1 – based on the microscopic results.

Table 1: Vaginal cells characteristics in dairy cattle

Cell type	Characteristics
Parabasal cells 	Parabasal cells are one of the smallest epithelial cells seen in routine vaginal cytologic samples. These cells have a high nuclear-to-cytoplasmic ratio. They have round nuclei and basophilic cytoplasm. These cells are typical for diestrus and anestrus. Large numbers of parabasal cells may be seen normally in vaginal smears of prepubertal animals.

Cell type	Characteristics
Intermediate cells 	The Intermediate cells have variety in size, but in general they have to be twice larger than parabasal cells. Their nuclear-to-cytoplasmic ratio is decreased. These cells have abundant amounts of blue to bluegreen (keratinized) cytoplasm. Depending on the amount of cytoplasm, there are two types of intermediate cells – small intermediate cells and large intermediate cells. Large intermediate cells are sometimes called superficial intermediate cells or transitional intermediate cells.
Superficial Cells 	The superficial cells are one of the largest epithelial cells seen in vaginal smears. These are dead cells, whose nuclei become pyknotic and then faded, often progressing to anucleate forms. The superficial cells have abundant amounts of light blue to bluegreen (keratinized) cytoplasm, and angular to folded cell borders. Superficial cells with pyknotic nuclei and anucleated superficial cells have the same physiologic significance. Superficial epithelial cells are commonly called cornified cells.
Mucin 	The Mucin is a neutral polysaccharide comprising glikoproteid. Mucin is a major part of the mucus, which is produced by the mucous glands and the epithelial cells of the mucosa. Mucus with mucin is normally found in routine vaginal cytologic samples from healthy animals.

Cell type	Characteristics
Polymorphonuclear cells (PMN) 	The neutrophil nucleus is elongate and separated into multiple lobules by invaginations of the nuclear border. Cytoplasm is clear, pale eosinophilic to faintly basophilic with a fine grainy texture and, rarely, contains a few small vacuoles.

Secondly the relationship between PMN%, VDS and pregnancy rate was calculated. The results are presented in table 2.

Table. 2: Relation between clinical condition, PMN percentage and pregnancy rate

Clinical condition	PMN %	VDS	PR
Subclinical endometritis	≤ 7.7	≤ 2	not affected
Subclinical endometritis	≥ 8*	≤ 2	affected
Clinical endometritis/metritis	≥ 20	≥ 3	affected
Normal	≤ 1	≤ 1	not affected

*p≤0,01
PMN – plymorphonuclear cells; VDS – Vagina Discharge score; PR – pregnancy rate.

Discussion

Neutrophils are the first and most significant inflammatory cell involved in endometritis, but are also foremost during normal uterine involution. The inflammatory cell response in cases of subclinical endometritis is widely believed to be quantifiably more severe than that associated with normal involution yet milder than clinical endometritis. Such cytological diagnostic approach is useful for both – normal and infected vagina/uterus with or without presence of discharge (LeBlanc 2008).

Vaginoscopy is a rapid and simple technique for the diagnosis of purulent vaginal discharge (PVD). The use of vaginoscopy for the diagnosis of clinical endometritis is based on the premise that purulent exudate present in the cranial vagina is probably the result of drainage from the uterus. The nature of the discharge is important. Clear mucus is normal, whereas purulent (>50 % pus) and mucopurulent (approximately 50 % pus and 50 % mucus) and foul-smelling discharge are indicative of disease. By utilizing vaginoscopy after 26 days postpartum, 44% of cows with an abnormal discharge are been identified that would have otherwise gone unnoticed if only palpation and external examination were used. By delaying the vaginal examination until approximately 1 month after calving, false positives (i.e., cows undergoing normal involution) will be less likely. Other ways of detecting uterine discharge have been studied, including the gloved hand and the Metricheck device

decribed by McDougall 2007 (Simcrotech, Hamilton, New Zealand). These alternatives are at least as efficacious as vaginoscopy and may offer the advantage of detecting exudate that would otherwise go unnoticed, especially in cases where the cranial vagina slopes ventrally. Another practical advantage is that it is much easier for the examiner to avoid being soiled. Those with larger hands and arms may find the gloved hand technique difficult to employ, whereas the Metricheck device is easy to insert and easy to clean between cows. Vaginoscopy, or a similar procedure, offers an immediate result, but fails to identify all cows at risk of poor reproductive performance due to endometritis. Subclinical endometritis cannot be diagnosed by inspection of vaginal exudate; however, if no other screening tests are being used, routine vaginal examination to detect mucopurulent or purulent exudate is a simple, reliable, and cost-effective way to identify cows at risk of impaired reproductive performance. Endometrial cytology, based on the presence of cellular evidence of inflammation, is currently considered to be the most accurate way to diagnose endometritis in cattle, both clinical and subclinical. Inflammatory cells may be recovered by either of two techniques: uterine lavage or cytobrush (LeBlanc 2002).

The microscopic examination supplied and easy and clear approach to the examined organs (vagina, uterus). All the cells discussed were clearly identified by two pathologists with 100 % agreement. Such success could be accepted as a proof for the value of this simple and cheap diagnostic method (McDougall et al. 2011).

The clinical significance of this diagnostic approach was visualized by the results of for the pregnancy rate, VDS and PMN % (table 2). Affected pregnancy rate is associated with both types of endometritis. Cthe clinical cases are easy to be identified with or without additional examination of the discharge. Opposite to the latter most of the subclincal cases persist unidentified. The threshold of 8% PMN in the smear is highly correlated ($p \leq 0{,}01$) low pregnancy rates in the examined animals.

Summary of the results

1. A distinct clusters of cell types are produced by cattle`s vagina and uterus.
2. Successful sampling and staining is possible to recognize the cell clusters in cattle`s vagina.
3. Cytology is valuable and inexpensive tool to diagnose the presence of inflammatory cells in cattle`s uterus/vagina.
4. Future examinations are need to develop successful confidential intervals for PMN in the EM cases.

Conclusion

The represented results are good basement for development of modified cytological methods for subclinical endometritis diagnostics based on the clear distinction in cell clusters and high correlation between PMN% and pregnancy rate.

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APPLICATION OF PLATELET RICH PLASMA (PRP) IN TREATING OF A COMPLICATED POSTOPERATIVE WOUND IN A CAT: A CLINICAL CASE

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ABSTRACT

Platelet Rich Plasma (PRP) therapy was used in treating postoperative wound complications in an 11 years old cat. After mechanical debridement of the wound, the wound edges were infiltrated three times at certain intervals with PRP. The wound healed completely 50 days after treatment. This clinicalcase indicates that autologous Platelet Rich Plasma (PRP) can be successfully used in treating postoperative wounds with complications in the cat.

Key words: PRP, postoperative wound, platelet, plasma, cat.

Introduction

Until recently it was thought that platelets have exclusively haemostatic function. Presently we have evidence indicating that platelets are responsible for different functions in the body. The first clinical study describing the use of the PRP method in accelerating healing of tissues was published in 1988 by oral surgeons [1]. In this case, autologous PRP was applied to cancellous bone grafts in reconstruction of mandibular defects in humans.

Platelets - anatomy and function

Platelets are small disc-shaped blood cells (approximately 1–3μ m) with average count in peripheral blood of 1.5–3.0 × 109 / L. Their usual life span is 5 to 9 days. Platelets are produced by the megakaryocytes in the bone marrow by cytoplasmic fragmentation after which they are released in the peripheral blood flow [2].

Platelets do not have nuclei; they have an extensive cytoskeleton, mitochondria, lysosomes and ribosomes [3]; modified smooth endoplasmic reticulum and multiple unique organelles [4]. Platelet function is determined by the size and age of the cells [5]; younger and bigger cells have more clearly defined hemostasis than smaller and older cells.

Platelets can express different substances depending on the stimuli used to activate them [6, 7]. Their internal structure includes three types of granules: alpha-granules, dense granules and lysosomes. Alpha-granules are most numerous and serve more important function than the other two. Alpha-granules contain more than 300 proteins, including clotting/coagulation factors, growth factors and other proteins most of which are synthesized or secreted by the megakaryocytes [8, 9]. Dense granules are relatively low in number and contain several small molecules such as serotonin, adenosine diphosphate (ADP), adenosine triphosphate (ATP), GDP, GTP, histamine, calcium, magnesium and polysulfide [9]. Platelet lysosomes are similar to those in other cells; it is unclear whether they perform platelet-specific functions [4].

In addition to their hemostatic function, platelets perform other functions such as anti-inflammatory, immune and tissue repair.

Growth factors in PRP

The alpha-granules of platelets contain polypeptide growth factors such as PDGF [10]; TGFb [11]; IGF-1 [12]; VEGF [13]; HGF [14]; EGF [15]; and bFGF.

Traditionally, platelets have been used therapeutically to treat the thrombocytopenia and platelet dysfunction [16]. Our current knowledge on the topic has been expanded; we now know that platelets also play a key role in restorative processes due to tissue traumas, as they contain platelet growth factors released by activated platelets.

Platelet growth factors initiate and sustain wound healing of bone and soft tissue [17, 18]. The presence of platelet-derived growth factors (PDGF) is essential to the restorative process. PDGF consist of heterodimeric A and B chains as well as A-A and B-B chain homodimers [19]. Other growth factors which play an essential role in the tissue and bone restorative processes include: transforming growth factor-beta (TGF-β 5), vascular endothelial growth factor (VEGF 6), minor quantity of insulin-like growth factor (IGF 7), epidermal growth factor (EGF 8) and connective tissue growth factor (CTGF-9).

It is hypothesized that accelerated wound granulation and epithelization requires four to five times greater platelet concentration than the baseline platelet numbers [1, 20].

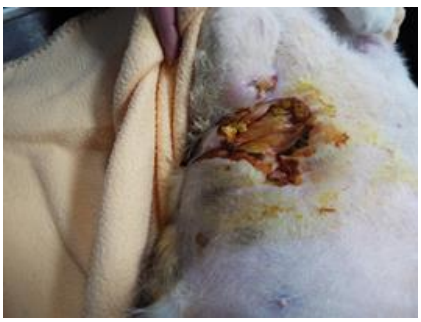
Wounds in animals usually do not heal or heal slowly due to poor blood flow, decreased oxygen supply, insufficient inflammatory response to trauma and others [18, 20].

The purpose of this case report is to describe our experience using PRP in treating complicated and difficult to heal wounds in cats.

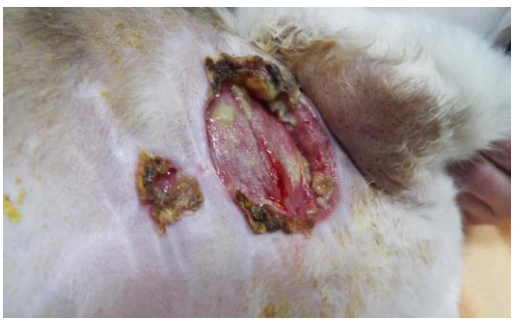
Clinical Case

The patient – a 12 years old female Turkish Angora cat named Kido – presented with a tumor formation on the right cranial and left caudal mammary glands.

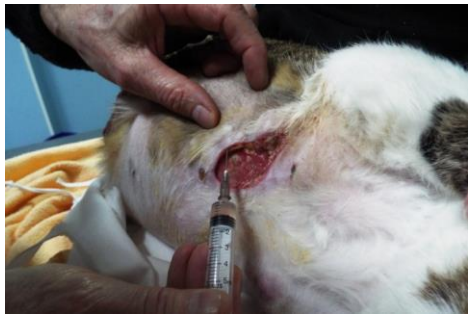
Both tumor formations were removed surgically on 12/07/2015 and the sutures were removed on 18.12.2015 at which time evidence of impaired wound healing was not visible. Ten days later, the skin in the area of the cranial mammary gland ruptured forming a wound measuring 4 by 6 cm. (Picture 1, 2). Conservative treatment, administered for the following ten days, did not produce any results. The wound had atrophic dry bottom, tapered wound edges, multiple pockets and was covered with necrotic tissue and scabs. On 08.01.2016, the wound was mechanically debrided and 2 ml of autologous PRP were infiltrated in the edges and bottom. Seven days after the treatment, a number of islets of granulation tissue were visible. The wound shrunk to 3.5 by 5 cm. (Picture 3).



Picture 1.



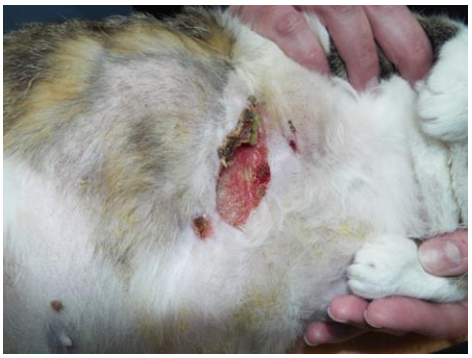
Picture 2.



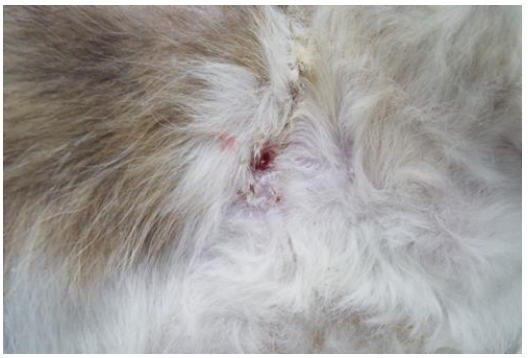
Picture 3.

A second course of PRP treatment as administered on 21/01/2016. The edges of the wound were covered with granulation tissue; necrotic tissue was present only in a few small areas. The wound measured 2.5 by 2.5 cm. Gradual reduction of the size and number of necrotic tissue areas was observed (Picture 4).

The adjacent smaller wounds healed without any treatment and the seborrhea significantly decreased. By 02/27/2016, the wound had completely healed (Picture 5).



Picture 4.



Picture 5.

PRP Preparation

16 mm of whole blood was drawn from the jugular vein using a 22 gauge needle. The blood was placed into 2 heparin vacutainers. The average time lapse between blood draws and PRP extraction was about 10 minutes.

Double centrifugation method

The blood was centrifuged for 20 min at 2800 rpm to achieve separation of cell layers. This procedure divides the blood into three basic components: red blood cells, platelet rich plasma (PRP) and platelet poor plasma (PPP). Each vacutainer yielded approximately 4–5 mL of PRP, 80 % of it was discarded.

The part containing platelets and mononuclear cells was carefully removed using a spinal needle attached to a syringe and re-suspended in 2 ml of the remaining plasma. The final solution, obtained by mixing the resulting PRP and plasma, was placed in sterile vacutainers and was centrifuged at 1300 rpm for 15 min for better separation of the platelet pellets from the supernatant layer

of PPP. The platelet pellets accumulated at the bottom of the container and the PPP on top. The PPP was removed; only the PRP was left in the containers. The platelet pellets were re-suspended within the remaining plasma with a vortex mixer; the final PRP was drawn up with a syringe.

Discussion

A number of clinical studies in animals and humans have shown the important role of platelets in the process of wound healing when applied topically. PRP accelerates healing due to release of growth factors (GF) contained in platelets.[18]

GF can stimulate the inflammatory and proliferative phase in wound healing.

In our clinical case, PRP was applied topically to treat a complicated postoperative wound in a cat. During the course of treatment with PRP, no other medications were administered either topically or internally (such as systemic antibiotics and/or anti-inflammatory agents).

There are different methods for PRP preparation: cuvettes, quadruplicate blood samples, manual preparation using open, closed or automated systems.[21] The double centrifugation method using vacutainers, employed in this study, is inexpensive, relatively easy to implement and does not require expensive or complex equipment.

PRP can be applied topically by continuous dripping or a spray in the form of a gel or injection in wound edges.

PRP stimulates faster wound healing and provides antibacterial and anti-inflammatory environment.[22.23.24] PRP exhibit antimicrobial properties against various microorganisms by inhibiting their growth.[22.25.26]

The results of this clinical case suggest that, the PRP method is an effective therapeutic method in treating atopic and slow healing wounds. Regenerative therapy can be applied in order to improve the quality of tissue regeneration and the rate of wound healing in cats.

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A CASE REPORT OF ECLAMPSY IN DOG

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ABSTRACT

Eclampsia known as puerperal tetany is an acute, life-threatening disease caused by low blood calcium levels (hypocalcemia) in dogs, during pregnancy and the first weeks of lactation after giving birth. Etiology is poor nutrition, hypoalbuminaemia, lactation, parathyroid glands disease. The purpose of this article is to explore the causes, various clinical aspects related to the progress of this disease and its treatment in the particular case.

Key words: eclampsy, hypocalcaemia, dog, therapy.

Introduction

Eclampsy is a life threatening condition in dogs and much rarely in cats caused by hypocalcemia. In dogs it is better described as puerperal tetany. Low calcium levels are typical. Sometimes it is misdiagnosed as `milk fever`. The low calcium levels a related to low free calcium in the blood serum. The condition normally develops in first 1–3 weeks post partum but it is described during pregnancy as well. The condition is triggered by calcium release with initial milk secretion and increasing needs of the puppies. The period between whelping and 40th day is crucial. Puppies normally do not develop signs of hypocalcemia, because the normal level in milk (Pathan M. M et al., 2011). The nursing animals are highly sensitive to changes in free calcium levels due to release in milk secretion (Moe S. 2008).

The metabolism of some nursing animals is not able to sustain high levels of calcium release in milk without affecting the blood levels. Animals with puerperal tetany show inability for fast calcium compensation in blood serum after release in milk. Small breeds with increased irritability are predisposed.

Material and Methods

The object of this study was a 5 years old female, jack Russel terrier, 6 kg body weight. Up to the presented information this is the first pregnancy of the bitch. The cycling period was normal – two times a year. The nutrition practice was poor – mostly homemade food with some mid class market food. Water was being supplied ad libitum.

At the time of hospitalization, a full anamnesis was taken and blood sample was collect from v. cephalica antebrachii using 22G catheter. The blood examination included complete blood count (CBC) supplied in SI units by Mindray BC-288 Vet automatic blood counting analyzer. Biochemical profile included total protein, albumin, blood sugar, bilirubin, cholesterol, aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT), urea, creatinine, calcium and phosphorus on semiautomatic analyzer Mindray BA-88A using reagents by Giesse Diagnostics, Italy.

ECG examination was made using three lead EKG machine Mindray DECG-03A. Semi quantitative urinalysis was made with UroTest-10" (Vet Expert, Poland). The nutrition was corrected using "Trovet puppy" (Netherlads) in amount, recommended by manufacturer.

The correction therapy of the Ca levels was managed using Calcium gluconate 10 % (Sofarma, Bulgaria). The supporative therapy includet Natrium chloride 0.9 % (B. Braun, Germany), Glucose 5 % (B. Braun, Germany), Natrium chloride 0.9 % + Glucose 5 % (B.Braun, Germany), and other additives as Catosal (Bayer, Germany), Vit. AD3E (Vet Prom AG, Bulgaria), Introvit B-complex (Interchemie, Netherlands) extra label-doses adapted for average dog dose of B vitamins, Flawitol for puppies, tablets (Dermapharm, Poland).

Results

During last normal cycle the owners took decision to breed the animal. The male dog is of same breed but much larger in size. On the 8th February 2016 (63 days after) at night time first contractions have been noticed. Until next morning the bitch has not delivered a puppy and shown restless and vocalization. The owners brought her to nearest clinic. At 9 am the first puppy was born and 30 minutes after the second, and third which was of large size for the breed. The ultrasound scanning showed the presence of another one fetus. Because of the lack of medicine treatment, a C-section was made and the last puppy was exteriorized dead and of normal size. After four days the animal is back in the clinic because of bloody vaginal discharge. A blood sample shown low calcium levels have been noticed. Calcium therapy was made without information about dose and medicine used. On the 17th Feb the dog stopped to eat and on 18th Feb developed some nervous symptoms. The animal is being brought in clinic and calcium and buscolisin have been administered.

On the next day a thorough examination was made and affected heart activity was diagnosed. The heart sounds were accentuated and tachycardia of 185 to 196 bpm was count. Respiratory rate was increased without any abnormal findings. Body temperature was normal 38.2–38.9 ºC during whole period af management in the clinic. The mobility of the animal was affected and she was not moving normally. Generally, the animal was dull and did not respond to vocal commands of her owner.

A bloody discharge was present during examination for around a week time and disappeared after that.

Blood sampling showed the following results: hypocalcemia total calcium 1,14 mmol/l, ionized calcium 0,82 mmol/l, increased AP – 508 U/L (for the whole 12-day examination period average 526 U/L), increased hematologic parameters – MCV 72.9 fl, MCH 26.3 (for the whole period MCV – average 73.9 fl and MCH – average 26.14 pg) and low increase in the levels of chlorine – 103.1 mmol/l. The levels of potassium (4.63 mmol/l), Sodium (145 mmol/l), Phosphprus (0.98 mmol/l), Magnesium (0.67 mmol/l) and the levels of the rest of biochemical analytes – total protein, albumin, glucose, bilirubin, cholesterol, ASAT, ALAT, GGT, urea, creatinine were normal. The changes in the levels of calcium and phosphorus are represented on table 1.

Table 1: Daily changes in blood levels of total calcium and phosphorus before calcium therapy.

Date / ***Parameter***	***18.02***	***19.02***	***20.02***	***21.02***	***22.02***	***23.02***	***24.02***	***25.02***	***26.02***	***27.02***	***28.02***	***29.02***
Ca, mmol/l	1.14	2.18	1.88	2.53	2.31	2.25	2.96	2.48	2.02	2.27	2.69	2.65
P, mmol/l	0.98	1.32	0.91	1.26	1.11	1.13	1.52	1.01	1.12	0.79	2.22	2.03

At the time of first visit at clinic the owner has been advised to discontinue ad libitum feeding of the puppies and to feed them adapted milk using a bottle and rubber teat. The nursing by the

mother to be only 5 minutes a day and to stop in 10 days period. To avoid overdose calcium a daily blood sample was checked for the level and the daily dose was adapted according to the result. After the morning treatment with 10% calcium gluconate (0.5–1.0 ml/kg) the blood level of calcium was normal for the next 12 hours. On the following morning the levels were lower (Table 1). The explanation of this fact we find in the massive milk release. We accepted the levels of high than 2.2 mmol/l but not higher than 2.9 mmol/l. The exact treatment plan was the following:

- Day 1: 5 % glucose solution – 180 ml, IV, for 2 hours; 10 % calcium gluconate – 6 ml, slow fractions for 60 minutes; Catosal – 3 ml, IV; Introvit B-complex – 0.5 ml, SC; Vit. АД3Е – 0.1 ml, SC.
- Day 2: equal volume of 5 % glucose and NaCl 0.9 %, total volume – 180 ml, IV, for 2 hours; 10 % calcium gluconate – 3 ml, slow fractions for 60 minutes; Catosal – 2 ml, IV.
- Day 3 to Day 7: IV infusion of glucose and sodium chloride – same dose and route as previous days; 10 % calcium gluconate – 5 ml slow fractions for 60 minutes; daily Catosal – 2 ml, IV and Introvit B-complex – 0.5 ml, SC.
- Day 8: equal volume of 5 % glucose and NaCl 0.9 %, total volume – 180 ml, IV, for 2 hours; 10 % calcium gluconate – 3 ml, slow fractions for 60 minutes; Vit. АД3Е – 0.1 ml, SC.
- Day 9: 5 % glucose – 180 ml, IV slow for 2 hours; 10 % calcium gluconate – 5 ml, slow fractions for 60 mins; Catosal – 2 ml, IV; Introvit B-complex – 0.5 ml, SC;
- Day 10 to Day 12: 0.9 % Sodium chloride 180 ml, slow IV infusion for 2 hours; 10 % calcium gluconate – 5 ml, slow IV fractions for 60 min.

After almost every infusion the dog urinated spontaneously with normal urine – gravity of 1.020 and pH 6.5 negative for glucose, bilirubin, urobilinogen, protein, ketones, blood and leucocytes. Normal amount of crystals were also noticed.

During initial examination and continuous calcium therapy we made ECG monitoring. On the ECG records there were no any abnormalities in cardiac function – pulse rate of 120 to 145 bpm, normal voltage and elements. The Q-T segment was shorter due to calcium injection. Its level was 0.17–0.18 s.

After the final examination we took the decision to release the animal because of its good status and successful therapy. The food was changed and vitamin/mineral tablets was assigned for two weeks – „Flawitol puppies“ (55.28 mg calcium). Control blood sampling after 3 days shown increased levels of MCV-73,7 fl, MCH-26,5 pg, AP-368 U/L, and norma levels of the rest of the analytes – calcium 2.55 mmol/l, ionized calcium – 1.4 mmol/l and phosphorus – 1.17 mmol/l. The nervous system activity and response was also normal at the time of the control examination.

Disscussion

The eclampsy is a condition connected to hypocalcaemia due to different predisposing factors.

In most of the cases the dog owners are not clear with the nutritive needs of their pets. Homemade diets are one of the paramount predisposing factors. They include mostly meet overwhelming the fact that they contain high protein. Such a diets affect negatively the Ca:P ratio, because the level of the digestible calcium amount in meat is less than that of phosphorus. In ideal case the ratio must be 1.2:1. Lots of available ingredients in homemade diets (liver for example) contain ratio of Ca:P

= 1:15 which cause a high disbalance in animal`s metabolism (Simpson, J. W. et al. 1993).The poor nutrition and calcium release with milk are the two main factors in our case.

The diet low in protein and increased release of albumin can also cause low calcium levels. This condition is typical for some kidney diseases, disrupted protein synthesis, and protein release in digestive system. In our patient such a conditions were not described, which brought us to exclude those conditions as predisposing problem (Mellanby R. J. et al. 2005).

Parathyroid problems can also affect the eclampsy in dogs and cats but those condtions are extremely rare (Pathan M. M et al., 2011).

In the period of increased milk production during day 10 and 30 post partum the animals abilty to sustain the calcium levels is stressed. This stress is increased in the next weeks because of the increased needs of the puppies for milk. The milk secretion is of higher priority and reduction in serum calcium levels is the consequence. The ionized calcium is responsible for neural symptoms and muscle dysfunction (cramps). Due to lack of information for any other predisposing factor we can conclude the lactation causes low calcium levels. Although only a few – the newborns were of larger size for the breed so they needed mor milk than average newborn – figure 1. The bitch had normal milk secretion. The puppies removal and their early weaning is milestone in successful treatment of such a case.

Figure 1:

Imbalance between loss of extracellular calcium and absorption by the organism connected to increased milk secretion is a key mechanism in puerperal tetany in bitches. A heavy hypocacaemia nd sometimes hypophosphoraemia coincides with peak lactation in animals with eclampsy as a result of disturbed ratio between excretion/absorption in extracellular calcium pool (Ettinger, 1983).

Intensive loss of membrane bound calcium leads to increased ion permeability of the membranes. This effect leads to lowering of action potential and clinically manifested neuro-muscular tetany and cramps.

Calcium supplementation or foods rich in calcium can affect negatively the parathyroid hormones. The result of the latter is decreased ability of the organism for calcium deposition in the bones, and absorption by the intestines. When the body is of high calcium need as in lactation there is no possibility to adapt quickly to the increased needs. When the calcium is released in the milk it serum levels decrease (Pathan M. M et al., 2011).

The eclampsy therapy normally starts with slow IV infusion of calcium under supervision of vet. In average cases volume 5- 10 ml 10% calcium gluconate daily is enough for dog with body mass of 5–10 kg the calcium must be administered slowly to avoid ventricular fibrilations and cardiac arrest. In eclampsy patients the conscious loss for only a few minutes can increase body temperature to 41–42 ºC such hypertherima affects the brain. Cold water in shower or in pool is a choice in such a cases. After a few minutes in cramps the glycogen in liver and muscles is finished and the animas are exhausted. Low blood sugar levels can also trigger the tetany and cramps in some patients. IV administration of 5 % glucose is indicated in those patients. In cases of sever tremor – sedation and barbiturates are the correct approach.

Conclusions

Corection in diet with correct Ca: P ratio or premium class food according to animals condition is the only prophylaxy.

Administration of 10 % calcium gluconate is an easy approach in practical treatment in eclampsy cases.

Early weaning of the puppies is an important supportive element in the whole eclampsy therapy.

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CLINICAL, HEMATOLOGICAL AND BIOCHEMICAL TESTS OF MALLARDS [ANAS PLATYRHYNCHOS, (L.)] FOLLOWING AN EXPERIMENTALLY INDUCED INTOXICATION WITH LEAD AMMUNITION

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ABSTRACT

Results from clinical, hematological and biochemical tests following an experimentally induced lead intoxication of mallards are presented. The clinical signs and the loss of body weight are proportional to the toxic exposition. Lower levels of red blood cells, hemoglobin and erythrocyte indices are registered. The results from the biochemical tests show elevated levels of liver transaminases, hypoproteinaemia, hypoalbuminaemia, and, also, significantly lower serum calcium levels.

Key words: mallards, intoxication, lead ammunition.

Introduction

The use of lead for manufacturing hunting ammunition dates back centuries and for thousands of years lead has been used in fishing. Nearly 150 species, representatives of the wildlife, that have been affected by lead intoxication due to ingestion of pellets, bullets or fragments of lead ammunition, are registered in scientific literature. Only in the late 19th century, hypotheses, evolving to indisputable facts, that proved that lead ammunition and fishing tackle are a major source of lead exposure expressed in toxicity with cumulative effect in game and fish, were formed. Despite the legal restrictions, significant amounts of lead ammunitions continue to be deposited in water and upland habitats thus entering the food chain up to the end consumer.

Lead is one of the most toxic metals and its negative effects range from mild biochemical and physiological disorders to serious pathological processes in which major organs and systems may be affected, with following functional and behavioral changes. The probability for a bird to be poisoned is determined by several factors, such as: time of retaining the lead elements, frequency of exposure, nutritional conditions, stress etc. There are documented cases of lethal poisoning as a result of only one pellet (Sanderson, G. C., and Bellrose. F. C. 1986). The gizzard of the bird, which contains swallowed sand from the river sediment (the so called gastrulates), is an ideal environment for the solubilization of lead. Furthermore, the gizzard combines acidic environment with strong contractibility of its cuticle, which as a result creates conditions for the abrasive action to lead, leading to its absorption for about 42 days (Pain et al., 2009). According to some scientists, lead fragments are readily consumed by the animals because of the salty-like taste of their oxidized surfaces, particularly when salt - deficient mammals and birds are concerned (Lewis et al. 2001). Most toxicologists determine levels of 16 mg/kg. BW lead from pellets as the cumulative lethal dose for birds (Dilov P., et al. 2005). Lead concentrations are the highest after direct absorption into the bloodstream, then in the kidneys and liver for days or months, and if the process becomes chronic the lead is deposited into the bones. If the intoxicated with lead birds are consumed by predators or saprophytes, the latter in turn absorb certain amounts of lead, which may result in their intoxication and death.

In terms of lead intoxication related to the use of lead hunting and fishing gear, in our country, there is no data of any conducted scientific studies with the exception of some popular scientific articles.

That is why we decided to investigate some of the toxic effects in waterfowl and in connection with their exposure to lead ammunition.

Material and Methods

16 clinically healthy mallards (Anas platyrhynchos) in the age range of 9 to 12 months and body weight range from 1050 to 1250 g were included in the experiment. The birds were divided by fours into 3 experimental and one control groups. They were kept separately, in groups, in closed aviaries with provided access to water for their physiological needs. Their feeding was organized with granulated feed mix for ducks provided twice a day. After a 7-day period of adaptation, the mallards were treated orally with lead pellets №3 with average weight of 0.26746 g as follows: 1st group – 3 lead pellets, 2nd group – 2 lead pellets, 3rd group – 1 lead pellet, and the 4th control group. The study was conducted over a period of 60 days after the treatment.

Bodyweight was recorded before treatment and then over seven-day intervals, respectively on the 7th, 14th, 21st, 28th, 35th and 42nd day.

Blood samples were obtained by venipuncture from v. subcutanea ulnaris before treatment, on the 7th, 14th, 21st, 28th, 35th and 42nd day. The obtaining of blood samples was performed using a closed system including needles MN-SV21Q (butterfly type) and 3ml tubes with Li- Heparin for hematology analysis and 5 ml tubes with Gel + Clot Act for biochemical analysis.

The hematology tests included: erythrocyte count (RBC), hemoglobin (Hbg), hematocrit (PCV), mean corpuscular volume (MCV), mean hemoglobin content in one erythrocyte (MCH), and the mean concentration of hemoglobin in erythrocytes (MCHC). The tests were performed with veterinary hematology analyzer Haemascreen 18.

The biochemical indicators included: serum levels of creatinine (Create), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (AP), albumin (Albumin) and calcium (Ca2 +). For their determination biochemical analyzer Screen Master LIHD 113 was used.

The analysis and statistical processing of the data was performed by a computer program SPSS 19.0. The data are expressed as mean plus standard error.

In the current study the assessment was made with guaranteed probability of 0.95 (significance level = 0.05), whereas $p < 0,05$ was considered the lowest level of statistical reliability.

Results and Discussion

During the first two days (24–48 h.) after the treatment, there were no visible changes in the general condition and behavior of the mallards.

On the seventh day after the oral administration of the lead pellets the experimental birds from group I exhibit the first symptoms, such as green-colored diarrhea accompanied by increased thirst (polydipsia), and a decrease in body weight (Table 1).

During the time interval between the 7th–14th day in addition to the continuing greenish diarrhea, there is also a distressed locomotor activity, the eyes are narrowed (photophobia), and the mallards demonstrates vocal changes, resulting in deaf hissing sounds. There is a new regression in terms of body weight (Table 1).

A similar clinical finding is observed in the next five days (from the 14th to the 19th day after treatment), with deepening signs of polydipsia, accompanied by manifestations of adynamia and prostration.

During the last days before death, refractory cachexia, photophobia, vocal changes, lack of appetite and completely distressed locomotor activity with paralysis of limbs, and reliable weight loss (Roscoe et al., 1979), were registered (Table 1).

The mortality rate in the mallards from grooup I and groups II was 100 % as a result of a severe anemic syndrome.

In group III the mortality rate was 25 %, where cachexia, lethargy, greenish diarrhea, adynamia and prostration, serofibrinous conjunctivitis as well as reliable weight loss on the 35th - 42nd days after treatment, were observed (Table 1).

The loss of body weight, as well as the severity of clinical symptoms, was proportional to the dose of the applied lead. The diversity and nature of the manifested symptoms observed within each group of birds are due to the different individual sensitivity to the administered toxic dose.

Table 1: Change in the body weight for the different groups of the tested mallards (Anas platyrhynchos), reported before, on the 7th, 14th, 21st, 28th, 35th and 42nd day after oral administration of lead pellets.

Day	group I n = 4 x ± SE	group II n = 4 x ± SE	group III n = 4 x ± SE	Control group n = 4 x ± SE
Before	1185.75 ± 29.856	1215.00 ± 32.787	1122.50 ±17.970	1088.75±21.348
7th	1110.00 ± 29.155	1130.00 ± 46.726	1088.75 ± 35.141	1122.25±19.063
14th	927.50 ± 46.615	1070.00 ± 39.158	1033. 25 ± 58.628	1164.50± 15.793
21st	822.50 ± 53.910**	970.00 ± 61.509*	933.75 ± 78.352	1192.00±12.403
28th	-	802.50 ± 80.661*	947.50 ±124.908	1209.25±12.665
35th	-	725.00 ± 74.442*	945.75 ±142.389*	1231.25±11.404
42nd	-		988.00 ±156.466*	1251.50±10.428

*p < 0.05; **p < 0.01; ***p < 0.001

Changes in the hematological parameters in mallards [Anas platyrhynchos, (L.)] following an experimentally induced lead intoxication.

There is a clear eritropeniya in the experimentally treated birds from group I on the 14th and 21st day, compared to the initial levels (Table 2).

In the birds from group II there is no registered statistical reliability, however, there is a tendentious decrease in the number of red blood cells (Table 2).

In the birds from group III a regressive trend in the total number of red blood cells up to the 28th day, followed by a gradual slow normalization correlating with cessation of clinical signs, was observed (Table 2).

In the control group, the fourth group, the number of red blood cells is maintained within the reference levels (Table 2).

A decrease in the concentrations of hemoglobin (Hgb) and hematocrit (PCV) indicators was observed (Table 3).

Significant regression in terms of hemoglobin is present mostly in the experimentally treated mallards from group I and group II, which correlates with the decreased levels of red blood cells (Table 2). This decrease is associated with the ability of the lead to inhibit the sulfhydryl enzymes involved in the cellular metabolism, leading to the deposition of hemoglobin precursors (Dilov P. et al. 2005; Nikov, 1977; Anderson, et al., 1985; Fisher et al., 2006).

Table 2: Effect of the experimental lead poisoning on the average numbers of erythrocytes, hemoglobin and hematocrit level in mallards [Anas platyrhynchos, (L.)].

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
I	RBC (10^{12}/L)	3.3450 ± 0.08180	2.0450 ± 0.07100	1.2400 ± 0.15226*	1.2550 ± 0.15300*	–	–	–
	Hgb (g/L)	197.00 ± 2.082	125.50 ± 4.406	72.75 ± 10.274**	59.50 ± 4.330*	–	–	–
	PCV (%)	52.600 ± 3.0586	34.600 ± 1.7903*	21.050 ± 3.0937	21.300 ± 2.0785	–	–	–
II	RBC (10^{12}/L)	3.1125 ± 0.59263	2.7075 ± 0.09031	2.4175 ± 0.11191	2.1125 ± 0.10451	1.6475 ± 0.09595	1.5225 ± 0.03425	–
	Hgb (g/L)	205.75 ± 14.203	160.75 ± 1.931*	133.00 ± 3.136	107.50 ± 7.511	79.25 ± 9.543	64.25 ± 2.562	–
	PCV (%)	55.600 ± 1.3620	48.925 ± 1.7433	44.225 ± 1.3028	34.600 ± 1.5039	26.250 ± 0.9474	22.575 ± 1.3143	–
III	RBC (10^{12}/L)	2.7300 ± 0.18471	2.2275 ± 0.40485	2.1600 ± 0.25103	2.0200 ± 0.30469	2.2950 ± 0.44558	2.2650 ± 0.43202	2.5225 ± 0.51367
	Hgb (g/L)	193.50 ± 12.926	127.00 ± 24.782	125.25 ± 19.163	118.75 ± 18.495	133.00 ± 31.115	146.25 ± 30.090	166.25 ± 40.463*
	PCV (%)	45.450 ± 3.7369	36.800 ± 7.0075	35.125 ± 4.6245	31.725 ± 4.6015	39.250 ± 10.3159	34.350 ± 6.1637	35.925 ± 6.6649
Contr.	RBC (10^{12}/L)	2.7425 ± 0.20882	3.2625 ± 0.34038	2.7300 ± 0.25096	2.5950 ± 0.03329	2.9450 ± 0.13629	2.9450 ± 0.05867	2.8525 ± 0.08693
	Hgb (g/L)	182.50 ± 7.599	187.50 ± 6.564	169.75 ± 13.269	195.75 ± 6.074	186. 74 ± 5.642	188.50 ± 5.694	177.25 ± 1.109
	PCV (%)	46.625 ± 4.2927	56.250 ± 6.0207	40.825 ± 3.2451	40.325 ± 1.1996	44.575 ± 1.3325	50.250 ± 2.0060	48.300 ± 0.8175

*p < 0.05; **p < 0.01; ***p < 0.001

Regarding the erythrocyte indices (MCV, MCH and MCHC), there is a characteristic decrease compared to the initial levels in groop I and group II, treated with a higher lead concentration (Table 3). The decrease of the values of the erythrocyte indices is an indication of the presence of microcytic anemia (Angelov 1999; Del Bono, 1973).

Table 3: Effect of the experimental lead poisoning on the erythrocyte indices (MCV, MCH, MCHC) in mallards [Anas platyrhynchos, (L.)].

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
I	MCV (fL)	169.00 ± 1.472	170.75 ± 1.887	140.50 ± 4.907***	162.50 ± 1.732**	–	–	–
	MCH (pg)	61.000 ± 1.1083	53.350 ± 4.3007***	52.700 ± 3.3919	45.100 ± 0.6351	–	–	–
	MCHC (g/L)	369.25 ± 7.521	312.50 ± 27.600***	328.00 ± 20.314	297.00 ± 9.238	–	–	–
II	MCV (fL)	162.15 ± 6.359	172.50 ± 2.754	160.25 ±3.425	158.75 ± 3.591	155.25 ± 2.689	145.50 ± 8.995	–
	MCH (pg)	68.750 ± 3.1071	56.050 ±1.1850	58.000 ± 1.8453	52.075 ± 3.0341	50.525 ± 2.0483	46.025 ± 3.8947	–
	MCHC (g/L)	431.75 ± 29.219	334.25 ± 13.762	366.25 ± 7.432*	330.00 ± 20.765	324.25 ± 15.456	319.75 ± 35.056	–

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
III	MCV (fL)	166.43 ± 4.084	165.75 ±3.568	159.00 ± 5.788	156.00 ± 6.843	153.50 ± 5.605	157.25 ± 1.377*	159.00 ± 2.121
	MCH (pg)	72.300 ± 7.6183	57.750 ± 4.3448*	55.300 ± 4.3507*	55.675 ± 2.0786**	49.675 ± 3.2268**	62.950 ± 6.0506	66.250 ± 7.2407
	MCHC (g/L)	439. 00 ± 55.562	348.50 ± 23.659*	347.00 ± 18.855**	372.75 ± 6.613***	408.50 ± 15.398**	406.75 ± 25.539*	393.50 ± 25.168
Contr.	MCV (fL)	167.43 ± 3.735	170.25 ±1.702	154.25 ± 4.785	154.75 ± 3.351	162.25 ± 2.175	167.25 ± 1.548	161.00 ± 1.683
	MCH (pg)	64.175 ± 5.1611	58.550 ± 1.5414	63.400 ± 1.3466	75.025 ± 2.6129	65.675 ± 1.3047	61.550 ± 1.2978	57.900 ± 1.6299
	MCHC (g/L)	342.50 ± 6.171	360.00 ±5.492	409.75 ± 5.893	478.00 ± 28.726	406.25 ± 7.642	362.00 ± 9.381	333.00 ± 9.764

*p < 0.05; **p < 0.01; ***p < 0.001

Changes in the biochemical parameters in mallards [Anas platyrhynchos, (L.)] following an experimentally induced lead intoxication.

When examining the transaminases ASAT and ALAT (Table 4) elevated levels of statistical reliability were observed, which is an indication of degenerative changes in the liver (Angelov, 1999; Mircheva, 2005; Mateo et al. 2003).

Alkaline phosphatase (AP) is an indicator of the osteoblastic activity and lead exposure is associated with alteration and mineralization of the bones in birds (Gangoso et al., 2009, Martinez-Haro et al. 2011).

In the mallards from group I, the increased concentration of lead in the blood is accompanied by reduced activity levels of the AP, where values remained within the reference levels (Mateo et al. 2003 b). In the experimental birds from group III (treated with lower doses) there is a statistically significant increase in the levels of AP which suggests a chronic process and deposition of lead in the bones.

Table 4: Effect of the experimental lead poisoning on the liver transaminases level in mallards [Anas platyrhynchos, (L.)].

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
I	ASAT (UI/l)	27.000 ± 9.1042	81.250 ± 13.7721	115.675 ± 7.4366	125.450 ± 5.2250	–	–	–
	ALAT (UI/l)	36.825 ±5.5552	59.450 ± 8.9245	77.575 ± 10.8992	78.600 ± 9.0067*	–	–	–
	AP (UI/l)	201.775 ± 29.6609	161.000 ± 32.2417	120.500 ± 22.9658	170.950 ± 52.3044*	–	–	–
II	ASAT (UI/l)	32.600 ± 0.0000	60.625 ± 14.4899*	104.975 ±7.4964**	117.225 ± 7.5260	96.950 ± 5.0419*	95.800 ± 6.1124*	–
	ALAT (UI/l)	34.225 ± 8.0257	47.525 ± 1.2776*	53.500 ± 3.0367	54.125 ± 7.2721	63.475 ± 5.2706	66.950 ± 5.5917	–
	AP (UI/l)	209.250 ± 15.7606	192.750 ± 20.7988	133.525 ± 30.4952	163.900 ± 19.7593	184.775 ± 12.2918	174.250 ± 8.9040	–
III	ASAT (UI/l)	18.100 ± 2.7249	68.900 ± 2.8499	101.700 ± 9.1310*	113.900 ± 11.3428**	99.875 ± 8.9141*	72.300 ± 18.2311	78.775 ± 16.0383
	ALAT	49.650	46.800	72.200	70.575	62.100	54.750	60.400

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
	(UI/l)	± 1.0524	± 3.5221	± 5.7881*	± 11.2970 **	± 13.5584 ***	± 12.4656*	± 10.3734*
	AP	182.100	180.125	270.500	217.625	186.525	205.100	218.725
	(UI/l)	± 3.2104	± 9.7643*	±14.1667*	± 50.9784*	± 35.6294*	± 29.7565*	± 33.9074*
	ASAT	22.325	43.750	39.875	23.925	33.825	40.475	37.550
	(UI/l)	± 0.9040	± 4.5688	± 4.5428	± 4.2074	± .3568	± 1.7542	± 0.9430
Contr.	ALAT	51.250	47.950	43.300	46.925	50.100	47.100	49.875
	(UI/l)	± 1.3525	± 1.9504	± 1.0320	± 1.2822	± 1.5061	± 0.8436	±1.4250
	AP	186.050	196.425	189.650	194.925	191.775	194.550	184.225
	(UI/l)	± 3.7078	± 7.3846	± 1.7576	± 2.1093	± 1.8621	± 2.4510	± 1.9491

*p < 0.05; **p < 0.01; ***p < 0.001

In the experimental birds from group I the creatinine levels are maintained within the norm, furthermore, there is even a visible decrease which is associated with the presence of an anemic syndrome (Angelov, 1999). Whereas, in group II and especially experimental group III there is an increase in the creatinine level which could be an indication of degenerative changes in the kidneys (Angelov, 1999; Mateo et all., 2003a). But, overall, the creatinine levels in this study were within the normal range (Table 5).

The examination of the serum proteins showed a slight but sustained decrease in the albumin levels, characteristic especially for the first and second experimental groups, in which the administered toxic dose was higher (Del Bono, 1973). This implies impaired resorption on behalf of the digestive system along with signs of anemia (Angelov, 1999; Mircheva, 2005).

There was a significant decrease of the levels of serum calcium compared to its initial values. This could be due to a series of biochemical regulatory mechanisms at the cellular level and the suppressive effect of the lead in relation to the calcium (Gangoso et al., 2009, Martinez-Haro et al. 2011).

Table 5: Effect of the experimental lead poisoning on the average numbers of creatinine, albumin and calcium levels in mallards [Anas platyrhynchos, (L.)].

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
	Creat.	48.600	41.975	25.925	25.900			
	(µmol/l)	± 15.6907	± 5.7686	± 4.4252	± 4.4336	–	–	–
I	Albumin	19.375	16.100	14.125	16.525			
	(g/l)	± 1.1302	± 1.7345*	± 1.5140	± 2.8482	–	–	–
	Calcium	2.000	1.900	1.475	1.350			
	(mmol/l)	± 0.1472	± 0.0816	± 0.1109	± 0.0645**	–	–	–
	Creat.	57.525	39.825	34.875	58.350 ±	48.100 ±	34.725	
	(µmol/l)	± 14.3325	± 7.4170	± 7.8711	22.1528	15.9399	± 8.2060	–
II	Albumin	23.575	19.250	20.225	18.975	15.350	13.500	
	(g/l)	± 2.9004	± 0.8549	± 1.2645	± 1.5526	± 1.4540	±0.5431	–
	Calcium	2.400	1.850	2.100	1.400	1.375	1.175	
	(mmol/l)	± 0.2160	± 0.1658	± 0.1581	± 0.0408	± 0.2926	± 0.1887	–
	Creat.	37.525	34.425	29.775	35.850	26.100	27.675	51.050
III	(µmol/l)	± 2.1124	± 3.6684	± 1.7143	± 4.0891	± 4.0301	± 4.2820	± 12.1442
	Albumin	19.650	16.575	19.075	20.250	18.825	17.825	18.550
	(g/l)	± 1.0300	± 0.8499	± 1.4221	± 2.1562	± 2.4356	± 2.2051	± 2.2897

Group	Index	Before n = 4 x ± SE	7th day n = 4 x ± SE	14th day n = 4 x ± SE	21st day n = 4 x ± SE	28th day n = 4 x ± SE	35th day n = 4 x ± SE	42nd day n = 4 x ± SE
Contr.	Calcium (mmol/l)	1.850 ± 0.1323	1.850 ± 0.1848	2.000 ± 0.2345	1.198 ± 0.0931	1.765 ± 0.3404	1.650 ± 0.2255	1.700 ± 0.2415
	Creat. (µmol/l)	37.775 ± 1.8085	38.800 ± 1.7335	34.650 ± 1.5174	31.125 ± 1.5151	33.850 ± 1.8012	41.000 ± 1.6477	48.225 ± 1.2526
	Albumin (g/l)	20.875 ± 0.9936	21.375 ± 0.3728	21.700 ± 0.6014	21.725 ± 0.6019	21.925 ± 0.5573	20.400 ± 0.4708	20.800 ± 0.1871
	Calcium (mmol/l)	2.000 ± 0.1080	1.900 ± 0.1291	2.200 ± 0.1080	1.800 ± 0.1080	2.250 ± 0.1555	2.100 ± 0.1080	2.075 ± 0.1250

**p < 0.05; **p < 0.01; *** p < 0.001*

Conclusions

The clinical signs and loss of body weight, proportionally correlates to the size of the administered toxic dose.

The results from the hematological and biochemical analyses show the presence of macrocytic (normochromic) anemia and possible liver and kidney degenerative changes as well as deposition of lead in the bones.

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INVESTIGATIONS ON THE PREVALENCE OF PATELLAR LUXATION IN DOGS

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ABSTRACT

The aim of the present study was to determine the prevalence of patellar luxation in dogs using the patient records of the Small Animal Clinic at the Faculty of Veterinary Medicine – Stara Zagora and Avicena Veterinary Clinic – Sofia.

From the studied cohort, the Pinscher, Pomeranian, and Spitz breeds were the most frequently affected. The disease was the most rarely encountered in Bolognеses. Female dogs were more commonly affected. In cases of unilateral patellar luxation, it was significantly more common in the left than in the right hindlimb. More than half of studied dogs (56 %) weighed between 2 and 5 kg, and 23 % were within the range 5–15 kg. In 91 % of dogs from studied breeds, medial patellar luxation was observed. The occurrence of the different patellar luxation grades was as followed: grade 1: 21 %, grade 2: 43 %; grade 3: 30 % and grade 4: 6 %.

Key words: dogs, patellar luxation.

Introduction

The dislocation of the patella is a common disorder in dogs, mostly congenital, with recessive polygenic inheritance. According to Hayes et al. (1994) the prevalence of congenital luxations of the patella is 82 %.

In 75 % of cases, the dislocation is medial, and bilateral luxation is encountered in 20–52 % of cases in small breeds and about 36 % of large breeds (Roush, 1993; Piermattei et al., 1997; Harasen, 2006, 2006A).

Miniature and toy breeds of dogs are most commonly affected. The number of young dogs from large breeds affected with patellar luxation as Boxers, Huskies, Labradors, Golden Retrievers, Akita etc. is also increasing (Remedios et al., 1992; Roush, 1993; Hayes et al., 1994; Piermattei et al., 1997; L'Eplattenier et al., 2002; L'Eplattenier et al., 2002A).

Traumatic patellar luxations are less common and are associated to damage of ligaments and periarticular structures (Roush, 1993; Denny et al., 2000; LaFond et al., 2002).

According to Roush (1993), female dogs are affected 1.5 times more frequently than males, while Harasen (2006, 2006A) affirms the existence of the opposite tendency.

There is no unanimous opinion about the pathogenesis of the disease, yet it is acknowledged that it results from anatomical deviations affecting the entire hindlimb. They begin from the hips with coxa vara (reduced angle between the femoral neck and femoral shaft) and decreased anteversion (cranial deviation) of the femoral head and neck vs the femur axis. This pathology results in medial displacement of extensors and particularly, of m. quadriceps femoris (Roush, 1993). As an element of the patellar mechanism, this displacement influences the distal femoral physis, slowing down the growth on the medial side but enhancing it from the lateral side (Hulse, 1995). The ultimate effect is medial bending and rotation of the distal femur and the proximal tibia. The patella exerts a relatively higher pressure on medial bone and soft tissue structures resulting in inadequate pressure

on trochlear groove, whose depth in growing animals remains insufficient (Roush, 1993; Hulse, 1995).

Congenital patellar luxation is definitely associated with abnormal development of extremities, with displacement of the quadriceps muscle complex (quadriceps muscle + patella + patella ligament/tendon) (Prose, 1984; Denny and Butterworth, 2000).

Lateral patellar luxation is rarely encountered, it is frequent in large dog breeds and associated to strong limb deformities – coxa valga. Such type of dislocation was observed in Pomeranians (Wangdee and Torwattanachai, 2010).

Traditionally, the classification of Singleton (1969) is used for evaluation of the extent of deformity and the needed treatment. A 4-grade system for classification of canine patellar luxations is created by Putnam (1968).

Although this grading system does not always correspond to clinical signs, they are useful for monitoring of the development of disease in young asymptomatic animals or for undertaking a specific type of surgery if the patient is lame. Such animals are at risk for developing degenerative joint diseases or cranial cruciate ligament rupture (Willauer et al., 1987; Wander et al., 1999; Langenbach and Marcellin-Little, 2010). Campbell (2010) observed a concomitant craniate cruciate ligament rupture in 41 % of patients with patellar luxation, while others (Piermattei et al., 1997; Denny et al., 2000) reported that 15–20 % of chronic patellar luxations could result in CCL ruptures.

The aim of the present study was to determine the prevalence, types, grades of patellar luxation in dogs using the patient records of the Small Animal Clinic at the Faculty of Veterinary Medicine – Stara Zagora and Avicena Veterinary Clinic – Sofia for the period 2011–2014.

Material and methods

The survey was made for the period 2011-2014 and included 3,167 dogs with surgical diseases (2,909 from the Small Animal Clinic, Faculty of Veterinary Medicine, and 258 from the Avicena Veterinary Clinic). From them, 203 dogs were diagnosed with patellar luxation (172 and 31 for both clinics respectively). The patient records of 169 dogs (218 joints) were complete and they were included in the survey. The diagnosis was made after clinical exam, and in more severe cases – after radiography in mediolateral and craniocaudal views.

Patellar luxations were classified according to the affected limb (left or right), dislocation type (medial and lateral), grade (first, second third, fourth), by breed and age.

The information was obtained from patients' records and data from primary and control exams.

Results

The distribution of patellar luxations by breed is presented on Fig. 1. The most commonly affected breeds in our survey were Pinscher, Pomeranian/Spitz (26 % and 25 % respectively) whereas in Chow Chow dogs, the disease was least frequently diagnosed (1 %). Female dogs (60 %) were more commonly affected than males (40 %) (Fig. 2). When the luxation n was unilateral, it affected more commonly the left hindlimb (40 %) than its right counterpart (31%, Fig. 3). The relatively high incidence of bilaterally affected dogs should be noted (29 %). More than half of dogs (56 %) weighed between 2 and 5 kg, and the weight of one quarter (23 %) of the studied cohort was between 5 and 15 kg. Dogs referred for examination and treatment of patellar luxation were most commonly between 1 and 5 years of age (50 %), and 41 of 169 (24 %) were < 1 years of age. The

proportion of dogs older than 10 years was insignificant (2 %, Fig. 5). In studied breeds, medial dislocation of the patella was established in 91 % of cases (Fig. 6).

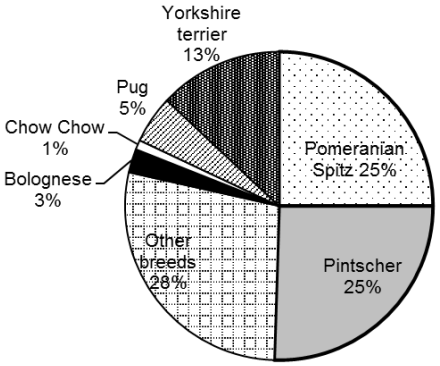

Figure 1: Distribution of dogs with patellar luxation by breeds.

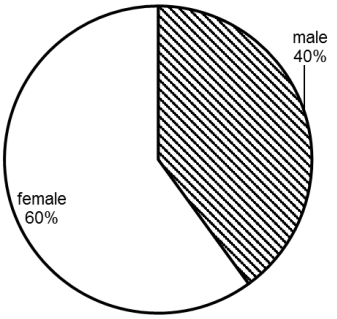

Figure 2: Distribution of dogs with patellar luxation by gender

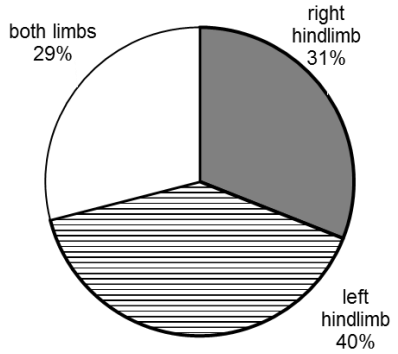

Figure 3: Distribution of dogs with patellar luxation by affected limb

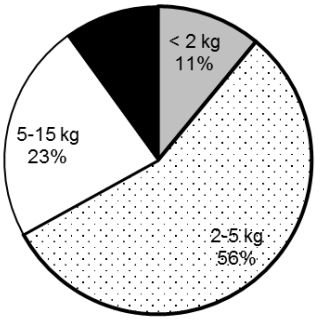

Figure 4: Distribution of dogs with patellar luxation by body weight

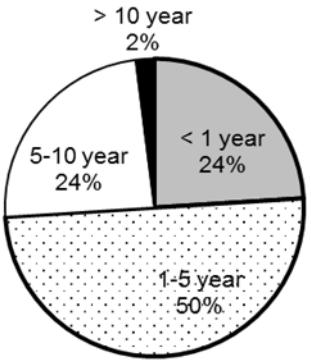

Figure 5: Distribution of dogs with patellar luxation by age.

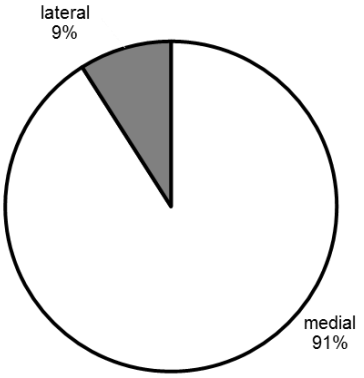

Figure 6: Distribution of dogs with patellar luxation by direction of the dislocation

The grades of observed patellar luxation cases are presented on Fig. 7. Grade II was the commonest (43 %) followed by grade III with 30 %, grade I – 21 % and grade IV – 6 %. Grades I and III were predominant in dogs between 1 and 5 years of age (40 dogs – 18.3 % and 34 dogs – 15.5 %, respectively) (Fig. 8). Grade II was the most prevalent among dogs younger than 1 year (21.6 %). The frequency of grade IV luxation was the same for these two age groups. There was a relationship between the body weight of patients and the grade of the kneecap dislocation. Grade I to III were mainly encountered in dogs weighing 2 to 5 kg, while those with body weight between 5 and 15 kg exhibited comparable percentages of all grades (Fig. 9).

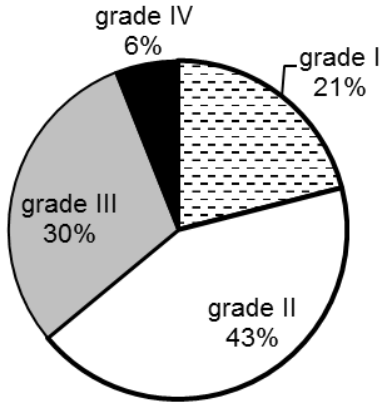

Figure 7: Distribution of joints with patellar luxation by grade of dislocation.

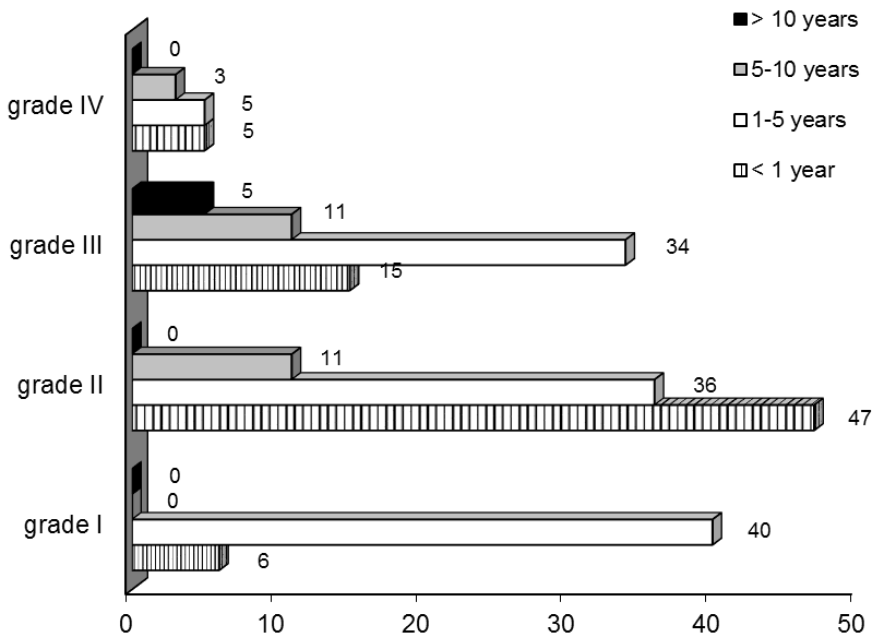

Figure 8: Relationship between patellar luxation grades and the age of dogs.

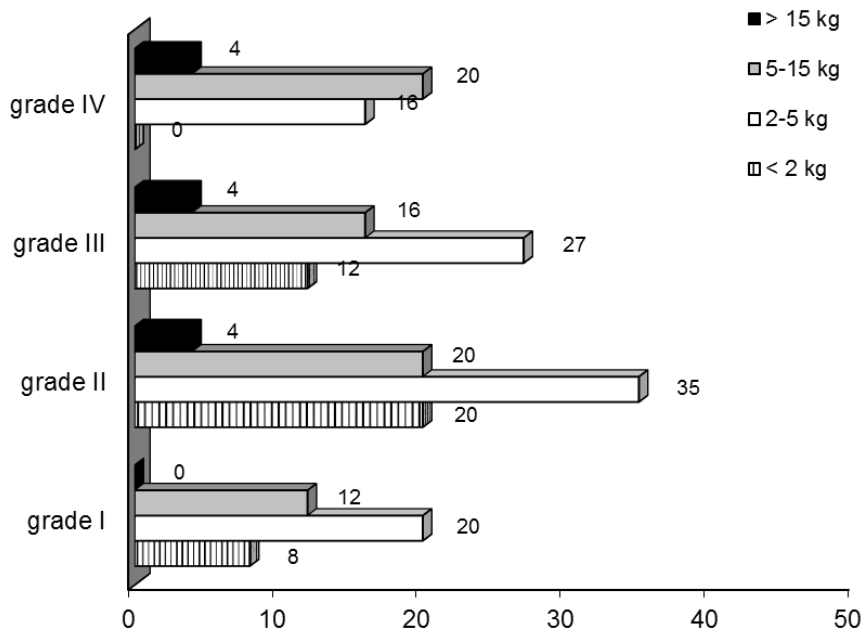

Figure 9: Relationship between patellar luxation grades and the body weight of dogs.

Discussion

The patellar luxation is a very common orthopaedic problem in dogs. Most patients are of small breeds – Mini Poodle, Yorkshire terrier, Pomeranian, Chihuahua, Pekingese etc. The number of young dogs from large breeds affected with patellar luxation as Boxers, Huskies, Labradors, Golden Retrievers, Akita etc. is also increasing (Remedios et al., 1992; Roush, 1993; Hayes et al., 1994; Piermattei et al., 1997; L'Eplattenier et al., 2002; L'Eplattenier et al., 2002A). In our survey, Pinschers and Pomeranians were most commonly affected, while Chow Chows – least frequently. The proportion of Yorkshire terriers with patellar luxation was also substantial. About Pekingeses, our results were different from previously reported data. Patellar luxation in large dog breeds was seen only occasionally.

Most luxations are congenital, but the mode of inheritance is not specified. There are, however, traumatic luxations, when the lateral part of the knee joint and the respective retinaculum are injured (Roush, 1993; Denny et al., 2000; LaFond et al., 2002). According to Hayes et al. (1994) 82 % of patellar dislocation are congenital. In our study, there was no history of trauma in patients' records, so we presume that all cases were congenital.

Female dogs are affected 1.5 times more commonly than males (Roush, 1993) although Harasen (2006, 2006A) reported the opposite tendency. In our clinical survey, the ratio of affected females to males was 1.5 as reported by the former researcher. There is not a plausible explanation about gender predilection to the disease (Roush, 1993; Harasen, 2006).

It was reported that the prevalence of bilateral patellar luxations ranged from 20 to 52 % in small breeds and about 36 % in large dogs (Roush, 1993; Piermattei et al., 1997; Harasen, 2006, 2006A). In this study, 29 % of cases were with bilateral luxation. The broad reported ranges were

probably due to the fact that many of grade I luxations remained unknown. The available literature does not provide information which hindlimb was more commonly affected in unilateral cases; in our patients, the frequency of luxations of the left hindlimb was 1.3 times higher.

Beyond any doubt, medial patellar luxation is considerably more frequently seen – in 75–80 % of cases according to the different reports. We established an even higher occurrence of medial luxation (91 %). This could be attributed to the fact, that large breeds where lateral patellar luxations prevail, are very rarely examined for this problem.

It is generally acknowledged that patellar luxation is a problem of juvenile (developing) skeleton. Congenital anatomical abnormalities resulting in altered angle of the femoral neck with the diaphysis in the axial plane or traumatic factors change the patellar mechanism (Roush, 1993) displacing the tension and compression forces in lateral or medial direction. This results in varus or valgus of the distal femur and proximal tibia and shallow trochlear groove (Roush, 1993; Hulse, 1995). Such deformities were observed in all dogs with patellar luxation grade III and IV and even in some cases with grade II.

Conclusions

1. Small dog breeds were predominant among the patients with patellar luxation in both clinics.
2. The left hindlimb was 1.5 times more frequently affected with patellar luxation that the right hindlimb.
3. Dogs with patellar luxation weighed most commonly from 2 to 5 kg – this was valid for luxations grade I, II and III. Grade IV luxations were the most prevalent in dogs weighing 5–15 kg.
4. Patellar luxation was most commonly diagnoses between 1 and 5 years of age. Within this age group, grade I dislocations were predominant, while in dogs < 1 years of age, grade II luxation was the most frequent.

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A TRIAL TO TESTIFY THE SAFETY OF VACCINAL MYXOMA VIRUS ON SPERMATOGENESIS IN RABBITS

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ABSTRACT

The aim of our study was to monitor in dynamics the impact and safety of attenuated vaccinal myxoma virus strain on some rabbit semen characteristics. Male rabbits of New Zealand White line were vaccinated (on D0) with attenuated myxomatosis vaccine with standard dose (group A, n=10) and compared in dynamic to control unvaccinated animals (group B, n=6). The samples were collected using an artificial vagina twice – on D20 and D50 post vaccination. Semen samples were analyzed using CASA system in order to evaluate the concentration, motility and velocity parameters of rabbit spermatozoa. Our study showed that there is no negative influence in basic spermatogenic parameters and the quality of rabbits' semen is not negatively affected by standard myxomatosis vaccination.

Key words: myxoma virus, rabbit, vaccination, spermatogenesis, CASA.

Introduction

The members from the Poxviridae family are large DNA viruses which can infect mammals and insects. These viruses are 69, divided in two subfamilies and in 28 genera, ten of which affect mammals (King et al., 2012). Typically, these viral infections include formation of skin lesions and papules but some of them are generalized potentially fatal infections (human's small pox, rabbit's myxomatosis).

Some of the poxviruses have the potential to affect also the male reproductive system and to cause orchitis, epididymitis, impaired spermatogenesis and infertility (smallpox virus, vaccinia virus, chickenpox virus, myxoma virus) (Riggs, Sanford, 1962; Mikuz, Damjanov, 1982; Dejucq, Jegou, 2001).

Myxomatosis is generalized virus infection which affects mainly Oryctolagus cuniculus. It has two different clinical forms – nodular myxomatosis which is characterized with formation of tumor-like myxomas in different parts of the body, and non-myxomatosed myxomatosis– mainly with respiratory symptoms (Best, Kerr, 2000, Farsang et al., 2003). Lethality in nonimmune rabbits affected from high virulent myxoma virus is more than 90 % (Fenner, Ratcliffe, 1965). It is known from long time that myxoma virus affect the male reproductive system (Hurst, 1937; Fenner, Woodroofe, 1953). There are inflammatory and necrotic changes in the testicles and the virus can be isolated from them and from the ejaculate. Six months after infection 50 % of the male rabbits are still sterile (Sobey, Turnbull, 1956). Recent trials proved that even field attenuated myxoma viruses can cause interstitial orchitis, epididymitis, impaired spermatogenesis and temporally infertility (Fountain et al., 1997). In non-myxomatosed form of the disease the virus cannot be isolated from nasal and conjunctival secretions, from monocytes, ovaries, but can be found in the testicles (Marlier et al., 2000). These date give us an idea for myxoma virus influence of the reproductive system.

Prevention from myxomatosis is based on vaccination mainly with attenuated mono- and bivalent vaccines (Lemiere 2000; Marlier, 2010). The EU legislations require proofs for safety and

immunogenicity of vaccines. Important part is lack of effects on the animal's reproductive system (Regulation (EC) No 726/2004, Directive 2001/82/EC, Directive 2009/9/EC).

The aim of our study was to monitor in dynamics the impact and safety of attenuated vaccinal myxoma virus strain on some rabbit semen characteristics.

Material and Methods

Animals

The trial was carried out in a rabbit farm in the Institute of Animal Science, Kostinbrod. The experiment included 16 clinically healthy mature male rabbits (5 months old) of New Zealand White line. Till the moment of the experiment the rabbits were unvaccinated but regularly dehelminthed. During the period the animals were in separated cages and bred under equal circumstances. The animals were fed with nourishing fodder, hay and root crops. Food and water were given ad libitum. All procedures followed the good clinical practice.

Experiment

The animals were divided in two groups: Group A /n=10/ with myxomatosis vaccine, single application, standard dose according to the manufacture requirement and Group B (n=6) – control group, with physiologic saline application. Spermogram parameters were investigated in dynamics on 20th (D20) and 50th (D50) post vaccination. The parameters between the two groups were compared.

Vaccine

The vaccine used was legalized, homologue, monovalent, lyophilized, attenuated myxomatosis vaccine, which includes in a single dose Poxvirus myxomatosae attenuatum – min 103.3 TCID50 max 105.8 TCID50. Vaccines were stored and diluted according to the manufacture requirements.

Semen collection, storage and transportation

Samples were collected with artificial vagina in D20 and D50. The proper storage of semen is of crucial significance when the samples are to be examined later than an hour after collection. The temperature shock should be minimized. A special storage solution is necessary for the vitality preservation. There are several suitable solutions mentioned in literature. In this experiment Tris Buffer was used (Boiti et al., 2005). After the sample was taken it was transferred in a warmed sterile Eppendorf cuvettes and diluted 1:1 with warmed Tris Buffer solution. The samples were stored and transferred to the laboratory under controlled temperature 18–20 ^{o}C.

Semen analysis

All samples were analysed using CASA (Computer Assisted Semen Analysis) system – Sperm Class Analyzer (Microptic, Spain) combined with microscope Nikon Eclipse E200 (Nikon, Japan) in no more than 2 hours after collection. We analyzed three different drops from every sample.

Statystical analysis

For statistical analysis and determination of significant differences was used SAS 6.02 statistical software (SAS Institute Inc., U.S.A.). The results are presented as means ± standard deviation (SD). P-values at $p<0.05$ were considered as statistically significant.

Results and Discussion

The following parameters from CASA analysis are presented: ejaculate concentration (106/ml); spermatozoa motility – static (%), non-progressive motile (%), progressive motile (%); velocity rate – rapid (%), medium (%), slow (%).

The experiment duration took into consideration the literature date about rabbit spermatogenesis. According to different authors this takes from 48 to 52 days (Swierstra, Foote, 1965; Morton, 1988).

Results from CASA analysis of some of the ejaculate parameters at D20 are presented in Table 1.

Table 1: CASA analysis of some of the rabbit ejaculate parameters, D20

	Group A, n=10	Group B, n=6
	X ± SD	X ± SD
Concentration 10^6/ml	604.43 ± 252.55	565.59 ± 206.8
Static (%)	58.5 ± 16.66	42.67 ± 22.11
Non-progressive motile (%)	36.8 ± 13.53	49.12 ± 16.47
Progressive motile (%)	4.61 ± 4.87	8.20 ± 5.85
Velocity – Rapid (%)	2.88 ± 3.09	7.74 ± 9.68
Velocity – Medium (%)	7.24 ± 5.74	16.64 ± 10.64
Velocity – Slow (%)	29.43 ± 7.03	32.89 ± 3.81

Statistically significant at $p<0.05$

There is no significant difference between experimental and control groups in both examinations. The literature date for spermatozoa count per milliliter shows reference rate – 250–600 106/ml (Boiti et al., 2005). The concentration in New Zealand White line is 416.72 ± 9.16 x 106 (Campos et al., 2014). The results from our examination and the literature date are similar. Normally only mature sperm cells are present in the ejaculate in about 40–42 days. During this long production period, the vaccine may show some negative effects on sperm quality, but such effects were not observed.

The percent of progressive motile spermatozoa is important parameter for male fertility. The reference rage in rabbits is 30–90 % (Boiti et al., 2005). The results in our examination showed severe aberration without clear explanation. The motility parameters show decreased per cent progressive motile in Group A in comparison to Group B in D20 but without statistically significance.

There is decreased percent of rapid and medium velocity spermatozoa in Group A in comparison to Group B in D 20 without statistically significance.

Results from CASA analysis of some of the ejaculate parameters at D50 are presented in Table 2.

Table 2: CASA analysis of some of the rabbit ejaculate parameters, 50 days

	Group A, n=10 X± SD	Group B, n=6 X± SD
Concentration 10^6/ml	437.1± 156.12	315.05± 103.63
Static (%)	49.52± 18.73	47.81± 8.84
Non-progressive motile (%)	45.12 ± 14.57	47.38± 5.26
Progressive motile (%)	5.38 ± 4.22	4.83± 3.62
Velocity – Rapid (%)	3.99 ± 4.11	1.52± 0.72
Velocity – Medium (%)	8.52 ± 4.96	8.56± 3.14
Velocity – Slow (%)	34.99 ± 5.24	42.12± 5.34

Statistically significant at $p<0.05$

The results from the experimental and control groups do not have any significant difference.

There are several possible explanations for the observed decrease in progressively motile spermatozoa only in D20. Vaccine can affect temporarily the mitochondrial function. Mitochondria are abundant in spermatozoa and provide adenosine triphosphate, necessary to maintain progressive motility (Evenson et al., 1982). It is known that retroviruses may affect mitochondrial function by causing mDNA depletion (Diehl et al., 2003). There is no such available date for poxviruses.

Different factors such as heat and cold exposure, pH and osmolality changes, oxidative damage can also affect motility (Castellini et al., 2003; Chrenek et al., 2011). It can be also affected by periods of sexual inactivity – male rabbits that have not ejaculated for prolonged periods often have poor motility on the first ejaculate, but much better on the second ejaculate collected soon thereafter. The same can be observed also in rabbits which are not still sexually active.

Conclusion

The trial testified that vaccination with attenuated myxoma virus in standard dose does not affect negatively ejaculate parameters. The vaccine application is safety for the male reproductive capability.

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INVESTIGATION OF THE BIOCIDAL EFFECT OF ELECTROCHEMICALLY ACTIVATED AQUEOUS SODIUM CHLORIDE SOLUTION ON STAPHYLOCOCCUS AUREUS

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ABSTRACT

Studies were carried out to determine the sensitivity of Staphylococcus aureus to electrochemically activated 3% aqueous sodium chloride solution (anolyte) in different concentrations – 100 %, 50 %, 25 % and 12.5 %. As a control was used the disinfectant Virkon S, applied at final concentrations of 1 %, 0.5 %, 0.25 % and 0.125 %. Two referent strains of S. aureus were used – ATCC and Kowan.

It had been found that the anolyte in concentrations of 50 and 100 % inactivates the cells of S. aureus ATCC in suspension at a density of 106 cells/ml within 5 min. After 10 minutes of impact and lower concentrations (25 and 12.5 %) had a bactericidal effect. The anolyte in all tested concentration (12.5 to 100 %) had a bactericidal effect on the cells of S. aureus Kowan in suspension with concentration of 106 cells/ml in 10-minutes. Shorter intervals tested (2 min and 5min) were not sufficient for achieving bactericidal action even at a concentration of anolyte 50 and 100 %, while after 10 min and even smaller concentrations (25 and 12.5 %) had such action. S. aureus ATCC showed slightly higher sensitivity to anolyte and Virkon S compared to the other tested strain Kowan. The effect of the control disinfectant Virkon S on the tested staphylococcal strains was completely analogous to that of the anolyte.

Key words: electrochemically activated solution of sodium chloride, anolyte, Virkon S, Staphylococcus aureus, antibacterial activity.

Introduction

One of the biggest problems in modern medicine is the increasing resistance of pathogenic bacteria to antimicrobial, as well as to disinfectants. This is a prerequisite to search for new effective antimicrobials, which while not be dangerous for patients and the environment. One possibility in this respect provides the technology for the electrochemical processing of water and the preparation of electrochemically activated aqueous solution (catholyte and anolyte). These can be used for disinfection of water and of other objects, as well as for the treatment of bacterial and viral diseases (Atanasov et al., 2014; Karadzhov et al., 2014; Ignatov et al., 2015). In Bulgaria Gluhchev et al. (2015) found a significant inhibitory effect of anolyte on Escherichia coli, as well as on the development of Classical swine fever in cell culture. In laboratory conditions Tasheva et al. (2010) found antimicrobial activity of electrochemically activated aqueous solutions (anolytes) of alkaline and alkaline earth metal salts on field strains of Candida albicans.

The activated water obtained by electrolysis acquire completely new properties and becomes effective acting ecologically clean disinfectant and means for prevention of many diseases. The fields of application of activated water are constantly increasing. The resulting solutions are also called "live" (catholyte) and "dead" (anolyte) water. Catholyte is an alkaline solution with pH between 10.7 and 11.1, with a low redox potential (RP) smaller than -400mV when is fresh and + 200 mV when is stored for several days. It has considerable detergent and washing properties. Anolyte has acidic to neutral pH (between 6.8 and 7.3), high RP (above + 900 mV when is fresh)

and a wide range of disinfecting properties. Its high redox potential determines its bactericidal action. Caroline with its alkaline properties proved an effective tool against free radicals in the body (Act Beauty, 2015; RADICAL WATERS, 2016). It was found that the activated water is not toxic and not dangerous both for external and internal use (Korodetski, 2011).

It turns out that such a water has occurred first in nature. In the bowels of the earth in the mineral springs it has layers with a large difference in the electrode potentials, which act as anode and cathode in an underground electrolyser (eg. copper or zinc and potassium and nickel, and zinc, in this case attaches electrons and copper accept them). Thus, the crust itself produces activated ingredients. Most mineral waters actually proved natural activated solutions and their healing effect is due more to this feature, rather than their mineral composition. This explains the fact that the mineral water at the very headspring has a much greater impact than bottled such, because the activated solutions over time lose its healing power (Korodetski, 2011).

Since staphylococci are one of the most spread bacteria and some of the most common causes of purulent infections with different location in animals and humans, while these are among the most resistant to antimicrobials Gram-positive bacteria, in this work we aim to investigate the effect of the anolyte on suspensions of Staphylococcus aureus.

Materials and methods

Anolyte (activated water). Tested was the effect of the anolyte containing Cl-, prepared by electrochemical activation of distilled water 3 % NaCl, applied in various final concentrations from 12.5 to 100 %.

Control. Virkon S was used in final concentrations of 1 % to 0.125 %.

Microorganisms. In the study were used suspensions with concentrations 106 cells/ml of two reference strains of Staphylococcus aureus (ATCC and Kowan).

Nutrient media. Culture media from Scharlau - Antisel, Bulgaria were used - agar of Mueller Hinton for the preparation of 24-hour cultures of the bacterial strains, Mueller Hinton broth, as well as Chapman Stone agar to determine the effect of the tested solutions for antimicrobial activity on S. aureus.

Scaffold. Twice increasing dilutions of the anolyte were prepared in sterile distilled water, as the obtained concentrations were respectively 100 % anolyte, 50 %, 25 % and 12.5 % in an amounts of 9 ml. To each of them was added a suspension of tested microorganism with concentration of 107 cells/ml in an amount of 1 ml, whereby it was achieved a final concentration of 106 cells/ml.

Enclosed were the following controls - sterile distilled water (without anolyte) with the same content of studies bacterial strain, as well as 100% anolyte without microorganisms.

After various time intervals for the influence of the anolyte (2 min, 5 min and 10 min) cultures were made from each of the samples in Mueller Hinton broth, which were cultured at 37 °C for 24–48 h under aerobic conditions. At cases of establishment of growth in liquid media, subcultures were made on the selective medium Chapman Stone agar to check for growth of the tested microorganisms.

Microscopic studies of preparations stained by Gram were made of materials from cultures in liquid and on solid media.

Results

The summarized results of the conducted researches are presented in Tables 1–4 and some of them – and in Figures 1–3.

Table 1: Growth (amount of colonies) of *S. aureus* ATCC with concentration 10^6 cells/ml after various intervals of exposure of anolyte, applied in different concentrations

Concentration of the anolyte in %	Exposure time - min		
	2	5	10
100	Single	0	0
50	Single	0	0
25	Single	Single	0
12.5	Single	Single	0
Control without anolyte	Many	Many	Many
Control (anolyte without bacteria)	0	0	0

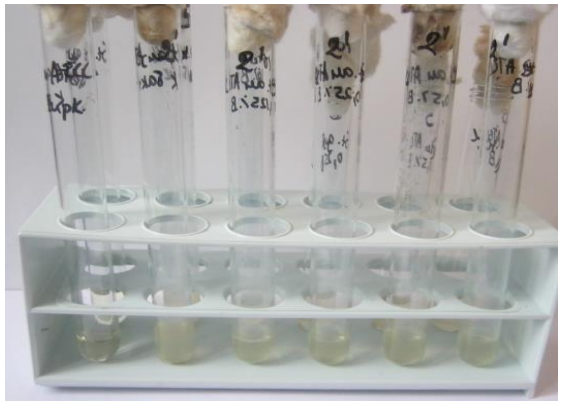

Figure 1: Growth in Mueller Hinton broth after impact of the anolyte, applied in different concentrations (100 %, 50 %, 25 % and 12.5) with a duration of 2 min on suspensions of *S. aureus* ATCC with concentration 10^6 cells/ml

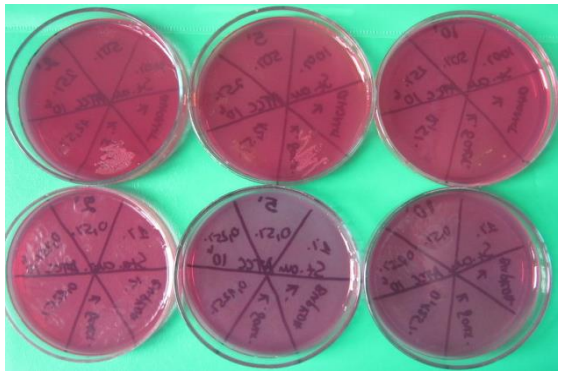

Figure 2: Growth of *S. aureus* ATCC after impact of the anolyte (above), applied in different concentrations (100 %, 50 %, 25 % and 12.5) and of Virkon S (below) at a concentration of 1 % to 0.125 % with duration of 2 min, 5 min and 10 min on suspensions with concentration10^6 cells/ml.

Table 2: Growth (amount of colonies) of *S. aureus* Kowan at concentration10^6 cells/ml after various intervals of exposure of anolyte, applied in different concentrations

Concentration of the anolyte in %	Exposure time - min		
	2	5	10
100	Many	Many	0
50	Many	Many	0
25	Many	Many	0
12.5	Many	Many	0
Control without anolyte	Many	Many	Many
Control (anolyte without bacteria)	0	0	0

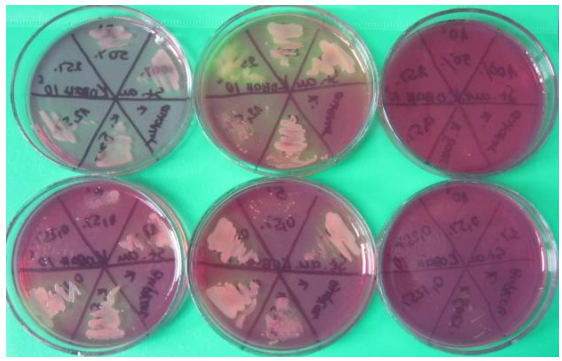

Figure 3: Growth of *S. aureus* Kowan after impact of the anolyte (above), applied in different concentrations (100 %, 50 %, 25 % and 12.5) and of Virkon S (below) at concentration of 1 % to 0.125 % with duration of 2 min, 5 min and 10 min on suspensions with concentration of 10^6 cells/ml.

Table 3: Growth (amount of colonies) of *S. aureus* ATCC with concentration 10^6 cells/ml after different intervals of impact of Virkon S, applied in different concentrations

Concentration of Virkon S in %	Exposure time - min		
	2	5	10
1	Single	0	0
0.5	Single	0	0
0.25	Single	0	0
0.125	Single	0	0
Control without Virkon S	Many	Many	Many
Control (Virkon S without bacteria)	0	0	0

Table 4: Growth (amount of colonies) of *S. aureus* Kowan with concentration 10^6 cells/ml after different intervals of impact of Virkon S, applied in different concentrations

Concentration of Virkon S in %	Exposure time - min		
	2	5	10
1	Many	Many	0
0,5	Many	Many	0
0,25	Many	Many	0
0,125	Many	Many	0
Control without Virkon S	Many	Many	Many
Control (Virkon S without bacteria)	0	0	0

The data in Table 1 and Figures 1 and 2 show that the anolyte in concentrations 50 and 100 % inactivated the cells of S. aureus ATCC in suspension with density of 106 cells/ml within 5 min. After 10 minutes of impact and lower concentrations (25 and 12.5%) have a bactericidal effect.

From the results presented in Table 2 and Figure 3 it is seen that in all tested concentration (12.5 to 100 %) the anolyte has a bactericidal effect on cells of S. aureus Kowan in suspension with concentration 106 cells/ml in 10-minute interval. The shorter intervals tested (2 min and 5 min) were not sufficient for achieving the bactericidal action even at a concentration of anolyte 50 and 100 %, while after 10 min and even smaller concentrations (25 and 12.5 %) had such action.

The results obtained when testing control disinfectant Virkon S on both used staphylococcal strains were similar to the anolyte. They can be seen in Tables 3 and 4 and in Figures 2 and 3. In all tested concentration Virkon S inactivated S. aureus ATCC within 5 min, but not for 2 min, while the other strain Kowan showed smaller sensitivity in tested concentrations of 106 cells/ml and died within 10 min.

Discussion

The results obtained by the current research show that the anolyte can be used as a reliable disinfection agent in the presence of staphylococci in amounts of 106 cells/ml in an aqueous medium. Due to differences in the sensitivity of the strains for a certain effect is required exposure not less than 10 min. In the presence of proteins slower action of the anolyte would be expected, as well as the use of higher concentrations of 50 % or most preferably 100 %. The effectiveness of anolyte is completely analogous to that of disinfectant Virkon S. Significant advantages, however, are the ecological safety of anolyte and the low price.

In some of the tubes with growth was proved that it is not due to bacteria studied, but because of entrance and development of spores of bacilli. However, for complete accuracy taking into account the time required for achieving the bactericidal action of the tested substances, it is preferable to use liquid media with subsequent subcultures on solid selective media, as this avoids completely some residual effect of the anolyte and Virkon S after direct inoculation on solid medium after the detected period of time before being absorbed into the agar.

S. aureus ATCC showed a slightly higher susceptibility to anolyte and Virkon S compared with the other tested strain Kowan. The resistance of staphylococci, however, turns out to be higher than that of P. aeruginosa, which under the same conditions are killed within 2 min (Popova et al., 2016), while staphylococci - for 5-10 min. In previous our studies (Popova et al., 2016) have established experimentally high antibacterial activity of the freshly prepared anolyte, which in a concentration of 100 %, 50 % and 25 % kills for a short time (2 minutes) suspensions and of other Gram-negative bacteria: Salmonella enterica, Escherichia coli and Pseudomonas aeruginosa with concentration of 106 cells/ml and suspensions of Salmonella enterica with concentrations of 108 cells/ml. Tasheva et al. (2010) found antimicrobial action of electrochemically activated aqueous solutions (analytes) of alkaline and alkaline earth metal salts on field strains of Candida albicans, while in two of the solutions had been observed suppress the growth of fungi on the 15th minute from the start of their action. Obviously for inactivation of Gram-positive microorganisms such as staphylococci and Candida albicans is needed more prolonged exposure of the anolyte in comparison with Gram-negative bacteria. What is the period of retention of the antibacterial activity, however, is not known, although it is important from a practical point of view? This requires further research.

Conclusions

The anolyte at concentrations of 50 and 100 % inactivates the cells of S. aureus ATCC in suspension with density of 106 cells/ml within 5 min. Smaller concentrations (25 and 12.5 %) have a bactericidal effect after 10-minute exposure.

In all tested concentration (12.5 to 100 %) the anolyte has a bactericidal effect on the cells of S. aureus Kowan in suspension with concentration of 106 cells/ml in 10-minute intervals. Shorter intervals tested (2 min and 5 min) are not sufficient for achieving the bactericidal action even of the anolyte with concentrations of 50 and 100 %.

The effect of the control disinfectant Virkon S on both used staphylococcal strains is similar to that of the anolyte. In all tested concentration Virkon S inactivates S. aureus ATCC within 5 min. S. aureus Kowan shows less sensitivity in the tested concentration of 106 cells/ml and dies within 10 min.

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EFFECT OF EXPERIMENTAL FASCIOLOSIS AND DIETHYLNITROSAMINE INTOXICATION ON TRACE ELEMENTS CONTENT IN RAT LIVER

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ABSTRACT

The aim of the work was to be investigated the trace elements content in rat liver after the effect of chronic fasciolosis and diethylnitrosamine (DENA) intoxication. The Mo, Rb, Br and Cu contents were near to the controls or slightly increased. The highly increased quantity of Cu and low values of Zn, Fe and Co were established. The obtained results pointed that the combined action of Fasciola hepatica and DENA leaded to a specific mineral imbalance in the liver, which might take place in the pathogenesis of the interaction between experimental fasciolosis and chemical intoxication.

Key words: Experimental helminthosis, chemical intoxication, liver, trace elements.

Introduction

The pathogenesis of the interaction between Fasciola hepatica-infection and diethylnitrosamine (DENA) treatment is not well clarified yet (Tsocheva, 1986). Fasciolosis and DENA initiated hepatocarcinoma are wide spread and very dangerous for humans (Kutikhin et al., 2013). The target organ of the both pathogenic factors is the liver. It is known that mineral imbalance corresponds to the structural changes of the tissue and to the biochemical disturbances of the cells, so the investigation of the changes in trace element content may reveal some of the mechanisms of this interaction.

The aim of the present study is to investigate the trace elements content in the rat liver tissue after the combined effect of chronic F. hepatica infection and DENA intoxication.

Material and Methods

The experiment was carried out on 24 male albino Wistar rats, 30 days old, divided in 4 groups: Group I - healthy animals – 6; Group II – F. hepatica infected animals – 6; Group III – DENA treated animals – 6; Group IV – F. hepatica infected and DENA treated animals – 6.

The rats were infected per os on the 1st day of the experiment with 15 methacercariae of F. hepatica. DENA was injected intraperitoneally 4 times at 7-day intervals at a dose of 100 mg/ kg body weight from the 6th week p. i. The animals were sacrificed on the 10th week of the experiment.

The experiment was conducted in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes and the current Bulgarian laws and regulations.

The trace elements content in the liver tissue was determined by a non-destructive method of neutron activation analysis (Gabrashanska & Damyanova, 1987). The contents of zinc (Zn), iron (Fe), copper (Cu), cobalt (Co), molybdenum (Mo), chrome (Cr), selenium (Se), rubidium (Rb) and bromine (Br) were determined in the rat liver tissue. The results were statistically processed after variation analysis and Student's t-test.

Results and Discussion

The results are presented in figures 1, 2 and 3.

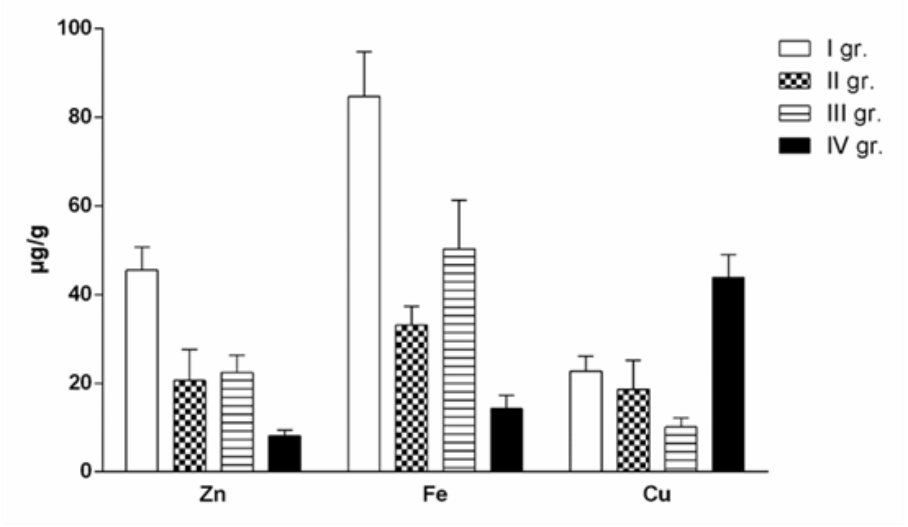

Figure 1: Content of Zn, Fe and Cu in rat liver after *F. hepatica* after *F. hepatica* infection and DENA treatment.

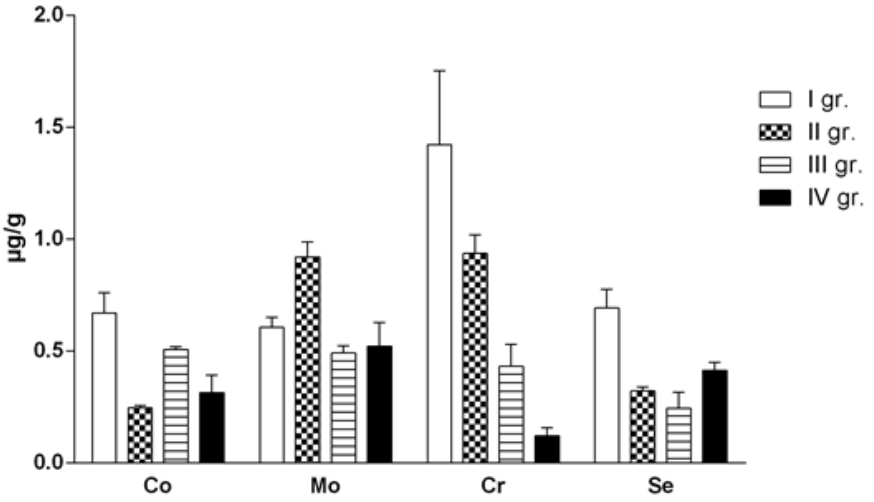

Figure 2: Content of Co, Mo, Cr and Se in rat liver after *F. hepatica* after *F. hepatica* infection and DENA treatment.

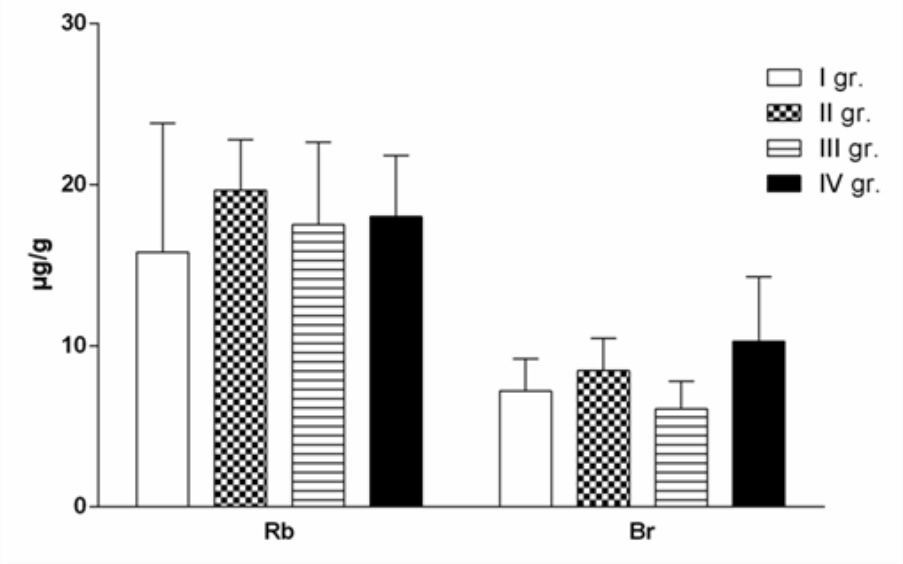

Figure 3: Content of Rb and Br in rat liver after *F. hepatica* after *F. hepatica* infection and DENA treatment.

The values of the quantitatively established trace elements in the normal liver tissue (Group I) are used as controls.

The study of the trace element status in the liver of F. hepatica infected rats (Group II) shows a significant decrease of Zn, Se, Co and Fe content compared to the control ($P < 0.001$) and slide decreased values for Cu and Cr ($P > 0.1$) (Fig. 1 and Fig. 2). The content of Rb and Br is near the control (Fig. 3) and the content of Mo is increased ($P < 0.001$) (Fig. 2).

The study of the trace elements spectrum in the liver of DENA-injected animals (Group III) shows a deficit of Zn, Cr, Se, and Cu compared to the control ($P < 0.001$). The Co and Mo contents are slightly reduced and the content of Rb and Br is similar to that of the controls (Fig. 1 and Fig. 2).

The study of the trace elements content in the rat liver after combined treatment with F. hepatica and DENA (Group IV) shows increased levels of Rb ($P < 0.01$) and Cu ($P < 0.1$) (Fig. 1 and Fig. 3). A decrease in the contents of Fe, Cr, Co and Zn ($P < 0.001$) is reported. The contents of Mo, Se and Br are similar to that of the controls (Fig. 1 and Fig. 2).

Our results for the trace element status in the liver of F. hepatica infected rats (Group II) support the literature data (Gabrashanska & Damyanova, 1987; Gajewska et al., 2005). The function and biochemical regulation of the liver cells are altered from the direct and indirect action of F. hepatica causing hepatitis, fibrosis, cirrhosis in the liver tissue. The decreased levels of Zn, Co, Mo, Cu, etc. depend of the intensity and stage of the parasitic infection (Gabrashanska et al., 2008; Breshahen & Tanumihardjos, 2014).

A decrease in the content of all studied liver trace elements is strongly expressed in the DENA-treated animals (Group III). It is possible due to the direct toxic effect of DENA. Other authors report a decreased level of Se in children with malignant tumors (Shabanov, 1981). Se-supplementation reduces oxidative stress in DENA-induced carcinoma in rats (Mohamed et al., 2011).

There are no literature data available for changes in the trace elements content in animals subjected to combined treatment with F. hepatica and DENA.

Our present study shows that the combined effect of the two pathogenic factors (Group IV) statistically changes but does not aggravate the status of the liver trace elements content. The Mo, Rb, Br and Cu contents are near to the control or slightly increased. The highly increased quantity of the liver Cu in this group may be discussed as a possible mechanism by which the inhibition of the DENA-induced liver carcinogenesis at the background of the chronic fasciolosis is realized, which has been established earlier (Tsocheva, 1986).

The low values of Zn, Fe and Co in this group show a disturbance of the oxidant-antioxidant processes in the liver cells and the increased permeability of the cell membranes (Evans & Halliwell, 2001). These data correlate with the structural changes in the hepatocytes in Group IV which have been established earlier (Tsocheva et al., 1988).

Our previous data show a stronger decrease of some liver drug metabolism parameters (heme, cyt b5, cyt. P450, etc.) After combined effect of chronic fasciolosis and DENA intoxication compared to the cases of their independent action (Tsocheva et al., 1992). These results correlate with the present data about the lowest Fe content in the rat liver in Group IV.

Conclusions

The results we obtained point that the combined effect of chronic fasciolosis and DENA intoxication leads to a specific mineral imbalance, which may take place in the pathogenesis of the fasciolosis and chemical carcinogenesis interaction.

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APPLICATION OF NONINVASIVE MOLECULAR – BIOLOGICAL METHODS FOR DIAGNOSTICS OF EIMERIOSIS IN DOMESTIC RABBITS (ORYCTOLAGUS CUNICULUS)

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ABSTRACT

The parasites of the genus Eimeria (Apicomplexa: Eimeriidae) are among the most common in rabbits. We use non-invasive molecular biological methods to determine the species of the genus Eimeria and confirmed the existence of five species in experimental infected domestic rabbits (Oryctolagus cuniculus). We consider that the obtained results could be used for species identification of parasites of the genus Eimeria in mixed populations.

Key words: Eimeria spp., Oryctolagus cuniculus, diagnostic, PCR, ITS

The species of the genus Eimeria (Apicomplexa: Eimeriidae.) are the most common parasites in rabbits. In world literature there are described eleven species Eimeria spp. found at domestic rabbits (Oryctolagus cuniculus) with clearly established morphological differences. In our country, we have scarce data about the diversity of species causing eimeriosis in rabbits. There are some studies on Burgas region (Meshkov, 1981, 1982) in which eight types of Eimeria spp. causing coccidiosis in domestic rabbit have been described: E. magna, E. perforans, E. irresidua, E. media, E. stiedai, E. intestinalis , E.piriformis and E.exigua.

In her PhD thesis (Kostova, 1989) after coproscopic examination of 1750 fecal samples from eight different regions in Bulgaria, she determined seven species Eimeria spp. in domestic rabbits by (Levine, 1973), including morphological characteristics as well.

There is some variability in biometric data of the oocysts, which can be explained completely by the effect of various external factors, such as intensity of infection, temperature, diet of the host, the endogenous period for development of the parasite, etc. (E. M. Cheissin, 1967; Milyauskaite & Arnastauskene, 1978). Even though oocyst morphology allows a good differentiation of Eimeria in rabbit, it is practically impossible to evaluate carefully thousands of oocysts just to assess if a given strain is really pure. Moreover, a small portion of oocysts may differ from the typically expected morphology and present a challenge to a correct diagnosis. In addition, all these biological features may present a variable level of overlap, hampering in some cases an accurate Eimeria species identification (Long and Joyner, 1984). The exact identification of the eimeriosis in rabbits is complicated in mixed populations, therefore more accurate species-specific methods for determining species of the genus Eimeria should be applied.

PCR-based molecular methods have been widely used in the diagnosis and characterization of Eimeria spp. (Lew et al., 2003; Oliveira et al., 2011). However, data obtained from regular PCR analyses cannot link species-specific genetic markers with oocyst morphology for species identification due to f. requent co-infections of multiple Eimeria species in the same host in the field (Wang et al., 2014)

Internal transcribed spacer (ITS) refers to the DNA segment located between the genes of the small ribosomal subunit RNA and a large ribosomal subunit in the chromosome rRNA. In eukaryotes there are two sections ITS. ITS1 is situated between the 18S rRNA genes and 5.8S. ITS2 unit is between the genes of the 5.8S and 28S rRNA.

A comparison of the ITS region sequences is widely used in taxonomy and molecular phylogeny. This is because it can be easily amplified, even small amounts of DNA, and because there is a high degree of variation even between closely related species.

The species of the genus Eimeria are host species-specific and localization-specific. In most cases they are present in mixed invasion with different pathogenicity.

Oliveira at all. (2011) describe molecular diagnostic methods for the differentiation of the eleven species of Eimeria in domestic rabbits. They determined the nucleotide sequences of ITS1 ribosomal DNA and model species- specific primers for each species, which we used in this study.

Aim

The purpose of this study is to implement new noninvasive molecular-biological methods for an accurate determination of the species from genus Eimeria based on ITS1 region.

To achieve the aim, we used oocysts from naturally infected with Eimeria domestic rabbits.

Material and methods

Parasite collection: Examination of 30 rabbit's fecal samples and collection of Eimeria spp. were conducted.

Coprological examination: Fecal samples were collected in polyethylene plastic labeled bags, and were examined during the same day of collection by the concentration floatation technique according to (Pritchard, M. H. and Kruse, G. O., 1982) and collected in 2% Potassium dichromate ($K_2Cr_2O_7$) solution.

Oocysts were mixed very well in water, and incubated at 15-30ºC for 24-120 hours (or 1-5 days). The culture was stirred every day for an aeration.

DNA extraction: The extraction of DNA from oocyst of Eimeria spp. was performed according to (Sambrook J. and Russell D. W. 2001).

Oocysts of Eimeria spp. were put in a sterile mortar and liquid nitrogen was used to disrupt the cells. The oocyst pellets were used in DNA extraction kit (Qiagen, Germany).

Polymerase chain reaction (PCR) assay: The oligonucleotide primers used in this study were selected from highly conserved sequences encoding rRNA, ITS1sequence of Eimeria spp.

For isolation and purification of the DNA was used solution of GeneJET Genomic DNA Purification Kit (Thermo scientific).

This primer set was used in the PCR assay for partially amplification of the DNA, ITS1 of Eimeria spp.

Table 1: Primers species-specific sequences used for general amplification of the ITS1 from Eimeria spp.

Eimeria species	Primer ITS1 Forward	Primer ITS1 Reverse	Product	Eimeria spp. Product
Eimeria spp. (all species)	GGGAAGTTGCGTAAATAGA	CTGCGTCCTTCATCGAT	Different for different species	
E. magna	TTTACTTATCACCGAGGGTTGATC	CGAGAAAGGTAAAGCTTACCACC	218 bp	457 bp
E. coecicola	AGCTTGGTGGGTTCTTATTATTGTAC	CTAGTTGCTTCAACAAATCCATATCA	256 bp	573 bp
E. media	GATTTTTTTCCACTGCGTCC	TTCATAACAGAAAAGGTAAAAAAAGC	152 bp	468 bp
E. exigua	GAATAAGTTCTGCCTAAAGAGAGCC	TATATAGACCATCCCCAACCCAC	280 bp	472 bp
E. perforans	TTTTATTTCATTCCCATTTGCATCC	CTTTTCATAACAGAAAAGGTCAAGCTTC	157 bp	473 bp
E. flavescens	GAATATTGTTGCAGTTTACCACCAA	CCTCAACAACCGTTCTTCATAATC	199 bp	463 bp
E. piriformis	ACGAATACATCCCTCTGCCTTAC	ATTGTCTCCCCCTGCACAAC	289 bp	451 bp
E. intestinalis	TGTTTGTACCACCGAGGGAATA	AACATTAAGCTACCCTCCTCATCC	241 bp	484 bp
E. stiedai	GTGGGTTTTCTGTGCCCTC	AAGGCTGCTGCTTTGCTTC	217 bp	563 bp
E. irresidua	TTTGGTGGGAAAAGATGATTCTAC	TTTGCATTATTTTTAACCCATTCA	226 bp	367 bp
E. vejdovskyi	GTGCTGCCACAAAAGTCACC	GCTACAATTCATTCCGCCC	166 bp	406 bp

PCR reaction was carried out in a total volume of 50 µl containing:

25 µl PCR Master Mix 2x (Thermo scientific) containing, (4 mM MgCl2; 0.4 mM deoxynucleotides triphosphates mixture (dATP, dCTP, dGTP and dTTP);0.05 u/µl thermos aquaticus (Taq) polymerase and reaction buffer;

5 µl of extracted parasite genomic DNA

2 µl for each primers and nuclease-free sterile double distilled water 16µl.

The obtained sample was amplificated via precise cycle in Veriti 96 Well Thermal Cycler from Applied Biosystems as follows:

Initial denaturation – 93 ºC for 5 minutes, 40 cycles – 93 ºC for 1 minutes, 56 ºC for 2 minutes and 72 ºC for 2 minute, followed by final extension at 72 ºC for 7 minutes.

The PCR amplification products (amplicons) were visualized: Received PCR amplicons (10 µl) were analyzed by dint of 1 % agarose gel electrophoresis. The DNA bands were visualized via ultraviolet transillumination (SCIE-PLAS Vision) after gel staining with ethidium bromide (0.5 µg/ml). Images were processed and analyzed with software program GelAnalizer 2010a.

Results

In our preliminary studies we used genus-specific primers to detect species from genus Eimeria. In agarose gel we proved the presence of parasites from this taxon.

The presence of two band in agarose gel gives us a reason to suppose that, there are more than one species in the sample, which proves mixed invasion (Fig. 1).

Figure 1: Agarose gel image with three columns: first is Ladder and two samples with dual bands with genus-specific primers in 451 and 573 bp.

Images of gel-documenting system were made, then they were analyzed by GelAnalizer2010b. Fallowing log-linear alignment of different columns from the first / weight ladder we differed two separate band in two different samples by 451 and 573 bp. After an alignment of the primers for Eimeria spp. with NCBI Nucleotide fragments we received the following products with base pairs in the last column (Table 1).

Used generic specific ITS1 primers could be used for primordial detection in copro samples from rabbit suspected of eimeriosis or for prevention.

To specify the species distinctly we start the test run with each pair of species-specific primers (Fig. 2).

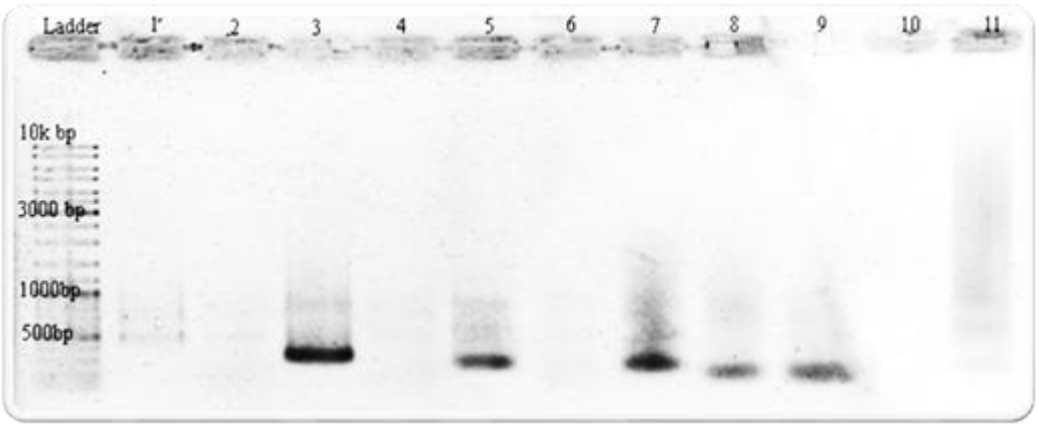

Figure 2: Agarose gel image with twelve column: Lader;
1: Eimeria stiedai; 2: E. coecicola; 3: E. exigua; 4: E. flavescens; 5: E. intestinalis; 6: E. irresidua; 7: E. magna; 8: E. media; 9: E. perforans; 10: E. piriformis; 11: E. vejovski.

Molecular biological methods make it easy to determine the species composition in the long and labor-intensive morphometric microscopic techniques. They help to overcome the subjective error or technical reasons based mainly on ITS1 conservative species specific gene sequences. Due

to the accumulation of more and more complete databases of genes in every organism on earth and the increasing interest in these methods, their implementation is becoming more available (both) methodically and economic. Undoubtedly they will impose as obligatory methods to prove the taxonomic identity when making diagnosis or choosing biological models for experiments.

We found five of the total eleven identified species of the genus Eimeria in domestic rabbits. It was confirmed the presence of E. exigua, species with morphometric smallest size, which was not identified by (Kostova, 1989).

There were not confirmed three of the eight morphometric identified species in Bulgaria, probably due to the small number of test animals.

To confirm the molecular biological results, we resorted to the classical histopathological methods.

After the histologic autopsy of the tested animals, we did not find hepatic pathological changes typical for the liver stage of eimeriosis – E. stiedai, which has been confirmed by their absence in the noninvasive molecular biological studies.

Discussion

The method is noninvasive and does not put test animals under stress during the coprosampling.

Through the methods used in this study we were able to confirm the presence of previously identified by (Meshkov, 1981, 1982; Kostova, 1989; Vladov, 2014) Eimeria species in domestic rabbits. We consider that the obtained results could be used for species identification of parasites of the genus Eimeria in mixed populations.

Conclusion

Conducted tests and analyzes would allow detailed studies with high sensitivity and specificity, that contribute to a better understanding of the epidemiology of this important group of parasites of the genus Eimeria.

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TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2016, vol. 1, No 1(1): 91–90

QUALITY AND SAFETY OF FEED USED IN FEEDING CATTLE

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ABSTRACT

This article addresses issues related to the quality and safety of feed and additives used in the feeding of cattle. The indicators characterizing the quality of feed - dry matter, energy, crude protein, digestible protein in the intestine, balance of protein in the rumen, calcium, phosphorus and raw fiber are described. Questions related to the requirements of dairy cows on their need for nutrients and levels of certain undesirable substances in feed (chemical, microbiological and physical) are reported. Legal norms and regulations concerning the quality and safety of food are considered and the influence of some genetically modified plants used as food for cattle are viewed.

Keywords: quality, safety, feed, requirements, pathogens, toxins, GMO – feed.

Introduction

Indicators characterizing the quality of feed are: chemical (moisture, crude protein, crude fat, crude ash and carbohydrates - crude fiber and NPN) physical form of feed crops, digestibility and consumption.

These key indicators determine the nutritional value expressed in dry matter content, energy, crude protein, digestible protein in the intestine, balance of protein in the rumen, calcium and phosphorus (Todorov et al. 2010, Simeonov et al. 2013 and Stoycheva et al. 2014).

On the basis of European regulations and directives is built the Bulgarian legislation related to the quality and safety of food.

Basic legal norms are: Feed low - last changed 13.02.2015, Regulation № 10 – 03.04.2009 from MAF for maximum levels of undesirable substances and products in feed and Law on GMOs – last changed 02.08.2013.

The purpose of this work is to summarize and analyze the indicators characterizing the quality and safety of feed used in cows and regulations affecting them.

Materials and methods

This study analyzed the literature related to indicators characterizing the quality and safety of feed and legal regulations of the Republic of Bulgaria on allowable concentrations of unwanted substances in them and the requirements concerning GMO products.

Discussion

The term "feed" feature all forage crops that are high in crude fiber. According to Morrison (1970) feed used for ruminants are rich in fiber, but low in other nutrients. To this group the author refers hay waste products from the production of corn-grain (cornstalks), the extraction of cereals (straw) and pastures with their variety in terms of botanical composition. This group includes waste from the milling industry (corn cob and bran) that are high in fiber and low at energy.

A comprehensive international classification is made (http://www.inra.fr/en), according to which feed is divided into 8 classes as follows: dry and coarse feed; green forage and pastures;

silages; high energy feed; protein and concentrated feed; mineral supplements; vitamins supplements and other additives.

The main indicators characterizing the nutritional feed are calculated based on the digestibility and chemical composition (Todorov et al. 2010). In high producing dairy cows it is very important the quality and utilization of rough feed. Many factors influencing consumption and utilization, more important are: health and physiological status, BCS and milk production, palatability of the feed, quantity and volume of ration and ambient temperature.

Appetite with which animals take feed determined amount of dry matter in feed (at libitum). The appetite is a factor influencing the uptake of large amounts of dry matter. In studies of Kirilov (2010) conducted with hybrid maize harvested during different phases of the growing season the dry matter content increases and is highest of waxed to full maturity. As a result, the author concludes that consumption is much higher in milky wax maturity to full maturity. Conservation of maize reduces consumption by 5-11% against the green corn. Differences in digestibility are not observed (Kirilov 2010).

In studies of Stoicheva (2015) with participation of grass mixtures fed through the various phases of the growing season has been found that increasing the DM (1 %) leads to a reduction of CP (0.55 %) at the expense of CF, which increased by 0.86 %.

Body condition scoring (BCS), milk production, pregnancy and technology are the main factors influencing the needs of nutrients in cattle. Raising the living mass in cows leads them to use more energy and protein to maintain life processes. The requirements to digestible nutrients and crude protein in the last three months of pregnancy are higher than previous ones. A similar trend is observed to the needs of crude protein and energy during lactation, as requirements increase with increasing milk production (Adams et al. 1996).

As a factor ambient temperature influencing the consumption of feed. In warm weather animals consuming small amounts of feed and vice versa, lower temperatures stimulate consumption (Todorov et al. 2007).

Feed with good quality indicators satisfy the requirements of cows for milk in terms of essential nutrients, which in turn provides a good health status through relevant physiological periods and productivity (Todorov et al. 2011).

Feed safety is determined by the presence or absence of adverse health of animals and humans substances. More important for cattle, respectively, the received productions are:

The alkaloids contained in plants affect physiological processes in the animal organism, as well as on the production. In most cases, their presence has a harmful effect on animals. Example is the pyrrolizidine alkaloids, which are a normal ingredient in some plants widespread in pastures and meadows. Although their main function is a protection against insects. This alkaloid cause liver disease and in some cases neoplasias on animals fed in larger amounts of plants which has it. Such plants prevalent in grass feed leads to a huge number of poisoning in livestock. Selection of grass fodder on pasture (contains the alkaloid) at grazing prevent the danger of poisoning, but feeding hay or silage can be dangerous (Stegelmeier et al. 1999 and Fu et al. 2001). The spread of pyrrolizidine alkaloid is the most widely in families Asteraceae and Leguminosae, where its concentration is highest. Complete descriptions of plant species containing it is given by Hartmann and Witte (1995). While some species contain only pyrrolizidine alkaloid, others may contain other alkaloids as well. The high concentration of the alkaloid is mainly in the seeds of plants which contain it. The most widespread is the alkaloid in the following plant species Heliotropium lasiocarpum, H. popovii and

H. europaeum, found in wheat crops and when harvest the grain mixed with them (Prakash et al. 1999).

Lactating ruminants, including cows received feed with the participation of the alkaloid secrete it with milk (Panter и James 1990).

According to Prakash et al. (1999) consequences of sub lethal doses were observed in cows reared on pasture in the presence of plants containing high amounts of alkaloids. The authors suggest that continued intake of alkaloids, but in small doses is not always fatal. The primary pathology that causes the alkaloid is on the hepatic veins that are blocked by growing connective tissue and lead to obstruction of the vessels.

Participation of ergot in feed animal feed also can lead to fatal consequences. Bush et al. (1997) suggest that feeding with forage containing alkaloids of ergot (Claviceps purpurea) can cause toxic effects in both animals and humans. Regulation 10 from MAF – April 3, 2009 describes the requirements for feed containing ergot.

The term "mycotoxin" is derived from the Greek word for fungus "mykes" and the Latin word for poison "toxicum". This concept means substances produced by fungi colonized the crops in the field and other feed. They represent a potential threat to animals and humans when used as food.

Each forage crop stored for more than a few days without proper conservation (drying or using chemicals) presents a danger expressed by mold predisposing to formation of mycotoxins. They are widespread in plants around the world and affect important crops such as cereals, nuts, dried fruits, spices, oil seeds and dried beans. Once formed remain very stable in structure, and therefore the best way to do something against them is prevention.

Mycotoxins as toxic metabolites produced by fungi have a wide range of chemical and physical properties that are toxic to animals and humans. Twenty – thirty of them have been studied by contaminated food of animal and human (Watson 1985).

The presence of mycotoxins in animal feed poses a risk to human health if they or their toxic metabolites pass in significant quantities in the production (Smith and Henderson 1991). The effect of various mycotoxins in different directions, some of them are carcinogenic, mutagenic or teratogenic, as well other affect negative on the immune system.

According to Douwes et al. (2003) mycotoxins can occur in the form of fungal spores in the atmosphere, which is considered as the cause of their spread in wetlands or barns.

Keith (2008) considers that the main types of fungi of the genus Fusarium, Alternaria and Aspergillus are most important. Also Penicillium can lead to contamination of crops after harvesting. The author describes the most important mycotoxins, which are: Aflatoxins B1, B2, G1, G2 (affect nuts, dried fruit, corn etc.); Aflatoxins M1, M2 (affect milk and milk products); Deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin и Zearalenone (affecting cereals) and Fumonisins B1, B2, B3 (affecting maize, maize products, etc.).

Field crops and those that are harvested for storage are very difficult to be decontaminated by contamination of mycotoxins. The prevention of the presence of fungi and their toxins is important to apply best practices in the cultivation, harvesting and storage of feed. It is also necessary to apply good practices and procedures of the system for analysis of dangerous and critical control points (HACCP) in the production of compound feed. Unfortunately, in countries with humid climate, there is an excellent environment for the development of these fungi and molds, with the result that produced contaminated feed pose a major problem for animal health and production received from them.

Aflatoxins are group of about 20 fungal metabolites. Only some of them (Aflatoxins B1, B2, G1, G2 and M1) affect forage used by animals. The main fungi producing aflatoxins are Aspergillus species and are found in grains, nuts, dried fruits and more. These kinds of fungus are found in cultures grown in countries with warm and humid climate.

Aflatoxin M1 and M2 are metabolites of aflatoxin B1 and B2, which are produced from cows or other ruminants fed with feed containing them. They are secreted with milk and can infect the dairy products. Smela et al. (2001) describes chemical and biological activity of aflatoxin B1, and Abbas (2005) the role of aflatoxins in safety and food quality. Aflatoxins are stable in foods that are contaminated. They are relatively resistant to the decontamination methods (Smith et al. 1994, Park 2002 and Scudamore 2004).

Climate is a major factor from which depends the development of aflatoxins. "Stress" at the plants associated with drought followed by heavy rainfall adversely affects them. From cereals corn is most vulnerable to infection. Other cereals used in the brewing industry also can become infected respectively to contaminate beer (Mably et al. 2005).

In the literature besides the described toxins are indicated much more: ochratoxin A; deoxynivalenol; trichothecenes; zearalenone etc.

Over the past 20 years was spoke wide about genetically modified organisms and GMO feed. There are a number of studies related to GM plants that are used as fodder.

Views of most researchers about GMO feed and the effects of their use are controversial.

Some authors (Flachowsky et al. 2006) describe studies of GMO feed crops and their relationship to food. There were 18 studies involving (16 of them) cultures from the first generation - Bt-corn, Pat-corn, Pat-beet, Gt-soy, Gt and Bt-potato and (2) with second-generation crops with altered chemical composition. The authors found that cultures of first generation do not modify noticeably the nutritional value of feed and there is not apparent transfer of recombinant DNA from plants to animals. Regardless of the results the authors reported negative attitudes in the public field on GMO products.

Research on the impact of GM crops in mammals and especially their reproductive function are limited. This provoked a number of researchers to conduct large-scale studies of their effects on reproduction, mortality in newborns and their weight development.

In studies with cross calves of Holstein-Friesian breed involving GMO corn (Bt11) Shimada et al. (2006) do not establish a negative effect on growth, hematology, blood biochemistry and function of rumen in calves.

In experiments with ruminants and their descendants held for three years, fed with participation of genetically modified maize (Bt176) not indicate harmful effects on health and productivity, as well as gene transfer to ruminal micro flora or tissues of animals. There were no differences in reproductive and hematological signs (Massimo et al. 2008).

The analysis of the data shows that the quality and safety of feed is crucial to the health and productivity of animals including cows. To comply with the indicators characterizing the quality and safety regulations and legislation are made (applying to all EU countries).

The legislation of the Republic of Bulgaria on the quality and safety of feed and permitted substances in them is regulated by Feed low - last changed 13.02.2015, Regulation 10 from MAF - April 3, 2009 and Law on GMOs – last changed 02.08.2013. Bulgarian legislation regulates the feed safety requirements and regulates the feed business (art. 25. (1) from Feed low). For performing them are developed guidelines and procedures for the implementation of best practices and follow the principles of the system of hazard analysis and critical control points (HACCP). According to

art. 26. (1) of the same low feed business operators must have sufficient and accurate information on feed and must be aware of their effects on animal health.

The maximum permissible concentrations of undesirable substances and products in feed are regulated in Appendix № 1 to Art. 2 para. 2 of Regulation 10 from MAF - April 3, 2009.

Law on GMOs - last changed 02.08.2013 regulates work release, marketing, transport, import, export and control of GMO products in order to protect human health and environment from possible adverse effects from them.

Conclusions

Quality of feed used to feed cattle depends on a number of factors and determined by many indicators, more important of which are: composition, digestibility, appetite and consumption.

The content of undesirable substances in feed can cause a number of diseases and toxic effects in animals, and affect the production.

Bulgarian legislation regulates the requirements and benchmarks of undesirable substances with a view to quality and safety feed.

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