FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE AND
PHARMACY IN KOŠICE — The Slovak Republic

ISSN 0015-5748

2

LVIII • 2014

FOLIA VETERINARIA is issued by the *University of Veterinary Medicine and Pharmacy in Košice (UVMP);* address: Komenského 73, 041 81 Košice, The Slovak Republic (tel.: +421 915 984 669, fax: +421 55 632 52 93, E-mail: Milada.Vargova@uvlf.sk).

IČO: 397 474

The journal is published quarterly in English (numbers 1—4) and distributed worldwide.

Subscription rate for 1 year is 120 €. Orders are accepted by *The Department of The Scientific Information — The Library of The University of Veterinary Medicine and Pharmacy in Košice* (UVIK), E-mail: Natalia.Palencarova@uvlf.sk; the subscription is accepted by the State treasure.

Bank contact: State treasure, Radlinského 32, Bratislava 15, The Slovak Republic; **account number:** 7000072225/8180.

Issued on March 25, 2014

FOLIA VETERINARIA, vydáva *Univerzita veterinárskeho lekárstva a farmácie v Košiciach (UVLF),* Komenského 73, 041 81 Košice, Slovenská republika (tel.: 0915 984 669, fax: 055/632 52 93, E-mail: Milada.Vargova@uvlf.sk).

IČO: 397 474

Časopis vychádza kvartálne (č. 1–4) a je distribuovaný celosvetove.

Ročné predplatné 120 €. Objednávky prijíma *Ústav vedeckých informácií a knižnice Univerzity veterinárskeho lekárstva a farmácie v Košiciach* (UVIK), E-mail: Natalia.Palencarova@uvlf.sk; predplatné Štátna pokladnica (na nižšie uvedené číslo účtu).

Bankové spojenie: Štátna pokladnica, Radlinského 32, Bratislava 15; **číslo účtu:** 7000072225/8180.

Dátum vydania: 25.3.2014

Tlač: **Univerzita veterinárskeho lekárstva a farmácie** Komenského 73, 041 81 Košice

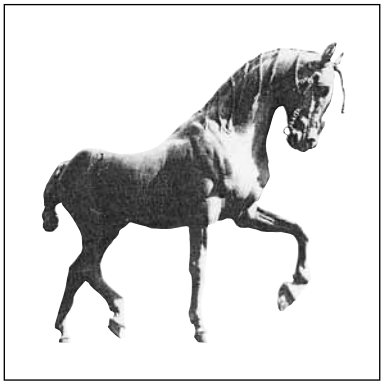
Sadzba: **Sapfo publishers**, Szakkayho 1, 040 01 Košice

EV 3485/09

For basic information about the journal see Internet home pages: www.uvm.sk; www.uvlf.sk

Indexed and abstracted in AGRIS, CAB, EBSCO

FOLIA VETERINARIA, 58, 2, 2014



57th STUDENT SCIENTIFIC CONFERENCE

April 9th, 2014

The aim of the 57th Student Scientific Conference (ŠVOČ), organised in the academic year 2013/2014, was to present results of scientific investigations carried out by students of the University of Veterinary Medicine and Pharmacy in Košice. The selected papers were presented in the following four sections:

I. Pre-clinical section. II. Clinical section.
III. Food and environmental section. IV. Pharmaceutical section.

UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE

FOLIA VETERINARIA, 58, 2, 2014

CONTENTS

FOLIA VETERINARIA, 58, 2: 61—65, 2014

GENETIC DETECTION AND ANALYSIS OF BVDV ISOLATES IN CLINICAL SAMPLES

Carmody, S., Slíž, I., Vilček, Š.

Department of Epizootiology and Preventive Veterinary Medicine
University of Veterinary Medicine and Pharmacy, Komenskeho 73, 041 81 Košice
The Slovak Republic

stephencarmo@hotmail.com

ABSTRACT

Bovine viral diarrhoea virus (BVDV) is a pestivirus which infects both domestic animals and wildlife species worldwide and is a huge economically important pathogen of cattle in the agricultural industry. The molecular epidemiology of the virus can be used in control and eradication programs, to direct vaccination development and usage and to trace likely infection sources. Phylogenetic analysis was conducted on 13 samples from Ireland based on the nucleotide sequences obtained from 5´-UTR and N^{pro} regions. It was confirmed that 9 of these samples belong to subgenotype BVDV-1a,of which 5 originated from Northern Ireland and 4 from the Republic of Ireland. Four of the 13 samples belonged to subgenotype BVDV-1b, all of which originated from Northern Ireland.

Key words: BVDV; Ireland; phylogenetic analysis; subgenotype; typing

INTRODUCTION

BVDV is one of the most widespread infectious agents in cattle, with a worldwide distribution and a high prevalence in virtually all host populations studied to date. The seroprevalence of BVDV is approaching 100 % in the Republic of Ireland (RoI) [5] and stands at 66 % in Northern Ireland (NI) [7] with serious economical implications.

BVDV infects its hosts either transiently or persistently; the latter creating persistently infected (PI) animals which is a characteristic feature of pestiviral infections and the main determinant of losses. Calves are born PI with BVDV following time specific in-utero exposure, predominantly due to the mother contracting the virus during gestation. The main source of infection is other PI animals. Epidemiological equilibrium determines that 1 % to 2 % of cattle are PI animals [8].

The successful control and eradication of BVDV infection requires the detection and the removal of PI animals. This entails both classical and molecular epidemiology. For *molecular epidemiologic* purposes, the *genotype/species* designations are considered the operational taxonomic unit, whereas the related *genotypes* are grouped as subgenotypes. The species and subgenotype designation of the virus determined in PI animals enables the use of molecular epidemiology to find relationship between old/existing and new cases and trace the origin of unexplained outbreaks. Traditionally, genotyping has been conducted in one region only, especially in the 5'Un-Translated Region (5'UTR) [1, 2]. More prevailing studies highlight the need to conduct genotyping in more than one region for verification or to conduct genotyping in the N^{pro} or other regions for more defined phylogenetic analysis and segregation of BVDV isolates into subgenotypes within species [18, 21].

The aim of our work was to type BVDV isolates from the RoI and NI by phylogenetic analysis in both the 5'UTR and N^{pro} genomic regions.

MATERIALS AND METHODS

The Central Veterinary Research Laboratory in the RoI provided 21 samples andthe Agri-Food and Bioscience Institute, NI, provided 20 samples. All samples were previously tested and confirmed to be from PI animals in Ireland. Previous analysis of these samples in Ireland had assigned preliminary subgenotypes.

The extraction of the total RNA employed the TRIzol® Reagent (Life Technologies, USA) and used the method according to the procedures as described by the manufacturer.

The cDNA was synthesised by reverse transcription; the first step of the two step RT-PCR process. The RT-PCR was done to obtain single and semi-nested products.

For the single RT-PCR, the viral isolates were amplified using primers selected from the 5'UTR. A 288 bp long (in the NADL strain of BVDV type 1) DNA fragment was amplified using primers 324 and 326. Primer 324 (5'-ATG CCC WTA GTA GGA CTA GCA-3') (W = A or T; position in BVDV NADL 108-128). Primer 326 (5'-TCA ACT CCA TGR GCC ATG TAC-3'; position in BVDV NADL 395-375) [19].

Viral isolates have also been amplified using primers selected from the N^{pro} genomic region for use in the semi-nested RT-PCR. For step I, amplification of a 738 bp long fragment, the primers BD1 (5'-TCT CTG CTG TAC ATG GCA CAT G-3'; position in BVDV NADL: 367-388) and BD2 (5'-TTG TTR TGG TAC ARR CCG TC-3'; R = A or G position in the BVDV NADL 1104-1085) were used [20].In step II, amplification of a 428 bp long fragment of the N^{pro} region, the primers BD1 as above and BD3 were used. BD3 (5'-CCA TCT ATR CAC ACA TAA ATG TGG T-3'; position in BVDV NADL: 795-771) [21].

The detection of the PCR products was conducted via agarose gel electrophoresis where the PCR products were separated in 2 % agarose gels with 1 x TAE as the running buffer.

The sequencing was done in both directions in both the 5'UTR and in the N^{pro} region using the primers as per RT-PCR. Sanger DNA sequencing was conducted by the company Microsynth AG, Switzerland.

Phylogenetic analysis involved proof reading the sequencing chromatograms using the SeqMan II (DNASTAR Inc.; USA).A comparison of the percentages of nucleotide and amino acid identity was conducted using MegAlign (DNASTAR Inc.; USA). The phylogenetic and bootstrap analysis was performed by using MegAlign and neighbour-joining method implemented in Mega 4.0 [16].

RESULTS

Of the 41 samples amplified in the 5'UTR, 32 positive and 9 negative samples were obtained. The results in the N^{pro} region yielded 26 positive, 6 negative samples and 9 were not done. Of the samples positive in both regions, 13 were selected for sequencing. Phylogenetic analysis was conducted on these 13 samples and the results are as follows. All 13 samples were identified as BVDV-1 in both 5'UTR (Fig. 1) and N^{pro} regions (Fig. 2).

DISCUSSION

We identified BVDV-1a and BVDV-1b to be circulating in NI and BVDV-1a in the RoI. The predominant subgenotype in both regions was BVDV-1a. The only other published work on BVDV subgenotypes in these regions by Graham et al. [6] also identified BVDV-1a as the predominant subgenotype. The results identified BVDV-1b in NI for the first time. This study is also the first to analyse samples from these regions in the N^{pro} part of the genome.

Initially 41 samples were amplified by RT-PCR but not all resulted in positive results. Poorer results (more negatives) were obtained from the samples originating from the RoI which were not sent to Slovakia on dry ice. Very low levels of virus in the sample may also account for negative results. Any sample negative in the 5'UTR region was not analysed in the N^{pro} region.

No BVDV-2 was detected in this study which is reassuring since the vaccines currently used in Ireland only confer good cross-protection against type 1 strains. BVDV-1 is the predominant genotype worldwide [13]. BVDV-2 occurs sporadically throughout Europe [10], [21], [22] and is common in North America [12], [14].With such a widespread geographical distribution and proximity, it may be seen to be only a matter of time before it is also detected in Ireland.

Pestiviruses are highly variable both antigenically and genetically, therefore, each species is further classified. To date fifteen subgenotypes of BVDV-1 (1a to 1p) and 2 subgenotypes of BVDV-2 (2a and 2b) have been identified, not all of which are internationally accepted [11], [13].Genetic typing of BVDV has traditionally been performed in the 5'-UTR, N^{pro} and E2 regions[1,2,14].Mostly 5'UTR nucleotide sequence analysis alone has been used for pestivirus genotype identification [3], [9], [10], [22].It is currently accepted that analysis in multiple regions or in the N^{pro} region is best for determining subgenotypes [18], [21]. The genetic diversity of BVDV isolates is important not only for taxonomy but also for laboratory diagnosis, vaccination design and molecular epidemiology. Typing in this study was obtained and verified through phylogenetic and nucleotide sequence analysis of the 5'UTR and N^{pro} regions for the selected 13 samples. Typing in these regions lead to identical grouping. No identical isolates were found on a genetic level. Bootstrap values of 99 % were obtained from analysis in the N^{pro} region, (Fig. 2) thus confirming the results obtained in the 5'UTR (Fig. 1).

Similar subgenotypes of BVDV-1 have been confirmed in Great Britain. In England and Wales viruses are predominantly BVDV-1a subgenotype but also circulating are BVDV-1b and BVDV-1i [17], [1].The most recent study by Booth et al. [4] also found BVDV-1a to be the predominant subgenotype in these regions. The diversity of BVDV-1 identified to date in Great Britain also includes BVDV-1b, BVDV-1e, BVDV-1i, BVDV-1d [4] and BVDV-1f [15] subgenotypes.

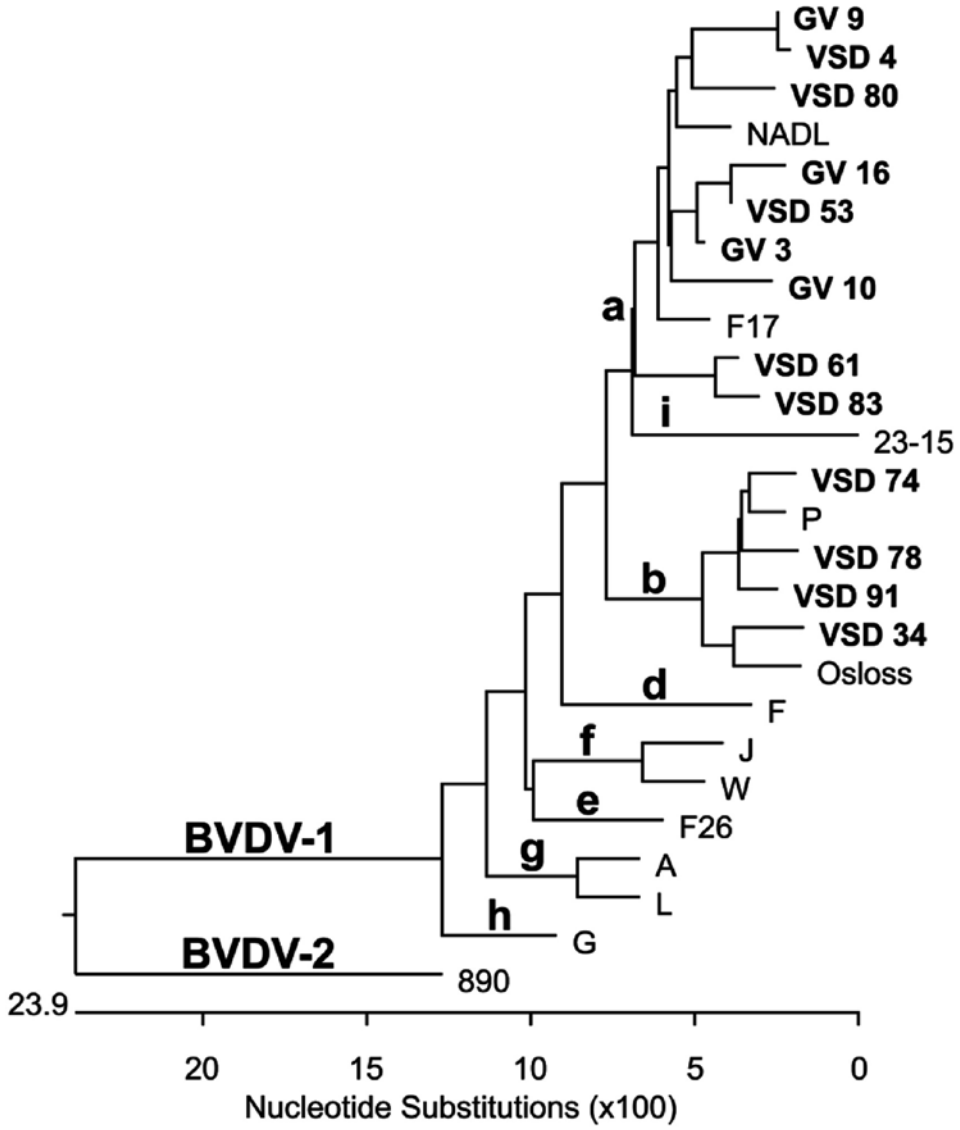


Fig. 1. Phylogenetic tree in 5'UTR

The small letters a, b, d, e, etc., represent subgenotypes of BVDV-1. The reference strains included are in their subgenotype according to those assigned in the study of Vilcek et al. [21]. All 13 sequenced samples in this study from Ireland are denoted in bold and assigned a subgenotype by comparison with the reference strains. Line lengths are proportional to genetic distance as indicated by the scale bar at the bottom

The frequency of the isolation of different subgenotypes differs by geographic regions. The minimal genetic diversity in regards to subgenotypes found in Ireland to date may be partially attributed to the highly regulated trade into Ireland and also based on the fact that Ireland is largely an export market of cattle. The apparent increasing heterogeneity in one of Ireland's biggest agricultural trading partners, Great Britain, highlights the necessity for continual monitoring of our BVDV status. The importance of molecular epidemiology was illustrated by Booth et al., 2013 [4] highlighted in the study by the authors' ability to clarify the routes of viral transmission. No comparison has yet been made between viruses found in Ireland and Great Britain but it is highly likely that in future studies related to viruses will be found. It is also likely that the subgenotypes present in Great Britain will be next to be identified in Ireland.

CONCLUSIONS

BVDV samples studied in this work are the first from Ireland to be analysed in over 13 years and the first from Ireland to ever to be analysed in both the 5'UTR and the N^{pro} genomic region. The results confirm that both subgenotypes BVDV-1a and BVDV-1b are present in Ireland. With further work and analysis of samples, such as in this study of mo-

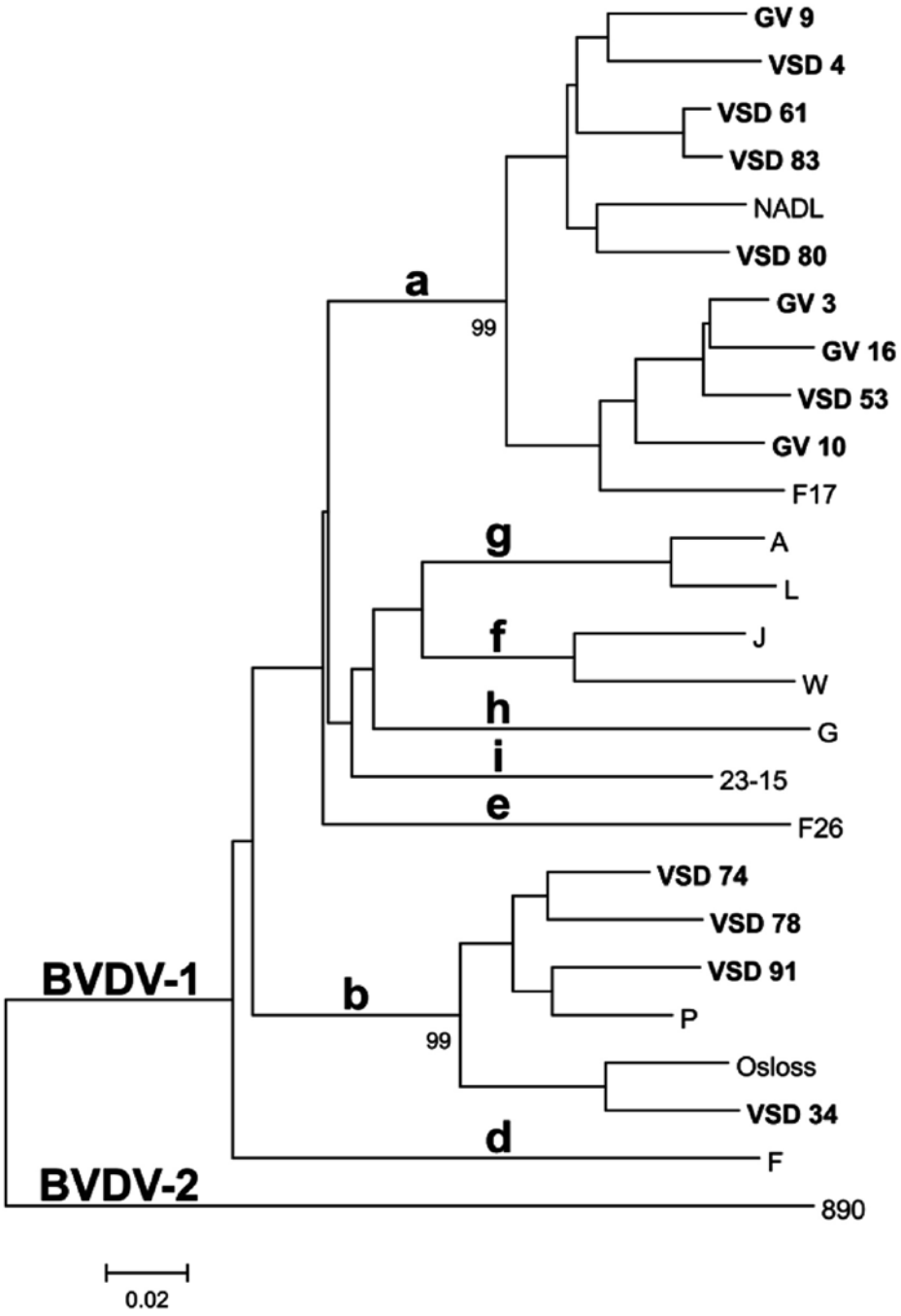


Fig.2. Phylogenetic tree in the Npro region.

The small letters a, b, d, e, etc., represent subgenotypes of BVDV-1. The reference strains included are in their subgenotype according to those assigned in the study of Vilcek et al. [21]. All 13 sequenced samples in this study from Ireland are denoted in bold and assigned a subgenotype by comparison with the reference strains. The tree was prepared using MEGA 4.0. The numbers close to the branches 'a' and 'b' represent the values in percent of 1,000 bootstrap replicates. Only values greater than 70 % are shown. Line lengths are proportional to genetic distance as indicated by the scale bar at the bottom

lecular epidemiology, it may be used to achieve control and eradication of BVDV in Ireland.

ACKNOWLEDGEMENTS

The collection of the samples was organised and sent by David Graham MVB PhD MRCVS, Animal Health Ireland, Dr. Rónan O'Neill, Department of Agriculture, Food & Marine Laboratories, Backweston and Dr. Maria Guelbenzu Agri-Food and Biosciences Institute, Belfast. This work was supported by the University of Veterinary Medicine and Pharmacy in Košice and Grant No. APVV-0379-10.

REFERENCES

1. Becher, P., Orlich, M., Kosmidou, A., Konig, M., Baroth, M., Thiel, H. J., 1999: Genetic diversity of pestiviruses: identification of novel groups and implications for classification. *Virology*, 262, 64—71.

2. Becher, P., Orlich, M., Shannon, A. D., Horner, G., Konig, M., Thiel, H. J., 1997: Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *J. Gen. Virol.,* 78, 1357—1366.

3. Becher, P., Orlich, M., Thiel, H. J., 2000: Mutations in the 5' nontranslated region of bovine viral diarrhea virus result in altered growth characteristics. *J. Virol.,* 74, 7884—7894.

4. Booth, R. E., Thomas, C. J., El-Attar, L. M., Gunn, G., Brownlie, J., 2013: A phylogenetic analysis of Bovine Viral Diarrhoea Virus (BVDV) isolates from six different regions of the UK and links to animal movement data. *Vet. Res.,* 44, 43.

5. Cowley, D. J., Clegg, T. A., Doherty, M. L., More, S. J., 2012: Bovine viral diarrhoea virus seroprevalence and vaccination usage in dairy and beef herds in the Republic of Ireland. *Ir. Vet. J*., 65, 16.

6. Graham, D. A., Laren, I., Brittain, D., O'reilly, P. J., 2001: Genetic typing of ruminant pestivirus strains from Northern Ireland and the Republic of Ireland. *Res. Vet. Sci.,* 71, 127—134.

7. Guelbenzum, M., Graham, D. A., 2013: *Prevalence of BVD in Northern Ireland Dairy and Suckler Herds*. Vol. D-41-08 RCF-01/2010. AgriSearch, Northern Ireland.

8. Houe, H., 1999: Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet. Microbiol.*, 64, 89—107.

9. Jones, L. R., Zandomeni, R., Weber, E. L., 2002: Quasispecies in the 5'untranslated genomic region of bovine viral diarrhoea virus from a single individual. *J. Gen. Virol.,* 83, 2161—2168.

10. Letellier, C., Kerkhofs, P., Wellemans, G., Vanopdenbosch, E., 1999: Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5'untranslated region. *Vet. Microbiol.,* 64, 155—167.

11. Nagai, M., Hayashi, M., Itou, M., Fukutomi, T., Akashi, H., Kida, H. et al., 2008: Identification of new genetic subtypes of bovine viral diarrhea virus genotype 1 isolated in Japan. *Virus Genes,* 36, 135—139.

12. Pellerin, C., Van Den Hurk, J., Lecomte, J., Tijssen, P., 1994: Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. *Virology*, 203, 260—268.

13. Ridpath, J. F., 2010: Bovine viral diarrhea virus: global status. *Veterinary Clinics of North America – Food Animal Practice*, 26, 105—121.

14. Ridpath, J. F., Bolin, S. R., Dubovi, E. J., 1994: Segregation of bovine viral diarrhea virus into genotypes. *Virology,* 205, 66—74.

15. Strong, R., Errington, J., Cook, R., Ross-Smith, N., Wakeley, P., Steinbach, F., 2013: Increased phylogenetic diversity of bovine viral diarrhoea virus type 1 isolates in England and Wales since 2001. *Vet. Microbiol.,* 162, 315—320.

16. Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007: MEGA4:Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Molecular Biology and Evolutio*n, 24, 1596—1599.

17. Vilcek, S., Drew, T. W., Mcgoldrick, A., Paton, D. J., 1999: Genetic typing of bovine pestiviruses from England and Wales. *Vet. Microbiol.,* 69, 227—237.

18. Vilcek, S., Durkovic, B., Kolesarova, M., Greiser-Wilke, I., Paton, D., 2004: Genetic diversity of international bovine viral diarrhoea virus (BVDV) isolates: identification of a new BVDV-1 genetic group. *Vet. Res.,* 35, 609—615.

19. Vilcek, S., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P., Paton, D. J., 1994: Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.,* 136, 309—323.

20. Vilcek, S., Nettleton, P. F., Paton, D. J., Belak, S., 1997: Molecular characterization of ovine pestiviruses. *J. Gen. Virol.,* 78 (Pt 4), 725—735.

21. Vilcek, S., Paton, D. J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A. et al., 2001: Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Arch. Virol.,* 146, 99—115.

22. Wolfmeyer, A., Wolf, G., Beer, M., Strube, W., Hehnen, H. R., Schmeer, N. et al., 1997: Genomic (5'UTR) and serological differences among German BVDV field isolates. *Arch. Virol.*, 142, 2049—2057.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 66—68, 2014

CANINE LEISHMANIOSIS IN THE MALTESE ISLANDS

Spiteri, C., Vella, A.*, Goldová, M.

Department of Epizootiology and Parasitology
University of Veterinary Medicine and Pharmacy, Komenskeho 73, 041 81 Košice
The Slovak Republic
***Qormi Veterinary Clinic**
Malta

Maria.Goldova@uvlf.sk

ABSTRACT

Leishmaniosis is a vector-borne parasitic disease caused by protozoa of the genus *Leishmania*. These parasites infect a variety of vertebrate animals and are transmitted by the bite of an infected sand fly; the Phlebototaminae genus *Lutzomyia*in the New World and *Phlebotomus* in the Old World. Dogs are the principal reservoir for this zoonosis, which causes a serious veterinary problem and also has an increasing adverse impact on public health.

Key words: blood smear; *Leishmania* spp.; lymph node aspirates; Malta; Phlebotominae; skin scrapings; Snap test; vaccination

INTRODUCTION

Canine leishmaniosis is a serious systemic disease with diverse clinical presentations resulting from an infection by diphasic protozoa. Diseases caused by various *Leishmania* species in people are divided into 3 forms according to their clinical manifestation, i. e., cutaneous leishmaniosis (CL), mucocutaneous leishmaniosis (MCL), and visceral leishmaniosis (VL).Canine leishmaniosis is often classified as a visceral one, as it is associated with some of *Leishmania* species that cause visceral leishmaniosis in humans. However, dogs usually have both the visceral and cutaneous involvements [3]. Canine VL is caused by *Leishmania infantum* in the endemic areas which include; Portugal, Southern France, Italy, Greece, Turkey and especially Malta. *L.infantum* is synonymous with *L.chagasi* found in Central and South America. *Leishmania tropica* is also an important agent. It causes cutaneous leishmaniosis in people in some parts of the Middle East and Africa. However, infection of *L. tropica* is rare compared with the more commonly found *L. infantum* infection [4], [5].

Only the female sand fly bites to take blood meals. The female sand fly becomes parasitized when it ingests *Leishmania* amastigotes contained in the blood of an infected host. The mammalian host cells are transformed into amastigotes, which multiply and develop in the anterior segment of the gut [2]. When a parasitized sand fly bites another vertebrate host, it inoculates the final metacyclic promastigotes form into the new host. The parasite is phagocytized by a macrophage where it continues to develop and multiply within the macrophage as a non-motile amastigotes which then invades other tissues and cells thus, completing the lifecycle [1].

The onset of clinical signs can vary from 1 month to several years. The transmission can be by direct contact, blood transfusions, or placental transmission. Both visceral and cutaneous manifestations maybe found simultaneously in dogs, whereas in humans, separate forms are usually seen. The clinical signs are variable and can mimic other infections. In symptomatic cases, common visceral signs include: lethargy, weight loss, decreased appetite, anaemia, splenomegaly, and local or generalized lymphadenopathy. Fever can be intermittent, but is absent in many cases. Bleeding disorders including epistaxis, haematuria and melena, can also be seen. Chronic renal disease is common in dogs infected with *Leish-*

mania infantum, which is usually the cause of death. Some animals may also have ocular, skin or mucosal lesions, sneezing, chronic diarrhoea, vomiting, and autoimmune disorders. Skin lesions are common in dogs with visceral disease, but can also occur separately. The most common cutaneous syndrome is a non-pruritic exfoliative dermatitis found especially around the eyes and on the face, ears, and feet. Cutaneous disease can also be manifested by nodules, ulcers, scabs which lead to secondary bacterial infections [6]. The prognosis of the disease depends on what stage it is in when it was diagnosed. Nowadays, with all the treatments available, it is no longer a death sentence and dogs can usually live normally with life-long treatment.

The Maltese islands are a group of low-lying islands at the centre of the Mediterranean basin having one of the highest population densities in Europe. The Maltese climate, with hot summers and short cool winters, makes it a suitable environment for sand flies, which require both relatively high temperatures and humidity. The best period for sand fly activity is between May and the first week of November [7].

The main objective of this study was to use the Snap test as a definite confirmatory test and then evaluate blood films together with total blood count, skin scrapings (from infected dogs) and lymph node aspirates by means of light microscopy in order to differentiate from *Leishmania* positive dogs and negative ones.

MATERIALS AND METHODS

Our study included 6 dogs of different ages from Malta. Patient No. 1: Springer Spaniel, 6 years old, male, lived outdoors. Patient No. 2: Pug, 8 years old, male, lived outdoors. Patient No. 3: Toy poodle, 8 years old, female, lived indoors. Patient No. 4: Labrador, 3 years old, female, lived outdoors. Patient No. 5: Cavalier King Charles, 2.8 years old, male, lived indoors. Patient No. 6: mixed breed, 13 years old, male, lived outdoors.

All dogs were examined for *Leishmania* species via the Snap test; blood smears were stained by Diff-Quick. Also, in all 6 dogs, the total blood count was analysed, after a blood sample was taken from a peripheral vein. In patient No. 1, lymph node aspirates were prepared. Skin scrapings were performed in patient No. 2.

RESULTS

Results of *Leishmania* species positive cases with amastigote stage: patients 1, 2, 4; and clinically healthy dogs, *Leishmania* species free, were found in patients number 3, 5, 6 (Tab. 1).

DISCUSSION

Patient No. 1 was initially checked due to shortness of breath after walking a short distance. Concurrent with the positive Snap test, a blood smear was evaluated which had a higher number of eosinophils, which is very suggestive of a parasitic infection. In the lymph node aspirate, the amastigote stage was identified within the macrophages, which was a further definite confirmation of *Leishmania* disease. As in every positive case, a total blood count was taken showing high eosinophils (6.6 %: normal range = 0—6 %) which indicated the possible presence of the parasite. High creatinine (2 mg.dl^{-1}: normal range = 0.4—1.4) and high BUN (93.1 mg.dl^{-1}: normal range = 9.2—29.2), meant that this was avisceral form of leishmaniosis with the kidneys being affected mainly. This patient was also diagnosed with *Ehrlichia* which

Table 1. Results of the Snap test and total blood count

Patient No.	Snap test result	Total blood count	Blood smear	Increased number of eosinophils (blood smear)	Skin scraping	Amastigote stage present (skin scraping)	Lymph node aspirate	Amastigote stage present (lymph node aspirate)
1	Positive (27/12/2013)	•	•	Yes		No	•	Yes
2	Positive (30/12/2013)	•	•	Yes	•	Yes		No
3	Negative (3/01/2014)	•	•	No		No		No
4	Positive (20/11/2013)	•	•	Yes		No		No
5	Negative (6/01/2014)	•	•	No		No		No
6	Negative (6/01/2014)	•	•	No		No		No

• — test was performed; Blank space — test was not performed

although, no real relationship is usually present with leishmaniosis, it is still quite often found simultaneously with positive leishmania cases in canines in Malta. In the blood count, *Ehrlichia* and *Leishmania* were both manifested by lower RBC (5.15×10^6.µl^{-1}: normal range = 5.5—8.5), lower hemoglobin (11.3 g.dl^{-1}: normal range = 13.2—19) and haematocrit count (37.5 %: normal range = 40—55), which are all indicative of an anaemia. The treatment for *Leishmania* was started immediately in this dog with Milteforan syrup (20 mg.ml^{-1}) administration for 1 month, Allopurinol tablets (twice daily) and Ferroglobin syrup. For *Ehrlichia,* treatment with Doxycycline was also administered.

Patient No. 2 was also diagnosed via the Snap test. Suspicion of CL was triggered by skin lesions and exfoliations on the elbows, hocks, nose and muzzle areas. This is indicative of a cutaneous form of leishmaniosis. In the blood smear, there were an increased number of eosinophils, which could be seen, again, suggesting the parasite presence. After the total blood count was taken, the eosinophils were higher (8.2 %). The WBC (4.7×10^3.µl^{-1}: normal range = 6—12) and RBC (3.11×10^6.µl^{-1}: normal range = 5.5—8.5) were also lower, suggesting there may have been some iron deficiency anaemia. The hemoglobin was lower (6.9 g.dl^{-1}: normal range = 13.2—19) which further confirmed the presence of anaemia. The haematocrit (23.5: normal range = 40—55) and creatinine (0.3 mg.dl^{-1}: normal range = 0.4—1.4) count were all lower than normal which indicated there was a combination of cutaneous and visceral leishmaniosis, which may result in a systemic organ disease, if left untreated. The skin scraping also indicated the presence of the amastigote stage of the parasite. Milteforan syrup (20 mg.ml^{-1}) for 1 month, Allopurinol tablets (once daily) and Ferroglobin syrup were administered as the treatment.

Patient No. 4 was positive for leishmaniosis by the Snap test and cutaneous lesions were found on both elbows, but predominantly on the left, indicating the presence of the cutaneous form of leishmaniosis. More eosinophils were seen in the blood smear of this patient, which was also confirmed by the blood results, where eosinophils were 9.7 %. Slightly lower hemoglobin (12.7 %) indicated the presence of anaemia. The rest of the values in this patient were in the normal range which indicated that the disease was still in the early stages. Organs had not yet been affected and there seemed to be no progression into the visceral form of *Leishmania.* Milteforan syrup (20 mg.ml^{-1}) for 1 month, Allopurinol tablets (once daily) and Ferroglobin syrup were administered as treatment. All the 3 affected patients lived outdoors; this clearly indicated that dogs which are outside are more likely to come into contact with the sand fly, with Malta being at the top of the list of such cases.

Patients No. 3, 5 and 6 gave negative results to the *Leishmania* Snap test, and their blood smears did not show any excessive amounts of eosinophils. This was also confirmed by the blood test, which demonstrated that eosinophils were in the normal range for all 3 dogs and no organs were affected. Patient No. 5 was diagnosed with visceral leishmaniosis in April 2012 and had received Milteforan syrup, Ferroglobin and Allopurinol tablets as treatment. Following this, re-tests were done every month. Re-testing about 1.5 years later, via the Snap test showed that the patient had completely recovered from this disease. Therefore, patients No. 3, 5 and 6 were considered *Leishmania* free cases. These patients lived mainly indoors, which makes them less likely to come in contact with sand flies.

CONCLUSION

From the results obtained, it clearly shows that the rapid Snap test is the most reliable form of a definite confirmation of *Leishmania* disease in canines. It is almost impossible to find any amastigote stages in blood smears from peripheral blood samples. However, changes in blood sample examinations can give a better picture in which form the disease is mainly manifested, in which stage it is at, and which organs are infected. It is recommended, that after the rapid Snap test, a total blood examination must be taken for positive cases.

In the lymph node aspirates, amastigote stages in macrophages are more easily detected than in skin scrapings. The main disadvantage of lymph node aspirates are that dogs usually require more secure restraints, and it is uncomfortable for the patient. New preventive measures being introduced in Malta have been considerably effective against *Leishmania* species. CaniLesh is a vaccine containing ESP (Excreted-Secreted Proteins) produced by several protozoan parasites including *Toxoplasma, Plasmodium, Babesia.* Thus, this acts as a better stimulator of cell-mediated immunity than whole parasite extracts. ESP can be used to prime the immune response against all stages of the parasite. Also, Scalibor collars and Advantix drops together with citronella ointments can act as repellents towards these persistent detrimental sand flies.

REFERENCES

1. Alvar, J., Canavale, C., Molina, R. et al., 2004: Canine Leishmaniosis. *Adv. Parasitol.*, 57, 1—88.

2. Bates, P. A., 2007: Transmission of Leishmania metacyclic promastigotes by phlebotomine sandflies. *Inf. J. Parasitol.,*37, 1097—1106.

3. Gramiccia, M., Gradoni, L., 2005: The current status of zoonotic leishmaniosis and approaches to disease control. *Int. J. Parasitol.,*35, 1169—1180.

4. Greene, C. E., 2006: *Infectious Diseases of the Dog and Cat.* 3rd edn., Elsevier, 1387 pp.

5. Nuttall, T., Harvey, R. G., McKeever, P. J., 2009: *Skin Diseases of the Dog and Cat.* 2nd edn., Royal Canine, 978 pp.

6. http://www.cfsph.iastate.edu/Factsheets/pdfs/leishmaniasis.pdf (April 15, 2014).

7. http://www.maltaweather.com (April 15, 2014).

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 69—71, 2014

GIARDIOSIS AND CRYPTOSPORIDIOSIS IN DOGS IN KOŠICE

Adamová, V., Štrkolcová, G., Goldová, M.

Department of Epizootiology and Parasitology
University of Veterinary Medicine and Pharmacy, Komenskeho 73, 041 81 Košice
The Slovak Republic

Maria.Goldova@uvlf.sk

ABSTRACT

Protozoan parasites of the genera *Cryptosporidium* and *Giardia* cause the so-called waterborne and foodborne diseases, i. e. infections spread by water and food. Most frequently they cause opportunistic infections, particularly in the young animals with incompletely developed immune systems, or occur as secondary infections. Their spread is cosmopolitan, the host spectrum is very wide and some genotypes show zoonotic potential. The oral-faecal transmission is frequent. In dogs, both protozoan infections occur most frequently in puppies or weak individuals and the prevailing clinical symptom is diarrhoea. From September 2013 until March 2014, we examined 68 samples of excrements from dogs originating from Košice and the surrounding area that were affected with diarrhoea of varying severity. *Giardia intestinalis* was found in 21 dogs (30.9 %) and *Cryptosporidium* spp. in 1 dog (1.5 %). Of the total number of dogs examined, 23 were kept in households and we diagnosed *Giardia intestinalis* in 6 samples (26.1 %). Of 45 samples of dogs excrements collected in the Union for mutual help between people and animals shelter in Haniska near Košice, 15 (33.3 %) were positive for *Giardia intestinalis* and one (2.2 %) for *Cryptosporidium* spp.; the last one involved a mixed infection with *G. intestinalis*.

Key words: cryptosporidiosis; giardiosis; stray dogs; dogs with gastrointestinal disorders

INTRODUCTION

Giardiosis and cryptosporidiosis are endemic protozoonoses spread by the oral faecal route, with clinical manifestations ranging from asymptomatic stages, up to acute diarrhoea with serious malabsorption syndromes. *Giardia intestinalis* isolated from human and other animal faeces are morphologically identical. We recognize seven main genotypes; A to G, of which A and B are zoonotic and C and D are genotypes described in dogs [4]. Genotype E has been confirmed in calves and ratites, F in cats, and G in rodents [2]. *Giardia intestinalis* is usually located in the distal part of the duodenum and the proximal and central sections of the jejunum. In immunocompetent individuals, the infection may be asymptomatic or subclinical, while in the young and immunosuppressed animals, we observe clinically apparent forms associated particularly with: watery foul diarrhoea, subfebrile states, abdominal cramps, nausea, anorexia, flatulence and abdominal distension, and loss of weight. With giardiosis, one can observe increased amounts of mucus and fat. The course of the primary infections in the young is more serious than repeated infections at later ages [9].

The genus *Cryptosporidium* includes small monoxenic coccidia with intracellular and extraplasmatic localisations, populating mostly the gastrointestinal tract. Individual groups of mammals possess host adapted genotypes that are currently classified as different species and include species with zoonotic potential, particularly, *C. parvum* and *C. hominis*. Additional zoonotic species, such

as, *C. meleagridis*, *C. muris*, *C. felis*, *C. canis*, *C. suis*, *C. andersoni*, *C. Ubiquitum* and *C. cuniculus*, occur less frequently [11]. The infection affects individuals of all age groups but most frequently the young and immunodeficient subjects and the course of the disease ranges from asymptomatic, up to slight or serious and can even result in fatalities [9]. The typical clinical symptoms are watery diarrhoea associated with colicky bellyache, nausea, vomiting, anorexia, and flatulence, weight loss and increased body temperature. While immunocompetent individuals will recover, even life threatening infections may develop in immunocompromised hosts, for example in humans with AIDS or cats with feline leukemia virus (FeLV). In these individuals, autoinfection with thin walled cryptosporidia oocysts plays an important role[4].

The genera *Giardia* and *Cryptosporidium* are considered opportunistic pathogens that cause serious infections with marked clinical symptoms particularly in immunosuppressed subjects. Risk groups include; the young, geriatric patients and individuals with weakened immunity, e.g. those with malignant tumours or transplanted organs. *Giardia* cysts and oocysts are very resistant in the external environment, particularly in wet and cold habitats and resist common disinfectants, such as chlorine.

Recently, several studies were carried out in Europe dealing with the prevalence of *Giardia intestinalis* and *Cryptosporidium* spp. According to a study conducted in Košice in 2011, the prevalence of *G. intestinalis* reached 69.1 % in young dogs below the age of 7 months and 36.9 % in adult dogs older than 7 months [8]. In the Czech Republic, 3.3 % of dogs from dog shelters were positive for *G. intestinalis*, compared to 2.2 % of dogs from the suburbs [5]. The prevalence of *G. intestinalis*: in team dogs in Poland reached 28 % [1]; in dogs from dog shelters in Northern Germany 1.2 % [3]; and in Romania, giardia cysts were found in 8.5 % of dogs from dog shelters [10]. In Hungary, *G. intestinalis* were diagnosed in faeces smears in 7.5 % of dogs, while the ELISA test showed 58.8 % positivity [12].The examination of faeces by the ELISA *Cryptosporidium* (faecal) method in Košice proved 7.2 % prevalence in young dogs and 1.4 % in adult subjects [8].

The aim of our study was to determine the prevalence of these intestinal protozoa in dogs with digestive problems and diarrhoea, kept under varying conditions in Košice and the surrounding area.

MATERIALS AND METHODS

The prevalence of *Giardia intestinalis* and *Cryptosporidium* spp. was investigated between September 2013 and March 2014 by examining 68 samples of dog faeces with diarrhoea of varying severity. The dogs were divided to two groups, the first one (45 samples) consisted of dogs from the Union for mutual help between people and animals shelter in Haniska near Košice and the second of 23 dogs from households in Košice and the surrounding area.

The samples of dog faeces were examined by the concentration flotation method according to Faust [6] for the detection of the cysts of *Giardia intestinalis*. In addition, 4 samples were examined by SNAP® *Giardia* test (IDEXX Laboratories). For the intravital diagnosis of cryptosporidiosis, we prepared faecal smears and stained them with the haematoxylin/Kinyoun stain [7]. The faeces of dogs from the shelter were examined by the commercial kit sandwich ELISA *Cryptosporidium* (faecal) (Diagnostic automation, Inc., CA, USA, 91302).

RESULTS AND DISCUSSION

Dogs from the first (shelter) group lived in an overcrowded shelter at a low hygiene level with a high numbers of dogs in individual pens. The veterinary care at the shelter was limited, environmental sanitation inadequate, and the nutrition was not always appropriate. All these factors contributed to the reduced immunity of these dogs. The prevalence of *Giardia intestinalis* in this group reached 33.33 % (15/45). This prevalence is considerably higher in comparison with that reported by other countries, for example 3.3 % in the Czech Republic [5], 1.2 % in Germany [3] and 8.5 % in Romania [10]. Samples of faeces in these cited studies were collected immediately after dogs were first brought to the shelter. However, the Czech study described an11-fold increase in the prevalence of *G. intestinalis* after the long-term stay of the dogs in the shelter [5].

The 23 dogs in the privately owned second group were kept in apartments or pens next to family houses and their owners reported that their dogs suffered from diarrhoea of varying severity. These animals were usually provided with adequate care. The examination of their faeces showed a 26.08 % (6/23) prevalence of *Giardia intestinalis* in this group. In animals kept in apartments, the prevalence reached 16.67 %, while in those in outdoor pens it was as high as 36.36 %.

Giardia intestinalis in young dogs (< 7 months of age) from the shelter reached 30.43 % (7/23) and in adult dogs 36.36 % (8/22). In the privately owned group, 27.27 % prevalence was observed in young dogs and 25 % in the adult dogs. Our results do not agree with those from 2011 [8], when a higher prevalence of *G. intestinalis* was observed in young dogs.

Cryptosporidium spp. was detected only by the ELISA test in one sample (2.2 %; 1/45) from the faeces of an adult dog from the shelter. All samples of faeces from the privately owned group, examined by the staining method of Kinyoun, were negative. According to this, the total prevalence of *Cryptosporidium* spp. in our study was 1.5 % (1/68).

CONCLUSION

The examination of 68 samples of faeces from dogs suffering from diarrhoea of varying severity, in Košice and the surrounding area, detected *Giardia intestinalis* in 21 dogs (30.9 %) and *Cryptosporidium* spp. in only one dog (1.5 %). The investigated intestinal protozoa show considerable degree of genetic diversity with some genotypes showing zoonotic potential and posing a risk mainly to individuals with immunodeficiency syndrome or those susceptible to secondary infections. Dogs, cats and domestic ruminants are considered to be the reservoir of infection for humans. We frequently fail to recognise these infestations due to the low efficacy of commonly used parasitological methods. Giardiosis and cryptosporidiosis of dogs gains on importance in relation to routine de-worming, because commonly used anthelminthics are ineffective against these intestinal protozoa. This should support the importance of this issue and the need for the use of special diagnostic methods.

ACKNOWLEDGEMENT

The study was supported by the project VEGA No. 1/0831/12.

REFERENCES

1. Bajer, A., Bednarska, M., Rodo, A., 2010: Risk factors and control of intestinal parasite infections in sled dogs in Poland. *Vet. Parasitol.*, 175, 343—350.

2. Ballweber, L. R., Xiao, L., Bowman, D. D., Kahn, G., Cama, V. A., 2010: Giardiasis in dogs and cats: update on epidemiology and public health significance. *Trends in Parasitology*, 26, 180—189.

3. Becker, A. C., Rohen, M., Epe, C., Schneider, T., 2012: Prevalence of endoparasites in stray and fostered dogs and cats in Northern Germany. *Parasitol. Res.*, 111, 849—857.

4. Bowman, D. D., 2009: *Georgis' Parasitology for Veterinarians*. 9th edn., St. Louis, Saunders Elsevier, 451 pp.

5. Dubná, S., Langrová, I., Nápravník, J., Jankovská, I., Vadlejch, J., Pekár, S., Fechtner, J., 2006: The prevalence of intestinal parasites in dogs from Prague, rural areas, and shelters of the Czech Republic. *Vet. Parasitol.*, 145, 120—128.

6. Faust, E. C., D'Antonio, J. S., Odom, V., Miller, M. J., Peres, C., Sawitz, W. et al., 1938: A critical study of clinical laboratory techniques for the diagnosis of protozoan cysts and helminth eggs in faeces. *Am. J. Trop. Med. Hyg.*, 18, 169—183.

7. Garcia, L. S., Bruckner, D. A., 1997: Diagnostic Medical Parasitology. 3rd edn., American Society for Microbiology press, Washington, 937 pp.

8. Goldová, M., Valenčáková, A., Mojžišová, J., Halánová, M., Letková, V., Ravaszová, P., 2011: Occurrence of *Giardia* and *Cryptosporidium* in dogs. http://www.researchgate.net/publication/225303078_Occurrence_of_*Giardia*_and_*Cryptosporidium*_in_dogs.

9. Jíra, J., 2009: *Medical Protozoology* (In Czech). Prague, Galén, 567 pp.

10. Mircean, V., Györke, A., Cozma, V., 2011: Prevalence and risk factors of *Giardia duodenalis* in dogs from Romania. *Vet. Parasitol.*, 184, p. 325—329.

11. Ryan, U., Xiao, L., Caccio, S. M., Widmer, G., 2014: *Cryptosporidium: Parasite and Disease*. Springer-Verlag Wien, 3—41.

12. Szénási, Z., Marton, S., Kucsera, I., Tánczos, B., Horváth, K., Orosz, E. et al., 2007: Preliminary investigation of the prevalence and genotype distribution of *Giardia intestinalis* in dogs in Hungary. *Parasitol. Res.*, 101, 145—152.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 72—74, 2014

THE INFLUENCE OF CATIONIC PEPTIDES AND DIETARY NUCLEOTIDES ON THE POST-VACCINATION IMMUNE RESPONSE

Bratičáková, M., Kulichová, L., Smrčo, P.

Institute of epizootiology and preventive veterinary medicine
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

peter.smrco@uvlf.sk

ABSTRACT

The aim of this study was to evaluate the influence of dietary nucleotides and cationic peptides on the proliferation of lymphocytes and antibody response of cats after vaccination. Two groups of cats were formed: group E (n = 6) animals were supplemented with a preparation containing dietary nucleotides and cationic peptides for 92 days; group C (n = 6) animals received no such preparation. During the study the animals were vaccinated against; feline parvovirus (FPV), feline calicivirus (FCV), feline herpesvirus (FHV), and rabies according to a standard vaccination scheme. The post-vaccinal production of antibodies was evaluated by a commercial ELISA test and the proliferation of lymphocytes by the BrdU test. Comparison of groups E and C showed significant changes (from P < 0.0001 to P = 0.0468) in proliferation activity of lymphocytes and (from P = 0.0013 to P = 0.0488) for the post-vaccinal production of antibodies. The animals from group E reached a protective level of antibodies earlier than those from group C. Our results showed that preparations based on cationic peptides and dietary nucleotides improve the specific post-vaccinal responses and played an important role in increasing the immunocompetence of cats as well as serving to prevent infectious feline diseases.

Key words: cats; cationic peptides; dietary nucleotides; immune response; vaccination

INTRODUCTION

Immunomodulators are substances which are capable of affecting the immune response of organisms in a positive or negative way. Nucleotides are low-molecular intracellular components. They: improve the development of the gastrointestinal and immune systems and response to vaccination; affect natural killer (NK) cells activity [1]; and influence the maturation, activation and proliferation of lymphocytes. In addition, they positively affect the antibody responses of the young [2], increase the production of cytokines by T helper cells (Th cells), and cause an increase in IL-2, IL-4, IL-10 and GM-CSF [5]. Cationic peptides are usually 12 to 50 amino acids in length. They are produced by all organisms as the major component of their immediate, effective, non-specific protection against infections. They stimulate chemotaxis of monocytes and neutrophils, support the release of histamine from lipid cells, inhibit tissue proteases and stimulate healing of wounds [3].

MATERIALS AND METHODS

Animals: group E consisted of 6 clinically healthy cats administered a preparation based on cationic peptides and dietary nucleotides (Aminex, UNIREGEN s. r. o., Czech Republic), at a dose of 10 drops/1 kg/day for 92 days; group C comprised 6 clinically healthy cats that received no such supplementary preparation.

Sampling of blood: blood samples were obtained by puncture of *v. jugularis*. The samples for serological assay and isolation of lymphocytes were collected on days 22, 43, 64 and 92 of the study.

Immunological analysis: blastic transformation of lymphocytes was evaluated by ELISA BrdU (colorimetric) test, using phytohaemagglutinin PHA-P (Sigma, USA) of concentration 20 $\mu l.ml^{-1}$. The level of blastogenic response of lymphocytes was expressed as a stimulation index (SI).

Serological analysis: specific antibodies to feline herpesvirus, feline calicivirus and feline parvovirus were detected by commercial ELISA assays.

Statistical analysis: results were processed by Mann-Whitney test using software GraphPad Prism5 and are expressed as means ± standard deviation.

RESULTS

The results of the proliferation activity of lymphocytes expressed as a stimulation index (SI) together with statistical comparison of groups are presented in Fig. 1. Comparison of the SI determined in groups E and C indicated significance of differences between groups (from $P = 0.0468$ to $P < 0.0001$) at all samplings.

The highest level of SI was recorded on day 92 in group E. In group C the stimulation index was below the physiological range (1.7—4.1) at first three samplings. The development of antibodies to feline herpesvirus-1 (FHV-1)in groups E and C, together with their protective level, is illustrated in Fig. 2. The titre 1:270 (OD ≥ 1.0) is considered protective and was reached 28 days earlier (day 64 of study) in group E compared to group C. The increase in production of antibodies in group E differed significantly from that in group C ($P = 0.0481 – 0.0013$). The post-vaccinal development of antibodies to feline calicivirus (FCV) in groups E and C, together with the respective protective level, is shown in Fig. 3. The titre 1:270 (OD ≥ 0.25) is considered protective and it was again reached 28 days earlier (day 64 of study) in group E compared to group C. The difference between groups E and C was significant ($P = 0.0031$). The post-vaccinal development of antibodies to feline parvovirus (FPV) in groups E and C, together with the respective protective level, is shown in Fig. 4. The titre 1:1350 (OD ≥ 0.25) is considered protective and similar to the previous observations, it was reached

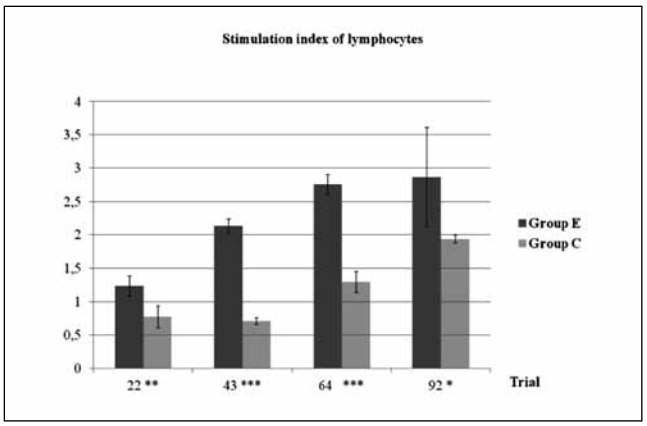


Fig. 1. Stimulation index of lymphocytes

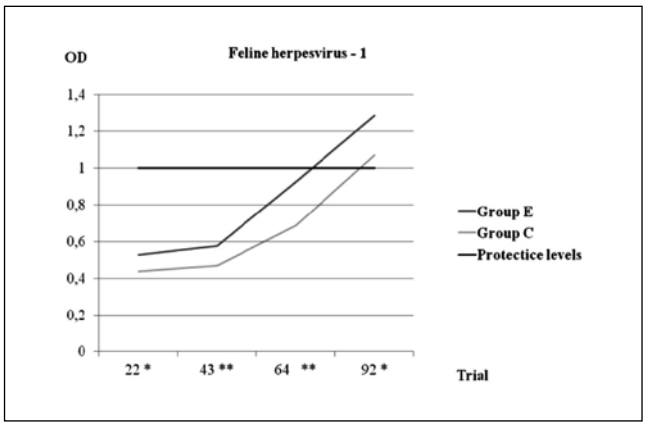


Fig. 2. Specific antibodies to feline herpesvirus

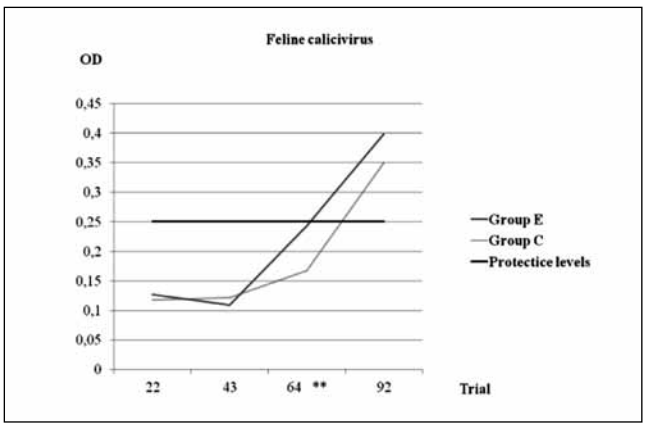


Fig. 3. Specific antibodies to feline calicivirus

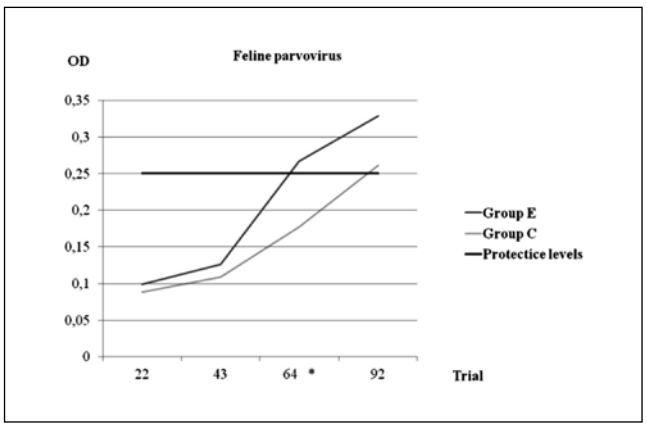


Fig. 4. Specific antibodies to feline parvovirus

28 days earlier (day 64 of study) in group E compared to group C. The difference between groups E and C was significant (P = 0.0488).

DISCUSSION AND CONCLUSIONS

Our study showed a significant increase in proliferative activity of lymphocytes throughout the study. Similar results were observed in mice that were fed diets supplemented with RNA, adenine or uracil [2]. Specific antibodies to FHV, FPV and FCV in group E were detected earlier and at higher titres compared to group C. The protective levels of specific antibodies to FHV, FPV and FCV were reached on day 64 of the study in animals from group E and on day 92 of study in group C. Similar results were reported in previous studies in the plasma of children who received food supplemented with nucleotides and showed a significant increase in non-specific IgM [4]. A study based on the oral administration of antimicrobial peptides from hen intestine showed an increase in the serum levels of IgG and IgM, and improvement in antibody response to virus of infectious bursitis of chickens 21 days post-vaccination [6].

Our results indicate that the administration of preparations based on dietary nucleotides and cationic peptides contributed to more rapid reaching of protective levels of antibodies and produced higher titres of specific antibodies to feline viruses.

ACKNOWLEDGEMENT

This study was supported by the project VEGA 1/0835/12, and Pharmacopola, s. r. o.

REFERENCES

1. Carver, J. D., Pimentel, B., Cox, W. I., Barness, L. A., 1991: Dietary nucleotide effects upon immune function in infants. *Pediatrics*, 88, 359—363.

2. Gil, A., 2002: Modulation of the immune response mediated by dietary nucleotides. *Eur. J. Clin. Nutr.*, 56 (Suppl. 3), 1—4.

3. Hancock, R. E. W., 2001: Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Inf. Dis.*, 1, 156—164.

4. Maldonaldo, J., Navarro, J., Narbona, E., Gil, A., 2001: The influence of dietary nucleotides on humoral and cell immunity in the neonate and lactating infant. *Early Hum. Dev.*, 65 (Suppl.), S69—S74.

5. Xu, M., Zhao, M., Yang, R., Zhang, Z., Li, Y., Wang, J., 2013: Effect of dietary nucleotides on immune function in Balb/C mice. *Int. Immunopharmacol.*, 17, 50—56.

6. Yurong, Y., Ybao, J., Ruiping, S., Qingqiang, Y., Kaisong, P, Huihui, B. et al., 2006: Effects of chicken intestinal antimicrobial peptides on humoral immunity of chickens and antibody titres after vaccination with infectious bursal disease virus vaccine in chicken. *Archives of Animal Nutrition*, 60, 427—435.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 75—77, 2014

IMMUNOPHENOTYPING OF INTRAEPITHELIAL (IEL) AND *LAMINA PROPRIA* LYMPHOCYTES (LPL) IN THE CHICKEN INTESTINE BY FLOW CYTOMETRY

Bucková, B., Revajová, V.

Institute of pathological anatomy
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

viera.revajova@uvlf.sk

ABSTRACT

Immunophenotyping is a technique used for the detection of membrane, cytoplasmic and nuclear antigens, either directly or indirectly, by fluorochrome labelled antibodies. Both immunophenotyping and immunodiagnosis are now routinely used, not only in immunological laboratories but also in other interdisciplinary research laboratories. Pathological studies use immunophenotyping mostly for qualitative examination of cells by fluorescence microscopy. The current procedures focus on quantitative evaluations. One of the methods that can provide information on the number of individual particles or antigens in the cytoplasm or on the cytoplasmic membrane of cells is flow cytometry. This method allows one to carry out multiple objective simultaneous measurements at the cellular level involving as many as 5000 cells per second. The output produces data in the form of graphic and/or numerical representations. The resultant graphs display the number of cells by the density of dots which are subsequently analysed and reported statistically, usually as relative percentages.

Key words: flow cytometry; chicken; immunophenotyping; mucosal immunity

INTRODUCTION

Flow cytometry involves the measurement of physical or chemical parameters of (biological) particles while the fluid flows through a measuring instrument. The commonly measured parameters are fluorescence and light scattering.

During the measurement, the individual cells which are suspended in an isotonic carrier fluid (sheath fluid) pass through a flow chamber, one after another, by hydrodynamic focusing at a flow rate of 10—30 $\mu l.min^{-1}$. After interaction with a monochromatic laser beam (most often an argon laser with an emission length of 488 nm), light refraction and reflection occurs. Subsequently, the physical characteristics of individual cells are analysed: Forward Scatter Component — FSC (refraction at a low angle), determines the size of cells, and Side Scatter Component — SSC (light refraction and scattered perpendicular to the beam), determines structures of the membrane surface and cytoplasm (granulation) of the cells [3].The data are presented graphically as a dot plot diagram which displays the number of cells by the density of dots, which are subsequently analysed and reported statistically, usually as relative percentages (Fig. 1).

The binding of fluorochrome-labelled monoclonal antibodies to the surface or cytoplasm of cells results in the generation of fluorescence signals (FL). It is possible to obtain simultaneously several fluorescence signals (FL1-FL4), as from: the membrane surface, the cytoplasm or nucleus, and the intensity of the individual fluorescence signals can also be compared. The signals are detected by photodiodes (FSC and SSC) and photomultipliers (FL1-FL4), digitalised and processed by a computer [1].

The reagents most often used in flow cytometry are monoclonal antibodies that serve for detection of cell antigens. They can be labelled by the direct or indirect immunofluorescence methods. The

main advantage of direct immunofluorescence is the simple processing of the specimen and shorter working protocol (Fig. 2) [3].

Because the current methods can analyse simultaneously two or more parameters of individual cells, it is possible to: determine the number of cells, distinguish differences in mixed cell populations, determine physiological indicators, such as intracellular pH and calcium; study processes such as phagocytosis and endocytosis; detect yeasts in yoghurts, and analyse relatively small cells, such as bacteria; or determine the presence of viruses in infected cells. Thus flow cytometry became a recognized and important method in various branches of science.

Flow cytometry based on the labelling of immunocompetent cells by specie-specific monoclonal antibodies in the blood and solid organs of various animal species has been used for many years by the Institute of pathological anatomy (IPA) of UVMP in various experiments with infectious, parasitic and toxic agents.

The aim of our study was to standardize the method of isolation and immunophenotyping of IEL and to introduce the method of isolation of LPL from the chicken intestine. The experimental model included supplementation of T-2 toxin to the diet of chickens and preventive individual peroral administration of glucan to experimental birds. Immunophenotyping was carried out after isolation of IEL and LPL from the jejunum of chickens.

MATERIALS AND METHODS

Animals

Forty one-day-old chickens of Lhomann Brown hybrid were placed in wire cages with solid floors. They were allocated to four groups: K, G, GT and T, with 10 chickens in each group, and fed a mixed feed prepared at IPA (corresponding to commercial feed for broilers BR1), free of antimycotics. The experiments lasted 28 day. Group K served as a negative control, chickens in groups G and GT were administered glucan perorally on days 11, 12 and 21 (a total of 3 mg per head). The feed that was given to chickens in groups T and GT between days 14 and 28, contained T-2 toxin (1,450 $mg.kg^{-1}$). Jejunum samples were collected from chickens slaughtered on day 14 before consuming feed with toxin, and on day 28, after consuming feed with toxin for 2 weeks.

Isolation of lymphocytes from jejunal mucosa

Lymphocytes were isolated and purified by the method of Solano, Aguilar et al. [4]. The removed jejunum section was cut lengthwise and placed into an ice cold buffered Hank's solution (HBSS, pH 7.2—7.3). The intestine was then cut into 0.5 cm pieces that were placed into individual 50 ml conical plastic tubes (Falcon, BD) containing 30 ml of HBSS solution 37 °C) with 5 mM dithiothreitol (HBSS-DTT). Dithiothreitol helps to remove mucin from the intestinal mucosa. The tubes were then placed into a thermostat (37 °C) for 15 min during which their content was mixed at 5 minute intervals. After incubation, the intestinal sections were rinsed 3 times in ice cold HBSS and incubated for 1 hour in HBSS (37 °C) with 0.1 mM EDTA with mixing at 5 minute intervals. EDTA releases intraepithelial lymphocytes. After the incubation, the supernatant was filtered through a nylon sieve (70 µm, BD, Germany) into a 50 ml conical test tube which was then placed on ice in a refrigerator.

Then RPMI-1640 (30 ml) was added to each test tube containing the intestinal sections, and the tubes were incubated in a water bath(37 °C) for 15 min to remove residues of the previous media. The sections were then rinsed and placed into a clean beaker containing RPMI-1640 with collagenase (Collagenase Type I, HP Biomedicals LLC, France; 15 $mg.30\ ml^{-1}$). During a 1 hour incubation, lymphocytes were released from the *lamina propria* by the collagenase. After incubation, the supernatant was filtered through a nylon sieve into a 50 ml conical test tube and stored on ice. The tubes with isolated IEL and LPL were centrifuged for 10 minutes at 600 g, the supernatant was removed and sediment re-suspended in 30 ml of 25 % Percoll. The tubes were centrifuged again for 30 min at 600 g, the supernatant was removed and the sediment (1 ml) re-suspended in 8 ml of 25 % Percoll (Sigma, Germany). Exactly 4 ml of the suspension of each sample was pipetted into 2 glass 15 ml test-tubes. Equal volumes of 66 % Percoll were introduced to each tube below the suspension layer. The tubes were then centrifuged for 30 min at 600 g. A layer of IEL or LPL in the form of a white ring developed at the interface of both layers. The lymphocyte ring was then transferred by a pipette into a clean test tube, rinsed twice with HBSS (with centrifugation for 5 minutes at 600 g) and the sediment was re-suspended in 1 ml HBSS. The number of lymphocytes was determined in a Bürker cell using a Türk solution (diluted 1 : 20). The concentration of lymphocytes was adjusted in all samples to $1\times10^{6}.50\ \mu l^{-1}$.

Immunophenotyping of IEL and LPL lymphocytes

Double labelling of lymphocytes was achieved by direct immunofluorescence. To 50 µl of lymphocyte suspension we added 2 µl of labelled monoclonal murine anti-chicken antibodies, namely CD-3FITC/CD45PE, CD4PE/CD8FITC and IgAPE/IgMFITC. Isotype controls were applied into control tubes according to need, namely murine IgG_1-FITC, IgG_{2b}R-PE, or IgG_{2b}FITC. The cells were incubated at room temperature, in the dark, for 15 minutes. After incubation, 1 ml PBS was added to the suspension and the contents were centrifuged (1400 rpm, 5 min). The supernatant was removed and the suspension diluted with 200 µl PBS with 1 % paraformaldehyde.

Measurement and analysis of lymphocytes

The cells were measured and analysed by a flow cytometer FACScan, with software Cell Quest (BD, Germany). 10 000 cells were collected in a live gate and evaluated in order to determine the proportion of individual subpopulations expressed in relative percentages.

Statistical evaluation

One-way ANOVA and Tukey test were used with software GraphPad Prism to determine mean values, standard deviations and significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Flow cytometry has many advantages and disadvantages. The advantages include: objective quantification of specific molecules, statistical accuracy, multiparameter cross-analysis of data, distribution information and subtype identification, dynamic measurements, sensitivity, rapidity and gen-

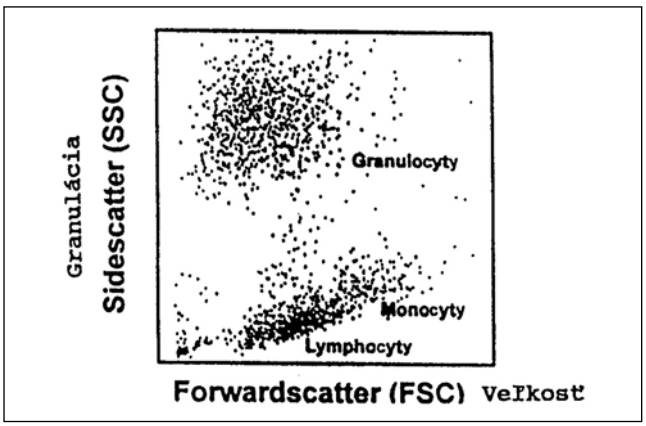


Fig.1. Density dot plot diagram — distinguishing the leukocytes by flow cytometry according to their size (FSC) and granularity (SSC)

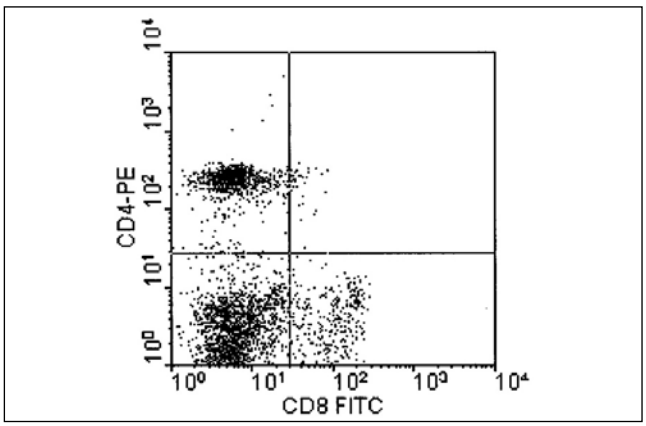


Fig. 2. Two-parameter dot-plot diagram — double labelling CD4+ and CD8+ of lymphocytes by direct immunofluorescence

eration of a great amount of data. The disadvantages include: loss of information regarding the position in solid tissues because results are obtained in a suspension [2].

We reached the set goals, i. e. standardization of the method of isolation of IEL and introduction of the method for isolation of LPL from the chicken intestine and their immunophenotyping. Our experimental model which involved the addition of T-2 toxin to chicken diet and preventive individual peroral administration of glucan to these birds, showed that glucan caused an increase in the levels of LPL T-lymphocytes and IgA+ intraepithelial lymphocytes. On the other hand, feeding the diet containing T-2 toxin (1,450 mg.kg^{-1}) without glucan resulted in a decrease of both LPL T-lymphocytes and IgA+ intraepithelial lymphocytes. The combined GT group showed an increase in both T-lymphocytes and IgA lymphocytes in the *lamina propria*, which confirmed the favourable effect of beta-D-glucan isolated from *Candida albicans* had on the immunocompetent cells with the parallel administration of T-2 toxin.

Another benefit of the study was that the described method of isolation of intraepithelial and *lamina propria* lymphocytes can be used for monitoring mucosal immunity in various experiments investigating issues affected by infectious, parasitic and toxic agents.

REFERENCES

1. Marinov, I., 2003: *Flow Cytometry in Clinical Haematology* (In Czech). TRITON, Prague, 11—16.

2. Revajová, V., 2006: *Immunopathology of Ascarioses in Paratenic Hosts* (In Slovak). Habilitation thesis, University of Veterinary Medicine in Košice, 151 pp.

3. Shapiro, H. M., 1988: *Practical Flow Cytometry*. Alan R. Liss, Inc. New York, 370 pp.

4. Solano-Aguilar, G. I., Vengroski, K. G., Beshah, E., Lunney, J. K., 2000: Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. *J. Immunol. Methods,* 241, 185—199.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 78—81, 2014

PATHOLOGY OF BONE TUMOURS — PRELIMINARY DATA AND EXTRASKELETAL OSTEOSARCOMA OCCURRENCE

Vilimová, Z., Revajová, V.

Institute of Pathological Anatomy
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

vilimova.zuzana@uvlf.student.sk

ABSTRACT

Bones consist of different mesenchymal tissues, and all of them are able to proliferate. Primary bone tumours may then arise from precursor cells of osteoid, chondroid, fibrous, adipose and vascular tissues. However, the most common cell lines of bone tumours are osteoid and chondroid. The frequency of these tumours is the highest in dogs, lower in cats and the lowest in other animal species. Unlike human with a benign prevalence of bone tumours, bone tumours of dogs have malignant tendencies. Osteosarcomas, osteoid mass producing malignant tumours, include 85 % of the cases in dogs, with a prevalence in large and middle breeds (boxer) and predominately in appendicular skeletal locations. Other species have different likely skeletal positions (head, ribs, and pelvis), and sometimes extraskeletal localisations (connective tissue, mammary gland) of bone tumours. Osteosarcomas metastasize by the haematogenous routes to the lungs. Common inflammatory lesions, pathological fractures and unusual reactions very often complicate the diagnosis. The complexity of diagnosis include; clinical history, radiography, gross appearance, cytological and histological findings, anatomical location, age of the animal and other vitally important data. The present paper summarises some pathological bone lesions observed during a 4 years period using 19 histological samples from dogs. The observations showed a predominance of malignant bone tumours in the larger breeds, the higher occurrence of osteosarcomas than fibrosarcomas and chondrosarcomas, with an appendicular skeletal predilection. An unusual occurrence of extraskeletal osteosarcoma with lung metastases in a dog is presented macroscopically and histologically.

Key words: dog; bone; tumour; inflammation

INTRODUCTION

The diagnosis of bone lesions and tumours is not easy and can be greatly aided by a combination of clinical, radiological, and pathological investigations [9]. Clinical history includes the duration and type of the clinical signs together with the location of the lesion. Fixation of previous fracture or longer limping and pain is mentioned in the history of dogs with bone tumours. From the point of anatomical distribution, there was a considerable prevalence of osteosarcomas in the appendicular skeleton (75.6 %), with the radius, ulna, femur and tibia being the most affected bones [2]. Radiography needs to be taken from two or more directions. Characteristic blastic and destructive lesions, for example sun-burst or hair on end pattern of matrix mineralization, periosteal reaction (Codman's triangle), or large soft tissue mass with maintenance of bone cortices are important radiographic information [7]. This helps to show the size, shape, and precise site of origin of the lesion [8] and is important for an optimal biopsy by the surgeon. The tissue samples must also be of sufficient size to include portions of both the growing margin and deeper parts of the tumour. Mainly in osteosarcoma, histological structure may vary widely in different parts and at the growing margin of which intercellular substances

such as osteoid material may be absent [8]. Pathological investigations of bone lesions are aimed at the typing of the tumour on the basis of histopathological changes and their differentiation from inflammatory reactions and dysplasias [3]. The most common tumorous changes are: bone lysis, disseminated osteoclasts, decalcification, cells included in the produced tumour tissue, malignant features of cells (anisocytosis, anisokaryosis, pathological mitoses), haemorrhages, infiltrative growth, and low degree of cell differentiation [7]. This will result in a more accurate diagnosis, in the sense of reactive changes, benign or malignant tumour. The diversity of tumours and tumour-like lesions in bone is shown in Table 1.

Because osteosarcoma may produce various kinds of extracellular matrix and have different degrees of differentiation, its histological pattern may vary significantly, not only from case to case, but also from area to area in the same case. Its classification into various subtypes is not only by the predominant histological pattern, but also by its anatomic location and sometimes by its histological grade [6].

In Slovakia, there have been no clinical-pathological studies on tumours and tumour-like lesions in the bones of dogs. The aim of this paper is to do a retrospective analysis of some cases using the data and histopathological findings from samples obtained from the Institute of Pathological Anatomy at the University of Veterinary Medicine and Pharmacy in Košice (IPA UVMP) during the years 2006 to 2009. One unusual case of extraskeletal osteosarcoma with lung metastases will be presented macroscopically and histologically.

MATERIALS AND METHODS

Animals

Nineteen cases of canine bone tumours and inflammatory lesions were detected in the histological samples from the IPA UVMP during the period of 2006—2009. The following data were recorded for the samples: age, sex, breed, topographic location of the lesion (axial or appendicular skeleton or extraskeletal sites).

Histopathology

Representative specimens of bone lesions were obtained from the dogs by biopsy, necropsy or surgery. The specimens were fixed in 10 % formalin and decalcified in Entkalker (Roth, Germany). Tissue sections (5 mm) were prepared, stained with haematoxylin and eosin (H & E), routinely processed for histology, and mounted on slides.

The criterion adopted for the diagnosis was based on the detection of cells, tissue matrix produced by the tumour cells and destructive changes.

Statistical analysis

The percentage of various changes was calculated on the basis of the cases examined.

RESULTS

On the basis of the history of the 19 dogs, it was possible to determine the age, sex, breed and topographic location of all bone lesions. Histological reports included the final diagnosis. The relevant available data are summarized in Table 2.

The analysis of cases showed a predominance of tumours over inflammatory lesions (84.2 *vs.* 15.8 %), and malignant over benign tumours (93.8 *vs.* 6.2 %), with osteosarcomas being the most frequent (60 %). The age of dogs ranged from 2.5 to 15 years, with a mean of 8 years. The sex of all 16 dogs with tumours was recorded, 8 of them being females and 8 males (50 %). The distribution according to breed showed

Fig 1. Extraskeletal location of osteosarcoma in *regio colli* (a); osseous spongy section plane (b); osteoblastic productive histological pattern (c); lung metastases (d); with the identical histological picture as in the mature tumour (H & E stain)

a predominance of large breeds (Table 2), with appendicular skeleton (60 %) and mammary gland (20 %) locations. With respect to the histological subtypes, combined osteosarcomas were more frequently observed then the pure pattern (Table 2).

Figure 1 presents extraskeletal osteoblastic productive osteosarcoma in a 5 year-old male Bishon, with subcutaneous position in *regio colli*, arising from the connective tissue, and metastases in the lungs. Fibroid and chondroid matrix was also observed in some areas. Necrotic changes and haemorrhages were spread across the tumour.

DISCUSSION

The preliminary results from the four year observations revealed a prevalence of malignant tumours in dog with 60 % occurrence of osteosarcoma with combined histological patterns. According to a study by Cavalcanti et al. [1] conducted on 52 dogs, osteosarcomas were presented in 85 % of cases with 52 % of a pure pattern and 42 % of a combined pattern. The highest incidence of malignant tumours in our study was found in large breeds such as Great Dane, German Shepherd and English Springer Spaniel. Hammer et al. [5] also reported a higher incidence of osteosarcomas in German Shepherds. Osteosarcomas mostly occur in adult and older dogs, with a median age of 8 years. Garjoaba et al. [4] reported the highest prevalence (of 136 dogs) in giant and large size breeds 7 and 9 years old (16.98 % and 24.53 %, respectively). The most frequent anatomical localisation of malignant tumours were in long bone metaphysis and also extraskeletal, and the mammary gland. Cavalcanti et al. [1] found 75.6 % of malignant tumours in the appendicular skeleton in the following order of frequency: radius and ulna (26.8 %), femur (24.8 %), tibia (20.3 %), scapula (14.3 %), and humerus (9 %). Extraskeletal localization of osteosarcoma was observed in three cases (7.3 %). The ratio of tumours in males and females was 1 : 1 in our study, but part of other studies reported a predominance among males while others reported a predominance among females.

REFERENCES

1. Cavalcanti, J. N., Amstalden, E. M. I., Guerra, J. L., Magna, L. C., 2004: Osteosarcoma in dogs: clinical-morphological study and prognostic correlation. *Brazilian Journal of Veterinary Research and Animal Science,* 41, 299—305.

Table 1. Histological classification of tumour and tumour-like lesions in bone [8]

Benign tumours	**Malignant tumours**	
Chondroma	**Central**	**Peripheral**
Feline osteochondromatosis	Chondrosarcoma	Maxillary fibrosarcoma (dogs)
Haemangioma	Fibrosarcoma	Periosteal osteosarcoma
Myxoma of the jaw	Giant cell tumour of bone	Periosteal fibrosarcoma
Ossifying fibroma	Hemangiosarcoma	Periosteal osteosarcoma
Osteoma	Liposarcoma	
Osteochondroma	Multilobular tumour of the bone	
Tumour-like lesions	Osteosarcoma:	***Tumour of bone marrow***
	a. poorly differentiated	
Aneurysmal bone cyst	b. osteoblastic	Malignant lymphoma
Epidermoid cyst of phalanx	• nonproductive	Plasma cell myeloma
Exuberant fracture callus	• productive	
	c. chondroblastic	
	d. fibroblastic	
	e. teleangiectatic	
	f. giant cell type	

Table 2. Summarized data of bone lesions and tumours in diagnostic cases

Years	Breed	Sex	Age	Location	Diagnosis
2006	Cocker Spaniel	female	11 y	right M_4 mammary gland	combine osteosarcoma
2007	Rottweiler	male	8 y	distal metatarsus	osteolytic osteosarcoma
	German Shepherd	female	7 y	right upper metatarsus	osteolyticosteosarcoma
	German Shepherd	female	12 y	right carpus	fibrosarcoma
	Golden Retriever	female	6 y	tendon	chondrosarcoma II. degree
	Bernese mountain dog	female	6 y	proximal epiphysis of tibia	osteomyelitis
2008	Rhodesian Ridgeback	female		mammary gland	osteosarcoma
	Irish Wolfhound	male	6 y	distal left antebrachium	teleangiectatic osteoblastic osteosarcoma
	Springer Spaniel	male	15 y	maxilla	poor-differentiated sarcoma
	Yorkshire terrier	female	14 m	end tail	osteomyelitis
	German Mastiff	male	8 r	sternum	hemangiosarcoma
2009	Labrador	male	3 y	distal femur	periosteal fibrosarcoma
	Dog	female	13 y	subcutis	osteochondroma
	Labrador	male	3 y	distal femur	periosteal fibrosarcoma
	Dog	male	-	distal radius	osteoblastic-fibroblastic osteosarcoma
	Springer Spaniel	female	-	mammary gland	combine osteosarcoma
	German Mastiff	male	2.5 y	distal right femur	osteosarcoma
	Crossbreed	male	8 y	nasal cavity	osteolysis and chondrolysis
	German Shepherd	female	10 y	os ilium dextrum	osteosarcoma

2. Cooley, D. M., Waters, D. J., 1997: Skeletal neoplasms of small dogs: a retrospective study and literature review. *Journal of the American Animal Hospital Association*, 33, 11—23.

3. Doige, C., 1988: Skeletal system. In: **Thomson, R. G.** (Ed.): *Special Veterinary Pathology*, Decker, Toronto, 467—507.

4. Garjoaba, I., Tudor, N., Soare, T., Tanase, A., Vlagioiu, C., 2009: Extraskeletal osteosarcoma in dogs: presentation of two cases. *Lucrari Stiintifice Medicina Veterinara, 42,* 103—105.

5. Hammer, A. S., Weeren, F. R., Weisbrode, S. E., Padgett, S. L., 1995: Prognostic factor in dogs with osteosarcomas of the flat or irregular bones. *Journal of the American Hospital Association*, 31, 321—326.

6. Klein, M. J., Siegal, G. P., 2006: Osteosarcoma anatomic and histologic variants. *Am. J. Clin. Pat.*, 125, 555—581.

7. Makai, F. et al., 2010: Tumours of the locomotor system (In Slovak). In **Jurga, M. et al.** (Eds.*): Clinical and Radiation Oncology* (In Slovak), Osveta, Martin, SR, 1200—1209.

8. Thomson, R. G., 2007: Bones and joints. In *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*, **Maxie, M. G**. (Ed.), 5th edn., Vol. I, Sauders Elsevier, 1—184.

9. World Health Organization, 1976: International histological classification of tumours of domestic animals. *Bull. World Health Organ.*, 53, 265—282.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 82—85, 2014

PREVALENCE OF HEMOPARASITES IN SNAKES FOUND IN SOUTH AFRICA

Terzer, P., Halan, M.

University of Veterinary Medicine and Pharmacy, Komenského 73, 04181 Košice
The Slovak Republic

milos.halan@uvlf.sk

ABSTRACT

Over a three week period in January 2013, 9 species of venomous snake provided 129 samples which were analysed for the presence of hemoparasites. The hemoparasites that were under investigation were; plasmodiids, hemogregarines and trypanosomatis flagellates. Blood samples were collected from 9 South African provinces; Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West, and the Western Cape. The most commonly encountered genera were *Naja* spp. (67), *Causus* spp. (19), *Hemachatus* spp. (15), *Dispholidus* spp. (14), *Bitis* spp. (8), and *Thelotornis* spp. (6). All blood samples were stained with Diff Quik and examined by light microscopy. From a total number of 129 samples, only 5 were positive giving a 3.8% infection rate. Of those 5 samples that were found to be positive, *Naja mossambica*, *Bitis arietans* and *Dispholidus typus* proved to be positive for *Haemogregarina* spp. and *Hemoproteus* spp.

Key words: Diff Quik; *Haemogregarina*; *Haemoproteus*; haemoparasites; microscopy; South Africa; snakes; venomous

INTRODUCTION

The majority of blood parasites found in snakes are intra-erythrocytic. In the last 15 years very little ongoing research has been carried out on parasites infecting South African snakes, with the only notable contributions made by Cook [3]. Given the unresearched diversity of blood parasites found within South Africa, it was considered essential that more research was conducted.

The aim of this study was to ascertain the prevalence of blood parasitic infections in venomous snakes within South Africa.

METHODS

A total of 129 blood samples were taken covering 9 venomous species of snakes. The blood parasites that were investigated were classified as Protozoa. All of the snakes that were sampled were venomous and indigenous to South Africa. All the samples that were taken came from wild caught individuals or individuals that were part of private collections or venom extraction units. It was also important to note that none of the snakes that were sampled were treated with anti-parasitics.

The majority of the samples collected were provided by Mike Perry from the African Reptiles and Venom unit [4], a company supplying snake venom for manufacturers of snakebite anti-venom and snake handling equipment and organising snake awareness, snakebite treatment and venomous snake handling courses on site and throughout Africa. The rest of samples were obtained from the wild over a three week period in the summer in the provinces of; KwaZulu-Natal, Gauteng, Free State, and Mpumalanga. All specimens were, identified, photographed, bagged at specific geographical coordinates recorded by a GPS. The blood samples (0.1 ml) were

taken from the ventral coccygeal vein [5]. All samples were air dried in South Africa, and then fixed and stained in the Department of Parasitology at the University of Veterinary Medicine and Pharmacy, Kosice, Slovakia. Slides were then screened with a Nikon-Lab photo 2 microscope and measurements (μm) were recorded.

RESULTS

Out of the 129 blood samples, only 5 samples were positive (3.8 %). All of the samples that were photographed, were done so at 100× magnification and this made them comparable to the photos found in other published research reports [3], [6], [7], [8]. Table 1, presents the overall results.

Table 1. Results for all 129 snakes, showing the number of positive samples

Snake species	Body condition	Number collected	Infested
Naja mossambica	Good	26	2
Hemachatus haemachatus	Good	15	0
Naja annulifera	Good	13	0
Naja nivea	Good	17	0
Naja melanoleuca	Good	11	0
Bitis arietans	Good	8	1
Causus rhombeatus	Good	19	0
Dispholidus typus	Good	14	2
Thelotornis capenis	Good	6	0

Fig. 1 shows a *Haemogregarina* spp. gamont within an erythrocyte of a *Dispholidus typus*. The parasite shows a slightly curved shape of the gamont. Both the posterior and anterior ends are rounded with the anterior end being slightly larger. It is enveloped in a cell membrane. The nucleus does not extend across the whole of the gamont's cytoplasm and can be seen to be centrally located. The size of the actual parasite within the erythrocyte ranges in size from 14.59—15.4×4.85—5.01 μm. By comparing the measured sizes to values stated in Telford for *Haemogregarina pelusiensi* 1962 (12.5—24×3.4–9 μm), which is the only *Haemogregarina* spp. to be described in Africa [7], both sets of values are comparable in size.

Fig. 2 shows an infected erythrocyte from a *Bitis arietans*. When comparing this photo to Telford, it can be said that this photo is of a *Haemoproteus* spp. macrogamete [7]. The macrogamete is seen to take up a large area of the host cell, and shows a thick plasma membrane with small black granules, microgametocytes [10].

DISCUSSION

The expectations of this study were very high as no single study into venomous snakes has taken place in South Africa in the last 15 years. Of the 129 samples, a total of 5 (3.8 %) were found to be positive for hemoparasites. Four samples (3.1 %) were positive for *Haemogregarina* spp. and 1 (0.7 %) sample was positive for *Haemoproteus* spp.

Naja mossambica and *Dispholidus typus* proved to be the species most commonly infected by *Haemogregarina* spp. These findings were supported by Pienaarin, that an African *Haemogregarina* ssp. was described in a *Naja* spp. [9]. *Bitis arietans* proved to be the only species to be infected by *Haemoproteus* spp. This appears to be the first time *Haemoproteus* spp. has been documented so far south in Africa [6]. Telford only described one *Haemoproteus spp*. that was found in *Naja n. nigercollis* in Northern Africa [10].

The average sizes of the infected erythrocytes by *Haemogregarina spp*. are (19.86×12.55 μm) and the average size of the normal erythrocytes are (16.64×12.55 μm). From this we can say that the parasite did slightly increase the length of the erythrocyte, but the width decreased. It can be said that this decrease in width was due to stretching of the erythrocyte.

It is widely recognised that nearly all wild/wild-caught snakes will harbour parasites (i.e. ectoparasites and/or endoparasites) of some sort [1], [2]. Hemoparasite infections can often go unnoticed as their hosts may never show any visible clinical signs. It has been determined that most of the life cycles are in fact indirect, so if none of the wild caught snakes are showing any clinical signs there in no reason for routine blood analysis as most healthy collections are vector free [3]. In carrying out this study it became very apparent that little is known about the infestation levels within snakes and reptiles in general within South Africa. Mainly the research has been focused on tortoises and turtles as they are rare and protected within the borders of South Africa. This has left a large void in the research that could possibly be undertaken.

CONCLUSION

It is difficult to gauge the significance of positivity from snakes as a whole as the sample pool was quite small when compared to all South African snakes. An important point to take into consideration when gauging the significance of the level of positivity depends on the actual number of snakes investigated relative to species, family and total number of samples examined. In this study, only 9 species were sampled, but there were approximately 6—26 samples for each species. The decision to sample in this way, in fact gives a

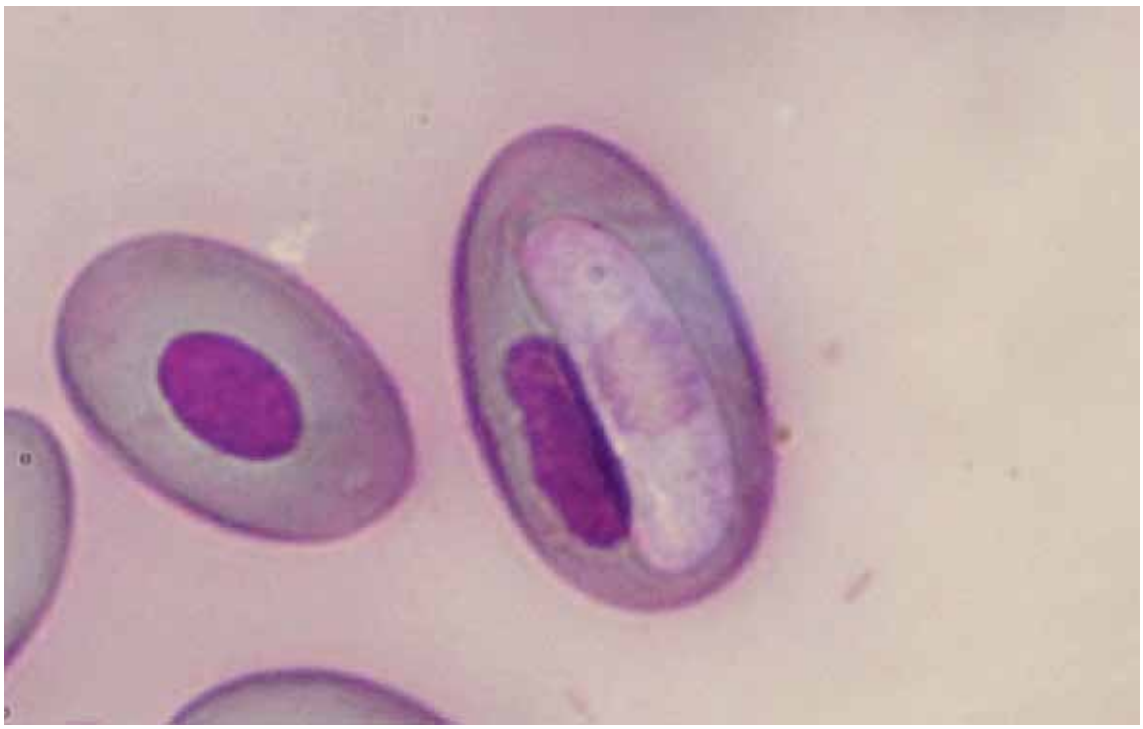


Fig. 1. ***Haemogregarina*** **spp. in a** ***Dispholidus typus*** **erythrocyte**

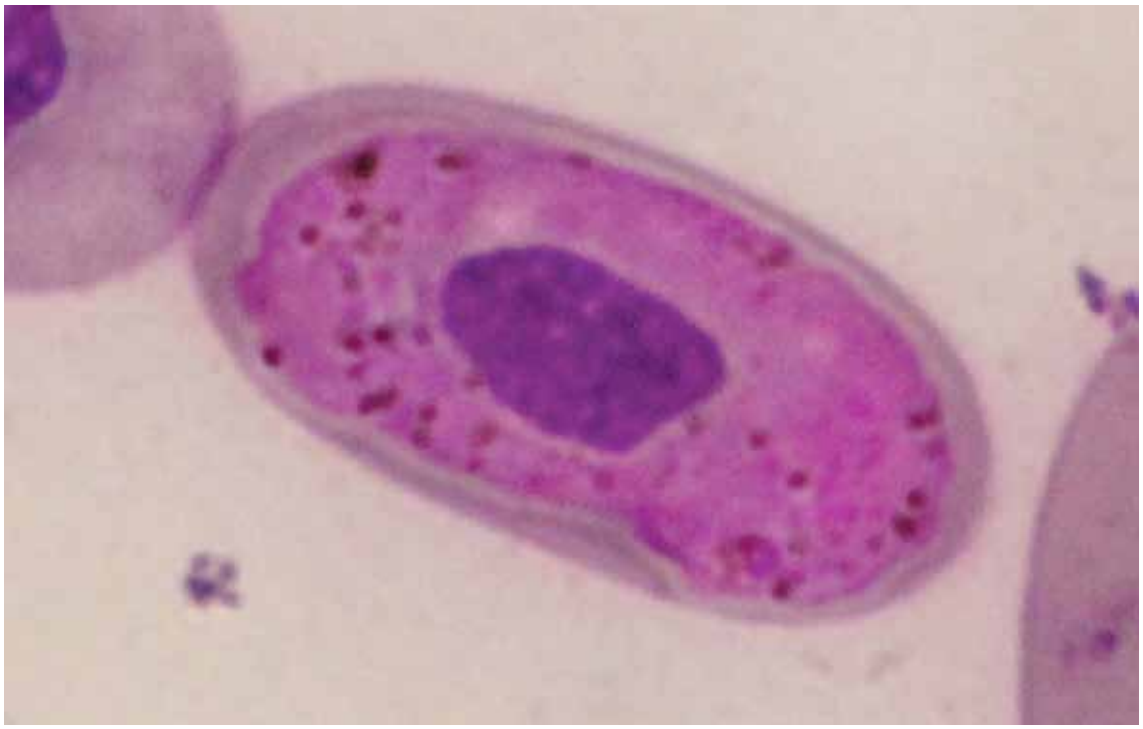


Fig. 2. ***Haemoproteus*** **spp. macrogamete found within a** ***Bitis arietans*** **erythrocyte**

better representation of the species infection rate rather than taking fewer samples from 1 species. This will invariably lead to a greater understanding of the species involved in hemoparasite infections in snakes and reptiles as a whole. Overall this study proved to be very positive with regards to adding significant data to hemoparasite research within South Africa.

ACKNOWLEDGEMENT

I would like to thank Dr. Milos Halan for his guidance and expertise and all the lab staff in the Department of Parasitology. As well as to Mike Perry at African Reptiles and Venom for his help and allowing me to collect samples from his venom extraction programme.

REFERENCES

1. Barnard, S. M., Upton, S. J., 1994: *A Veterinary Guide to the Parasites of Reptiles: Protozoa.* Krieger Publishing Company, Malabar, FL, 62—63.

2. Bush, A. O., Fernandez, J. C., Seed, W., 2013: *Parasitism. The Diversity and Ecology of Animal Parasites*. Cambridge, UK, 556.

3. Cook, C. A., Smit, N. J., Davies, A. J., 2009: A redescription of *Haemogregarina fitzsimonsi* Dias, 1953 and some comments on *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) from southern African tortoises (*Cryptodira: Testudinidae*), with new host data and distribution records. *Folia Parasitol*. (Prague), 56, 173—179.

4. Perry, M.: Johannesburg, South Africa, http://africanreptiles-venom.co.za/

5. Stacy, N. I., Alleman, A. R., Sayler, K. A., 2011: Diagnostic hematology of reptiles. *Clinics in Laboratory Medicine,* 31, 87—108.

6. Telford, S. R., 2009a: Hemoparasites of the reptilia. In *The Plasmodiid Parasites,* 1st edn., CRC Press, New York, 199—202.

7. Telford, S. R., 2009b: Hemoparasites of the reptilia. In *The Plasmodiid Parasites, Appendix,* 1st edn., CRC Press, New York, 376.

8. Telford, S. R., 2009c: Hemoparasites of the reptilia. In *Kinetoplastida,* CRC Press, New York, 275.

9. Telford, S. R., 2009d: Hemoparasites of the reptilia. In *The Hemogragarines*. CRC Press, New York, 251—252.

10. Telford, S. R., 2009e: Hemoparasites of the reptilia. In *The Plasmodiid Parasites*, 1st edn., CRC Press, New York, 176, 362.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section II, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 86—88, 2014

IMPACT OF PARASITIC INFECTIONWITH *ISOSPORA* SPECIES ON BLOOD CALCIUM IN THE LIZARD *POGONA VITTICEPS*

Mitrová, K., Halán, M.

Institute of Parasitology, Department of Epizootiology and Parasitology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

mitrova.k@gmail.com

ABSTRACT

Isospora is a worldwide distributed disease of the gastrointestinal tract of reptiles. *Isosporaamphiboluri*is a pathogen that frequently occurs in large breeding colonies of bearded dragons (*Pogona vitticeps*) among which it spreads relatively rapidly due to its simple developmental cycle. Infection results in the destruction of the intestinal mucosa and subsequent nutrient resorption disorders. Lizards are sensitive to the decreased intake of minerals which frequently causes skeletal damage. Our study focused on the blood levels of calcium in the bearded dragons infected with the parasite *Isospora amphiboluri*. Flotation methods allowed us to prepare an accurate infective dose which was then used to infect 6 dragon lizards. The presence of infection was confirmed by the examination of the faeces. The serum calcium levels were determined in the infected and control lizards. Our investigations showed that *Isospora amphiboluri* may cause severe problems in growing and immunodeficient lizards. *Isospora amphiboluri* caused no clinical changes nor affected the levels of serum calcium in previously healthy lizards kept in adequate hygiene.

Key words: bearded dragon; calcium; *Isospora amphiboluri*; lizards; *Pogona vitticeps*

INTRODUCTION

Recently an increased interest in lizards has been observed. Veterinarians more frequently face lizards suffering from deficient nutrition, reproduction problems and infectious or parasitic diseases. Problems are particularly prevalent in the bearded dragons which are purchased from large-scale breeders or some dealers who do not care or are unaware about the proper handling of these animals. Infections with parasites with direct developmental cycles spread rapidly among bearded dragons kept in large colonies. Such parasites include *Isospora amphiboluri*, an agent of isosporosis in *Pogona vitticeps*. This agent causes problems mainly in young, rapidly growing lizards, causing direct destruction of intestinal mucosa resulting in nutrient resorption disorders. Bearded dragons, especially the young, are very sensitive to a decrease in calcium levels, which prevents the proper development of the skeleton and muscles. Monitoring of calcium in the blood of infected bearded dragons allows one to increase its intake by adjusting the dietary calcium level and prevent irreversible skeletal damage.

The aim of this study was to determine the blood levels of calcium in bearded dragons infected with the parasite *Isospora amphiboluri*.

MATERIALS AND METHODS

We used the infected faeces of bearded dragons from an external breeding colony to obtain an accurate infective dose neces-

sary to infect one lizard by means of the flotation method [4] using Darling and McMaster's counting cells. We infected 6 bearded dragons (*Pogona vitticeps)* perorally by a syringe with a 0.3 ml dose containing approximately 13,688 sporulated oocysts of *Isospora amphiboluri*. The faeces of the infected lizards were examined by the flotation method [4]. After detection of *Isospora amphiboluri*, plasma calcium levels were determined on day 21 post-infection and then 2 more times in 2 week intervals. The levels in the infected lizards were compared with those in the control group (non-infected bearded dragons, n = 6) and with the levels reported by other authors [1], [3], [5]. The clinical state of lizards was monitored throughout the infection.

Calcium levels in blood plasma were determined using the biochemical analyser Cobas C 111 (Roche, Switzerland).

RESULTS AND DISCUSSION

Presence of *Isospora amphiboluri* was detected after the infection in the faeces of all six experimental lizards. One of them died on day 20 post-infection (No. 4). Shortly before death, the infected lizard No. 4 showed clinical signs of infection (somnolence, anorexia, and dehydration). The microscopic examination of the large intestinal contents from this animal showed the presence of *Pharyngodon* species. The presence of *Isospora amphiboluri* in the small intestine content was not detected, despite inflammatory changes in the intestinal mucosa. Macroscopic changes were observed in lungs, kidneys and liver in the form of small, white-yellowish nodules. The microbiological examination was positive for the bacterium *Morganella morganii*.

Samples of blood withdrawn on day 21 post-infection from the 11 remaining lizards, and then two more times in 2-week intervals, were examined for calcium and the results are presented in Table 1 and Fig. 1. According to the reference values presented by Knotek [3] and Diethelm and Stein [1], all results were within the standard range. When considering the normal values reported by Molnár [5], the level of calcium was increased at all three samplings. Hypocalcaemia was not recorded in any of the lizards. The levels of calcium in the infected and non-infected animals were relatively similar without any significant deviations. The infected animals showed apathy, vomitus and overall weakness approximately at 2 weeks post-infection. These signs persisted for several days and gradually disappeared completely. Bloody diarrhoea reported by Jarofke and Lange [2] was not observed.

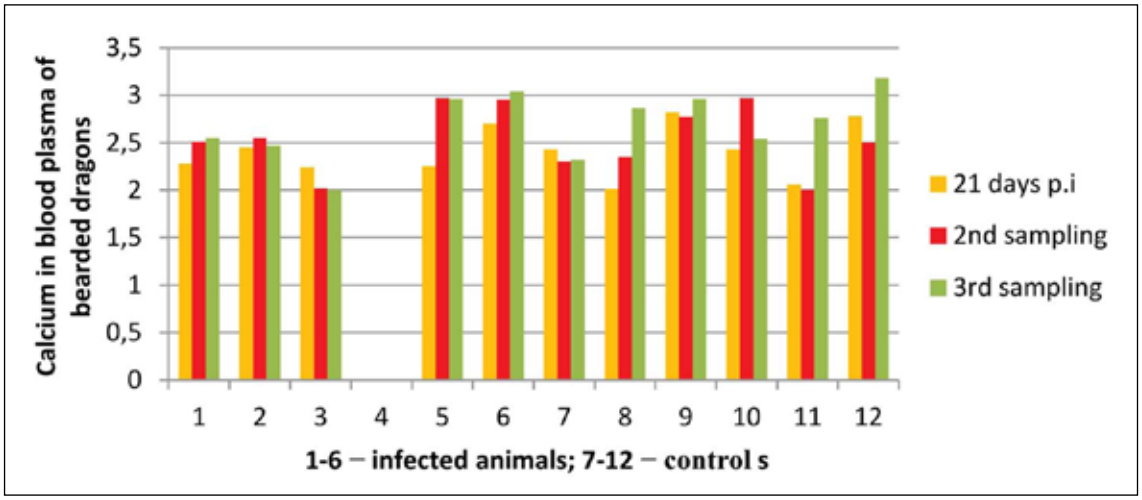


Fig. 1. Calcium levels in blood of experimental and control bearded dragons (mmol.l^{-1})

Table 1. Calcium blood levels in bearded dragons infected with *I. amphiboluri* (samples 1—6) and in control lizards (samples 7—12) in mmol.l^{-1}

Sampling	Sample No.											
	1	2	3	4	5	6	7	8	9	10	11	12
1st	2.28	2.45	2.24	–	2.25	2.70	2.43	2.01	2.82	2.43	2.06	2.75
2nd	2.51	2.55	2.02	–	2.97	2.95	2.30	2.35	2.77	2.97	2.00	2.50
3rd	2.55	2.47	2.00	–	2.96	3.04	2.32	2.86	2.96	2.54	2.76	3.18

CONCLUSIONS

Infected lizards showed clinical signs only for several days. Bloody diarrhoea described in other literary sources was not observed in our study. The levels of blood calcium in the infected animals did not differ considerably from the levels in the non-infected ones. All animals were provided high-quality diet and were kept in adequate hygiene conditions.

REFERENCES

1. Diethelm, G., Stein, G., 2006: Hematologic and blood chemistry values in reptiles. In **Mader. D. et al.**: *Reptile Medicine and Surgery*. Saunders Elsevier, St Louis, 1103—1110.

2. Jarofke, D., Lange, J., 1999: *Reptiles Diseases and Breeding* (In Slovak), Hajko and Hajková, Bratislava, 92—102.

3. Knotek, Z. et al., 1999: *Diseases of Reptiles* (In Czech). Czech Association of Small Animal Veterinarians, Brno, 15—244.

4. Letková, V., Čisláková, L. et al., 2010: *Laboratory Diagnostic Methods in Veterinary Parasitology* (In Slovak). University of Veterinary Medicine and Pharmacy in Košice, 110 pp.

5. Molnár, L., 2011: Snakes, tortoises, lizards (In Slovak). In **Mojžišová et al.**: *Practical Veterinary Handbook*, KVL SR, Bratislava, 258 pp.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section II, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 89—91, 2014

THE HELMINTHFAUNA OF CAPTIVE AND WILDBIRDS OF PREY IN PRIEVIDZA DISTRICT, CENTRAL SLOVAKIA

Jantošková, K., Halán, M.

Institute of Parasitology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

milos.halan@uvlf.sk

ABSTRACT

This study investigated species diversity of parasites of captive and free living birds of prey. The aim was to detect and identify propagative stages of parasites coprologically, by means of the flotation method. In addition, we also examined the feather and skin of the respective birds for the presence of ectoparasites. Parasitological post-mortem examinations of dead birds were carried out focusing on the parasites of the digestive and respiratory tracts.

Key words: birds of prey; parasites; parasitological examination

INTRODUCTION

Exact evidence on the time and place where the art of falconry may have begun is not available but we assume that the hunting of wild quarry by means of a trained bird of prey may have existed for more than 4000 years. Within the Slovak Republic, the relevant CITES regulations and Comission Regulation (EU) No, 101/2012, amending Council Regulation (EC) No 338/97 on the protection of species of wild fauna and flora are applied which regulate the trade of birds of prey. All European species of birds of prey and owls are included in the list "A" of Regulation 101/2012. These species are under the threat of extinction,, i.e. species included in the Appendix I of the CITES Convention, and natural wild species living in EU countries and which are protected by EU legislation; this also includes birds of prey in the Appendix II of CITES Convention. This means that all Slovak species of diurnal birds of prey and owls are subject to most strict regulations of trade.

The aim of this study was to investigate the species diversity of parasites of captive and free living birds of prey in the Prievidza district of Eastern Slovakia.

MATERIALS AND METHODS

Samples of faeces were collected during the years 2011—2013 from birds in the falconry club Aquila in Bojnice, i.e. ZOO in Bojnice and Wildlife rehabilitation centre situated on the premises of Bojnice ZOO. The samples were collected periodically from animals kept permanently in captivity, always before regular deworming, which was performed twice a year. Samples from the birds in the quarantine were collected before preventative deworming. We examined altogether 70 birds of prey included in 3 orders and 5 families.

The samples were examined by a flotation method [2] involving the concentration of propagative stages of parasites on the surface of flotation liquid according to different specific weight of parasite eggs and respective flotation solutions.

Bird cadavers were examined by a partial parasitological *post mortem* examination, involving the respiratory and gastrointestinal tracts of the birds.

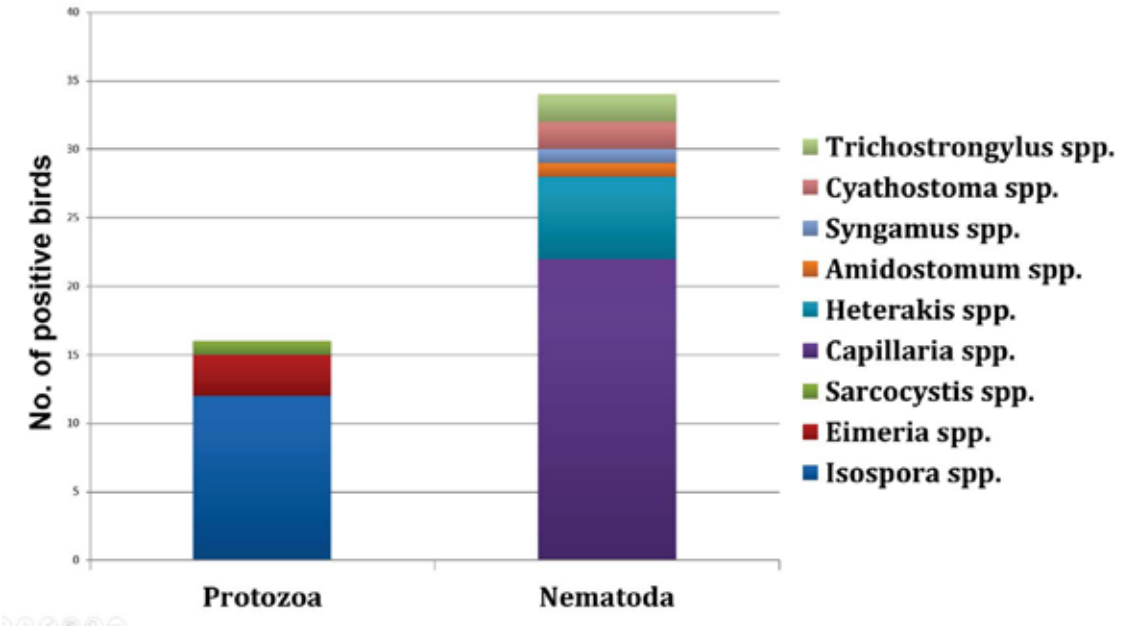


Fig. 1. Findings obtained by the flotation method

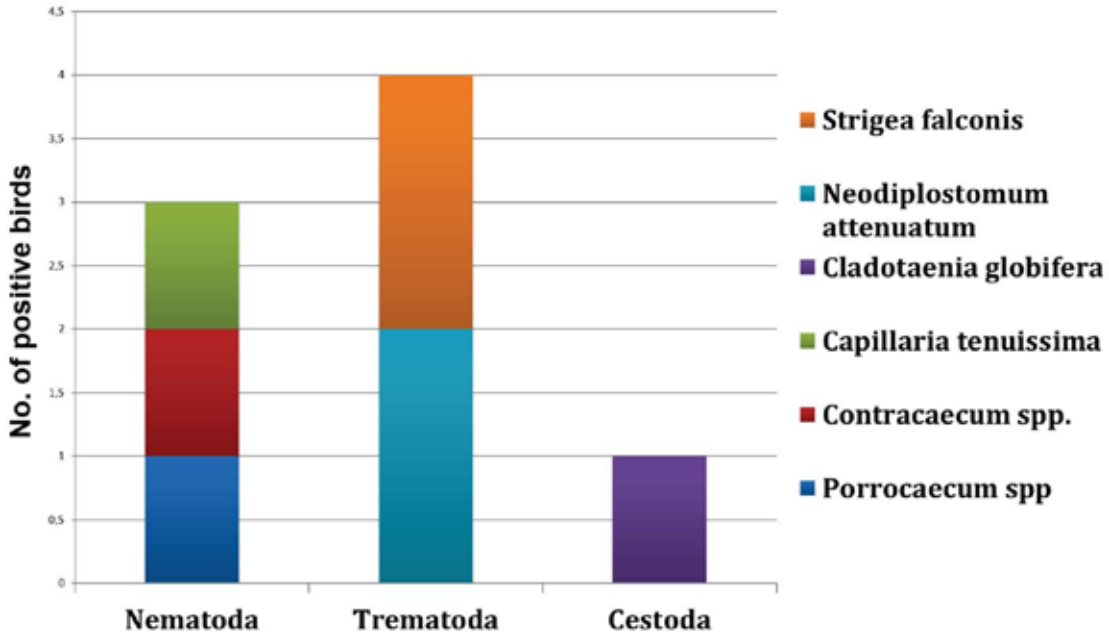


Fig. 2. Parasites found in dissected birds

RESULTS AND DISCUSSION

Of the 70 birds examined by the flotation method and parasitological dissection, 45 were infected (64.8 %; n = 70). We diagnosed mono-infections, but some birds were infected with several parasite species.

The only diagnosed ectoparasite was the bird louse fly *Ornithomyia auricularia*, found under the wings of an originally wild living common buzzard (*Buteo buteo*) which was brought to the falconry club Aquila in Bojnice. Besides the species *Ornithomyia auricularia,* also *Ornithomyia anchineuria, Pseudolynchia canariensis* and *Icosta spp.* may parasitise wild birds [1].

Unicellular parasites infected 16 birds (35.5 %; n = 45). Parasites of *Isospora spp.* were diagnosed in 12 birds (26.6 %; n = 45) of species: Eurasian eagle-owl, steppe eagle, snowy owl, Ural owl, western marsh-Harrier, common barn-owl, red-tailed hawk and the common kestrel. The oocysts of *Eimeria* spp. were detected in the faeces of the common kestrel, Harris hawk and saker falcon (6.6 %, n = 5). *Sarcocystis spp.* were found only in one saker falcon (2.2 %, n = 45). Diseases caused by *Eimeria* spp. and *Isospora* spp. can be manifested as diarrhoea, loss of weight, inappetence, vomiting and even acute death [5].

On the basis of the flotation method, we diagnosed 6 nematode species in 34 birds of prey (75.5 %; n = 45). The findings are summarised in Fig. 1. Twenty two birds (48.89 %; n = 45) of various species (red-tailed hawk, common buzzard, Eurasian eagle-owl, steppe eagle, Harris hawk, common kestrel, western marsh-Harrier, common barn-owl, long-eared owl, northern goshawk) were infected with *Capillaria spp.* In 6 birds (13.33 %; n = 45) of various species (Ural owl, northern goshawk and Eurasian eagle-owl), we diagnosed *Heterakis spp.* The faeces of the snowy owl and Eurasian sparrowhawk were positive for eggs of *Trichostrongylus* spp. (4.4 %; n = 45). Harris hawk and peregrine falcon were infected with *Cyathostoma* spp.(4.4 %; n = 45). Eggs of *Syngamus* spp. were found in the faeces of the long-eared owl (2.2 %; n = 45) and of *Amidostomum* spp. in the golden eagle (2.2 %; n = 45). Nematodes are considered the most frequent parasites in the birds of prey [1]. Many authors agree that the most common parasites of the birds of prey are members of the subfamily *Capillarinae.*

Four common buzzards were subjected to parasitological dissection. Parasites found in dissected birds are presented in Fig. 2. In the first buzzard we diagnosed the flukes *Strigea falconis* and *Neodiplostomum attenuatum* together with a nematode *Porrocaecum* spp. In the second buzzard, nematodes *Contracaecum* spp. and *Capillaria tenuissima were found*. In the third bird we found: a tapeworm, *Cladotaenia globifera,* and in the last buzzard, the flukes *Strigea falconis* and *Neodiplostomum attenuatum.* Tenora and Lusk [4] and Škarda [3] also reported *Strigea falconis* in the common buzzard (*Buteo buteo*) from the Slovak Republic. The tapeworm *Cladotaenia globifera* is the most common tapeworm found in European birds of prey [1].

CONCLUSION

Our results agree with the studies of other authors. There is very little information on parasitic infections of the birds of prey in the Slovak Republic. It is therefore very important to investigate the current situation, collect relevant data and develop effective preventive and therapeutic procedures against parasites occurring in these birds.

REFERENCES

1. Cooper, J. E., 2002: *Birds of Prey: Health and Disease.* 3rd edn., Blackwell Science, Berlin, 432 pp.

2. Letková, V., Čisláková, L. et al., 2010: *Laboratory Diagnostic Methods in Veterinary Parasitology* (In Slovak). University of Veterinary Medicine and Pharmacy in Košice, 110 pp.

3. Škarda, J., 1964: The helminth fauna of some wild birds in Czechoslovakia. *Acta Universitas Agriculturae*, Brno B, 269—293.

4. Tenora, F., Lusk, S., 1960: Beitrag zur näheren Kenntnis der Helminthenfauna bei Raubvögeln(*Falconiformes*) in der Tschechoslowakei. *Acta Universitatis Agriculturae*, Brno A, 205—211.

5. Upton, S. J., Campbell, T. W., Weigel, M., McKown, R. D., 1990: The *Eimeriidae* (*Apicomplexa*) of raptors: review of the literature and description of new species of the genera *Caryospora* and *Eimeria. Canadian Journal of Zoology,* 68, 1256—1265.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section II, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 92—97, 2014

ABDOMINAL ULTRASONOGRAPHY IN HORSES

Ďurčová, D., Valocký, I.

Equine clinic, University of Veterinary Medicine and Pharmacy
Komenského 73, 041 81 Košice
The Slovak Republic

igor.valocky@uvlf.sk

ABSTRACT

This study provides a topographic overview of the important structures of the abdominal regions of horses which can be distinguished immediately by transabdominal ultrasonography (USG). The dynamics of peristaltic movements in the digestive tract can be observed by analysis of the B-mode signal, produced by the movement of the tubular walls of the gut during the contraction and distension phases. Doppler ultrasound distinguished peristaltic P-waves from non-peristaltic P-waves. The influence of selected medicines affecting the motility of the digestive tract can be followed. Transabdominal ultrasonography methods are suitable for testing the sensitivity or non-irritability of digestive tract segments to prophylactic and metaphylactic medicines, and feed additives, but also the reactions to invasive examination methods and surgical procedures may be illuminated.

Key words: abdominal USG; Doppler; horse; intestinal motility

INTRODUCTION

Rectal examination is restricted to approximately 30—40 % of the caudal abdomen, depending on the horse's body frame and the palpation limit of the examiner's arm length [1]. Percutaneous sonography provides valuable information on density, topography, dislocation, mutual topographic relationship between organs of other organ systems and digestive organs and vessels and motility of abdominal organs [2]. Ultrasonography helps clinicians to decide between conservative and surgical treatment of colic [3]. Images of abdominal structures are obtained using either 2-D B-mode or Doppler modes and 3–3.5 MHz transducer projected to a depth of 10–25 cm [4]. Non-invasiveness and repeatability are the major advantages in the examination of functional processes.

The aim of this study was to obtain additional information concerning abdominal USG in horses because in the current Slovak situation, we lack an overview of acoustic windows (approaches) limiting the transducer position for obtaining images of internal structures. We also wanted: to obtain new knowledge on the functional dynamics of the motility of the digestive tract segments; validate the effectiveness of quantitative measurements of peristalsis of the segments of the tubular parts of GIT after the administration of selected prokinetics and relaxants; and to recommend a protocol for Fast Localised Abdominal Sonography of Horses (FLASH) for abdominal USG.

MATERALS AND METHODS

We examined 7 clinically healthy warm-blooded Slovak breeds of horses (n = 4), as well as English warm-blooded (n = 2) and Hutsul (n = 1) breeds of horses. The horses were between 6—17 years old; 3 were geldings and 4 were mares. The body weight of the examined horses was as follows: 1–735 kg; 2–678 kg; 3–535 kg; 4–563 kg; 5–612 kg; 6–462 kg; 7–385 kg.

Table 1. Effect of administration of drugs on peristalsis in the duodenum

DUODENUM					
Patient	**Fed horse[a]**	**Fasting horse[b]**	**Xylazin**	**Neostigmin**	**Lidocain[c]**
1	5/min	2/min	3.5/min	4.5/min	6.5/min
2	7/min	4.5/min	6/min	6/min	9/min
3	4.5/min	4/min	3.5/min	4/min	6/min
4	3/min	2.5/min	2.5/min	3/min	4.5/min
5	8/min	3.5/min	6/min	6/min	11/min
6	5/min	2.5/min	4/min	4.5/min	6.5 /min
7	8/min	5/min	7/min	6.5/min	9.5/min
Mean	**5.79/min-**	**3.46/min**	**4.64/min**	**4.93/min**	**7.57/min**
Σ	**1.77**	**1.05**	**1.55**	**1.18**	**2.13**

ab — P < 0.05; ac — P < 0.05; Fed horses — feeding with hay 15 min before USG; Fasting horses — fasting for 8 h before USG

Table 2. Effect of administration of drugs on peristalsis in the jejunum

JEJUNUM					
Patient	**Fed horse[A]**	**Fasting horse[B]**	**Xylazin[C]**	**Neostigmin[D]**	**Lidocain[E]**
1	9/min	7/min	7.5/min	8/min	10.5/min
2	11/min	6/min	9/min	8.5/min	12/min
3	5.5/min	3.5/min	4/min	5/min	7/min
4	7/min	5/min	5/min	6/min	9/min
5	9.5/min	5.5/min	7/min	6.5/min	10/min
6	15/min	9/min	11/min	12/min	16/min
7	12/ min	7.5/min	8.5/min	11/min	13.5/min
Mean	**9.86/min**	**6.21/min**	**7.43/min**	**8.14/min**	**11.14/min**
Σ	**2.93**	**1.66**	**2.21**	**2.4**	**2.76**

AB — P < 0.05; AD — P < 0.05; AC — P < 0.05; AE — P < 0.05; Fed horses — feeding with hay 15 min before USG;Fasting horses — fasting for 8 h before USG

Individual acoustic windows or approaches for ultrasonography were defined according to Reef [5]. We selected ultrasonographical sites which were most suitable for distinguishing the parenchymatous tissues from the tubular organs.

The Doppler pulsed wave (PW) mode was used to record the propulsion rate of fluid at the sonographic imaging 58° (2–4 MHz linear transducer) and 50° (5–10 MHz curvilinear transducer) angles. The activity of the respective regions was expressed as a number of contractions per 60s. Peristalsis was observed in fasting horses (fasting for 8 hours before USG) and fed horses (fed hay 15 min before USG).

The following selected drugs were administered to horses postprandially before USG examination: Xylazin (Rometar 20 mg.ml^{-1}) 0.6 mg.kg^{-1} body weight i. v.; Neostigmin (Konstigmin 2.5 mg.ml^{-1}) in 3 ml pro toto s. c.; Lidocain (Lidocain 2 %) 1.3 mg.kg^{-1} as a bolus, slow i. v. administration for 15 min by means of an injection pump.

Table 3. Effect of administration of drugs on peristalsis in the caecum

CAECUM					
Patient	**Fed horse[f]**	**Fasting horse[g]**	**Xylazin**	**Neostigmin[h]**	**Lidocain**
1	1.5/min	1/min	1/min	2/min	1.5/min
2	1.5/min	1.5/min	1/min	2/min	2/min
3	2/min	1/min	2/min	2.5/min	1.5/min
4	5/min	2/min	3.5 /min	6/min	4.5/min
5	3/min	2/min	2/min	4.5/min	3/min
6	2/min	1.5/min	1.5/min	3.5/min	2.5/min
7	4.5/min	2.5/min	3/min	5.5/min	4.5/min
Mean	**2.79/min**	**1.64/min**	**2/min**	**3.71/min**	**2.79/min**
Σ	**1.33**	**0.51**	**0.89**	**1.53**	**1.19**

fg — $P < 0.05$; fh — $P < 0.05$; Fed horses — feeding with hay 15 min before USG; Fasting horses — fasting for 8 h before USG

Table 4. Effect of administration of drugs on peristalsis in the large colon

LARGE COLON					
Patient	**Fed horse[F]**	**Fasting horse[G]**	**Xylazin**	**Neostigmin[H]**	**Lidocain**
1	2/min	1/min	1.5/min	2.5/min	2/min
2	1.5/min	1.5/min	1/min	2/min	2/min
3	1.5/min	1/min	1.5/min	2.5/min	1.5/min
4	3/min	2/min	2/min	4.5/min	2.5/min
5	3/min	2.5/min	2.5/min	5/min	3.5/min
6	2.5/min	1/min	2/min	4/min	2/min
7	1.5/min	1.5/min	1.5/min	3/min	1.5/min
Mean	**2.14/min**	**1.5/min**	**1.71/min**	**3.36/min**	**2.14/min**
Σ	**0.64**	**0.53**	**0.45**	**1.06**	**0.64**

FG — $P < 0.05$; FH — $P < 0.05$; Fed horses — feeding with hay 15 min before USG; Fasting horses — fasting for 8 h before USG

RESULTS

The right kidney was imaged between the 14th and 17th intercostal spaces (ICS) or up to the cranial edge of the paralumbar fossa, at the level of coxal tuber. The USG image located the right kidney between the 16th ICS up to the caudal edge of the paralumbar fossa, at the level between the coxal tuber and tuber ischiae. The stomach was imaged on the left, medial to the spleen, between the 8th and 13th ICS, at arm level. The duodenum was imaged on the right, as a 20 cm band, from the 8th ICS up to the cranial edge of the paralumbar fossa, 5 cm ventrally to the line joining the elbow and sacral tuber. The liver was located on the right side, between the 6th and 16th ICS, left to the 6th up to 9th ICS. The USG window for the spleen was located on the left side of the abdomen, from the 8th ICS up to the paralumbar fossa, medial

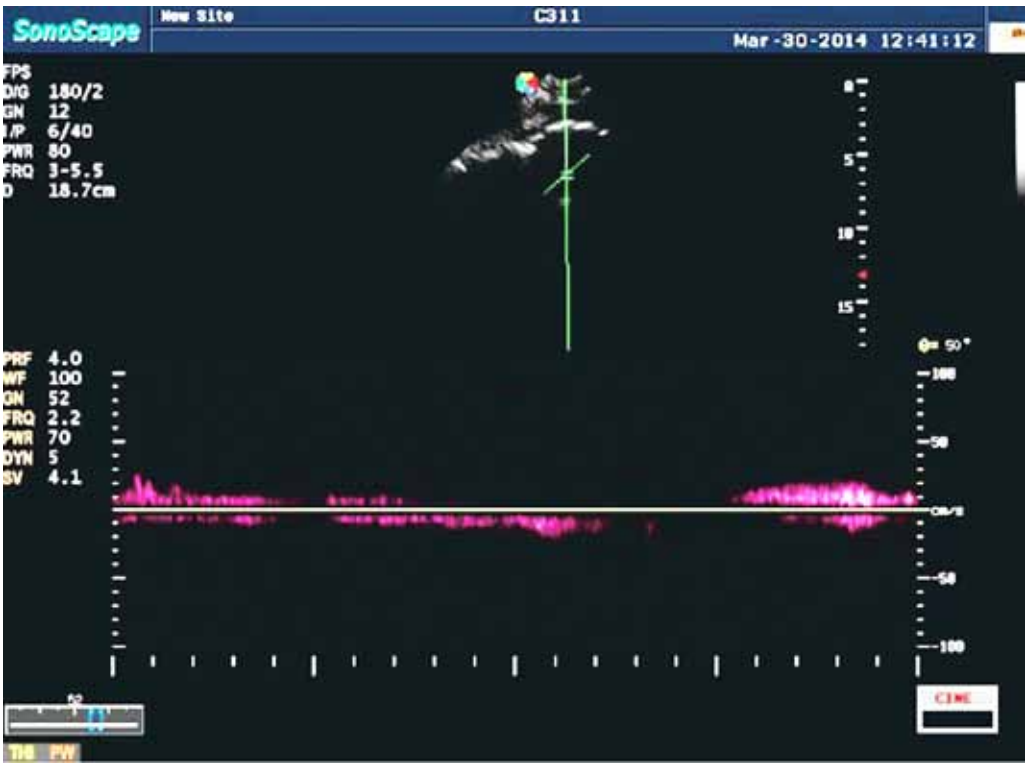


Fig. 1. Non-peristaltic activity

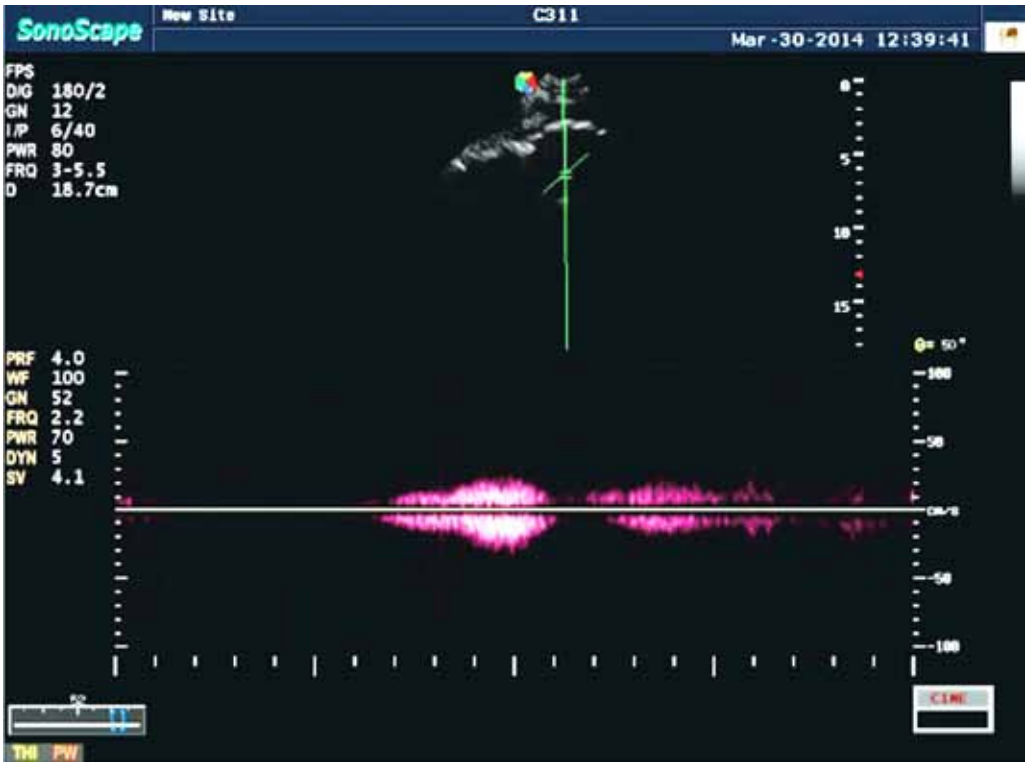


Fig. 2. Intermittent peak-producing activity

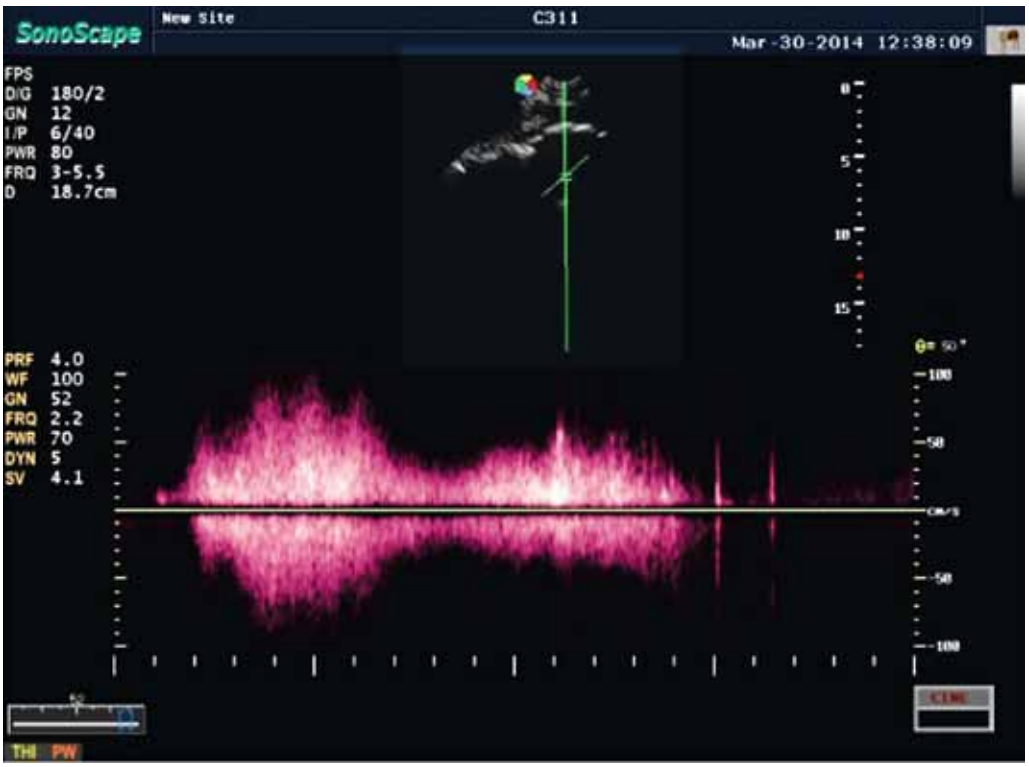


Fig. 3. Peak-producing activity

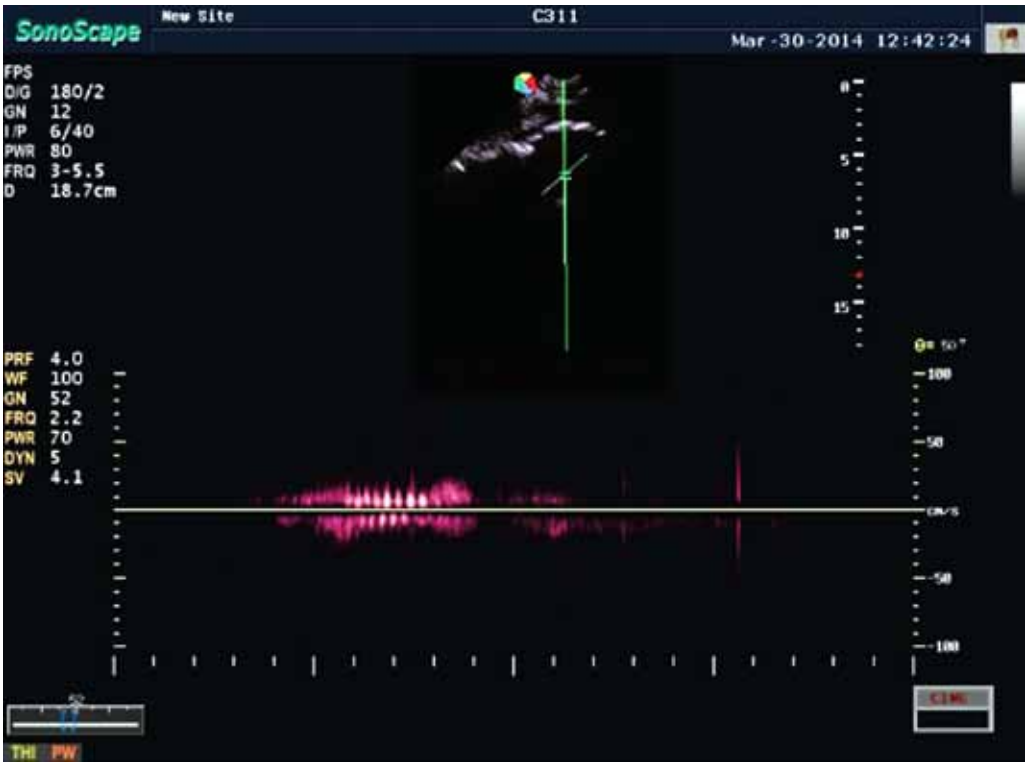


Fig. 4. Irregular phase

to the liver. The caecum was imaged along the right body wall in the dorsal and caudal parts of the abdomen. The large colon was located on both sides of the body along the ventral abdomen.

Doppler signals were recorded as peristaltic and non-peristaltic. Peristaltic activity manifested as a complex of spectral narrow laminar bands forming a track which deflected in both directions vertically to the baseline. Non-peristaltic activity produced a peak-lacking signal (Fig. 1). Peristaltic activity manifested as an intermittent peak- producing activity (Fig. 2) and peak activity (Fig. 3), was induced by contractions of smooth muscles which caused the propulsion of the digested feed. After this stage, an irregular bridging phase occurred (Fig. 4).

In the fasting horses, peristalsis slowed down in all segments ($P < 0.05$) (Tab. 1, 2, 3, 4). After the administration of Xylazin, the peristalsis slowed down in all segments but was insignificantly higher in the large intestine (Tab. 1, 2) and significantly higher in the small intestine ($P < 0.05$) compared to the fasting horses. After the administration of Neostigmin, the peristalsis slowed down insignificantly in the small intestine (Tab. 1, 2) and increased significantly in the large intestine ($P < 0.05$) (Tab. 3, 4). After the administration of Lidocain, the peristalsis in the small intestine accelerated significantly ($P < 0.05$) (Tab. 1, 2), but was not affected in the large intestine (Tab. 3, 4).

DISCUSSION

Doppler PW modality is a potential identificator of peristalsis of the gastrointestinal tract of horses. Quantitative and qualitative analysis of graphical visualisation allows one to measure propulsive motility and identify peristaltic contractions in a minimally 4 phases and the movement of digested feed orally/aborally [6], [7]. Our observations agree with those presented by Mitchell et al. [3] and Gimondo and Mirk [6]. Similar to Koenig and Cote [7], we observed a pro-kinetic effect of Neostigmin and Lidocain on the selected segments and relaxation effect of Xylazine. USG acoustic windows delineating the position of the transducer for distinguishing relevant internal structures, reported by Reef [5] were supplemented with specification for duodenum, stomach, colon and caecum. This facilitates in-field application of ultrasonographic techniques, testing of drugs for pharmaceutical companies and rapid orientation in patients with acute abdomen.

CONCLUSION

New information on the position of USG-transducer acoustic windows, suitable for obtaining images of internal structures, functional dynamics of segments of tubular parts of the digestive tract and pharmacological modulation of segments of these parts, obtained by duplex USG, constitutes a detailed review suitable for application of FLASH protocol.

REFERENCES

1. Porzuczek, A., Kielbowicz, Z., Haines, G., 2012: The use of percutaneous abdominal ultrasound examination in diagnosis equine small intestinal disorders. *Polish Journal of Veterinary Science*, 15, 759—766.

2. Dietrich, CH. F., Braden, B., 2009: Sonographic assessments of gastrointestinal and biliary functions. *Best Practice and Research Clinical Gastroenterology*, 23, 353—367.

3. Mitchell, C. F., Malone, E. D., Sage, A. M., Niksich, K., 2005: Evaluation of gastrointestinal activity patterns in healthy horses using B mode and Doppler ultrasonography. *Canadian Veterinary Journal*, 46, 134—140.

4. Bunsoni, V., De Busscher, V., Lopez, D., Verwilghen, D., Cassart, D., 2007: Preliminary evaluation of a protocol for fast localized abdominal sonography in horses (flash) admitted for colic. In *Proceedings of the Annual Meeting of the Association Vétérinaire Equine Francaise, IVIS, Deauville*, 354 pp.

5. Reef, V. B., 1998: *Equine Diagnostic Ultrasound*. 1st edn., Saunders, Philadelphia. 560 pp.

6. Gimondo, P., Mirk, P., 1997: A new method for evaluating small intestinal motility using duplex Doppler sonography. *American Journal of Roentgenology*, 168, 187—192.

7. Koenig, J., Cote, N., 2006: Equine gastrointestinal motility — ileus and pharmacological modification. *Canadian Veterinary Journal*, 47, 551—559.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section II, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 98—100, 2014

INFLUENCE OF OBESITY ON THE OESTRAL CYCLE OF MARES AND ON METABOLIC PARAMETERS OF HORSES

Izsáková, E.[1], Pošivák, J.[1], Novotný, F.[1], Valocký I.[1], Pošiváková, T.[2]

[1]Equine clinic, University of Veterinary Medicine and Pharmacy Komenského73, 041 81, Košice
[2]Department of Ecology, Faculty of Humanitarian and Natural Sciences University of Prešov in Prešov, 17thNovember, 081 16 Prešov The Slovak Republic

eduska181@azet.sk

ABSTRACT

The correct feeding of animals has become an increasingly important public issue. Appropriate animal nutrition, and especially the prevention of dietary problems and related diseases are inevitable aspects of a modern veterinary practice. The aim of this study was to investigate the influence of obesity on the oestral cycle of mares and on selected metabolic parameters in horses in general. The mean insulin level in the plasma of obese horses was considerably higher compared to non-obese horses (14.343 ± 0.843 mmol.dl^{-1} and 6.700 ± 0.600 mmol.dl^{-1}, resp.). On the contrary, the mean serum glucose level was decreased in obese horses in comparison with the non-obese animals (84.229 ± 0.471 mg.dl^{-1} and 93.857 ± 1.257 mg.dl^{-1}, resp.). The mean serum triglycerides level was also higher in obese horses in comparison with non-obese horses (33.40 ± 0.70 mg.dl^{-1}and 26.471 ± 0.971 mg.dl^{-1}, resp.). Clinical and ultrasonographic examinations also showed changes in the reproductive tract of mares and in their cyclicity. Both follicular and luteal phases were prolonged in mares which showed insulin resistance and obesity. Oestrus intervals were considerably longer in obese mares and reached 35.5 days in comparison with 24 days in non-obese mares. The dioestrus period lasted 32 days in obese mares compared to 15 days in non-obese animals. The difference within breeds was less pronounced but indicated potential breed predisposition.

Key words: insulin; mare; obesity; reproduction; stallion; triglycerides

INTRODUCTION

Obesity in animals, such as the horse, has become a popular topic of concern lately. First, it is necessary to analyse various factors contributing to obesity and then consider the relevant therapy and, preferentially, its prevention. Obesity is a pathological state involving many factors, especially food and performance. Unsuitable feeding regimens, the composition of the rations, insufficient exercise, and working load, all result in the development of obesity.

A natural predisposition to obesity has been observed in primitive horse breeds and most ponies. It is a well known fact that ponies and some horse breeds, such as Morgans, Paso Finos and Fjord horses, are affected most frequently [1]. Today, a high proportion of „modern" horses are used only for recreational purposes and although their load is nil or very low, they are still provided high energy feed. These horses are kept as hobby horses. It is not only the locomotor system that suffers in obese horses, but also affected are cardiovascular, thermoregulative and hormonal systems, and eventually also reproductive processes in horses. Current studies indicate that obesity in horses is related, not only to intake of high-energy rations based on concentrate feed, but also to excess intake of bulk and green feed and hay. The principal sources of energy for horses are carbohydrates and fat. A high proportion of obese horses suffer from the so-called equine metabolic syndrome related to insulin resistance. Insulin resistance is characterised by decreased ability of insulin to stimulate the passage of glucose into tissues sensitive to insulin, such as muscle and adipose tissue [2]. One of the reasons that justify prevention and treatment of obesity and insulin

Table 1. Level of insulin in blood plasma and of glucose and triglycerides in the blood serum of obese and non-obese horses

Horse	Insulin [mmol.dl^{-1}]		Glucose [mg. dl^{-1}]		Triglycerides [mg. dl^{-1}]	
	Obese	Non-obese	Obese	Non-obese	Obese	Non-obese
1	13.5	7.3	84.7	92.8	32.8	27.1
2	14.5	7.2	83.9	94.5	33.5	26.6
3	14.3	6.8	84.4	93.4	34.1	25.5
4	14.8	6.3	84.3	94.2	33.6	27.2
5	14.6	7.0	84.2	92.6	32.9	26.7
6	14.5	6.1	84.1	94.5	33.9	26.0
7	14.2	6.2	84.0	95.0	33.0	26.2
Mean	**14.343 ± 0.843**	**6.700 ± 0.60**	**84.229 ± 0.471**	**93.857 ± 1.257**	**33.400 ± 0.70**	**26.471 ± 0.971**

Table 2. Oestrus interval in obese and non-obese mares

	Horse	Days		Horse	Days
Obese	1	33	**Non-obese**	1	24
	2	35		2	23
	3	38		3	25
	4	36			
	Mean	**35.5**		**Mean**	**24**

Table 3. Dioestrus length in obese and non-obese mares

	Horse	Days		Horse	Days
Obese	1	30	**Non-obese**	1.	14
	2	33		2.	14
	3	34		3.	17
	4	32			
	Mean	**32.2**		**Mean**	**15**

resistance is that obesity and insulin resistance have been associated with increased risk of laminitis, the most frequent disease of horses [4].

The aim of this study was to investigate the influence of obesity on the oestral cycle of mares and on selected metabolic parameters in horses.

MATERIALS AND METHODS

Our investigations included 14 horses, 7 of them mares, 8 of the Nonius breed and 6 Hutsuls. They were between 8—16 years old. The horses were allocated to two groups, one group comprised obese horses and one served as control (non-obese horses). Of the 7 mares, 4 were obese. The body weight was assessed according to a body condition score (BCS) scale. This system assesses subcutaneous fat deposition in six specific areas of the body [3]. Animals with BCS ≥ 6—8 are considered obese and those with BCS 4—6 are non-obese. Obese horses had ad libitum access to pasture and were fed concentrate feed 1—2 times per day. They were kept only for recreational riding and had access to pasture throughout the year. Non-obese horses were fed balanced rations, were given hay twice per day and a total of 2 kg of concentrate feed per day. They had regular exercise and training and had access to a pasture for maximally 2 hours per day.

Blood (5 ml sample) was withdrawn from v*ena jugularis* in the morning hours. Plasma was obtained from the blood collected in Vacutainer tubes with anticoagulant heparin. Serum was obtained from blood collected in tubes without anticoagulant. Immediately after sampling the blood, it was centrifuged at 1800—2000 r.p.m. for 15 min. For the determination of glucose, the blood must be centrifuged within one hour to prevent a decrease in the glucose level and obtaining inaccurate results. Plasma and serum were stored in micro-centrifugation tubes (Eppendorf) at –20 °C. The glucose and triglyceride levels were determined by an immunochemical analyser COBAS e 411, and insulin by the ELISA method (set EIA 2340, Roner).

The diagnosis of ovulation and observation of changes caused by obesity was carried out in the field, at the owners of horses, using an ultrasonographic machine Aloka SSD — 500 V, Tokyo Mure Hitaka — SH Co, LTD, with linear probe UST — 588 — U of frequency 5 MHz, and another USG apparatus SonoScape, Model C352 CurvedArray.

RESULTS AND DISCUSSION

All results obtained in our study were processed and are presented in Tables 1—3. Table 1 shows the changes in obese horses in comparison with non-obese controls. Tables 2 and 3 compare cyclicity in both investigated groups.

Insulin was increased significantly in obese horses compared to non-obese ones. Due to defective insulin receptors, glucose did not enter the cells and accumulated instead in the extracellular space, which induced an increased insulin response of the pancreas. The insufficient energy supply of cells due to lack of glucose caused their dysfunction. The accumulated glucose was converted subsequently to triglycerides in the liver. These processes resulted in an increased level of triglycerides and decreased levels of glucose in the blood of the affected horses. Reproduction was also affected by obesity. The oestrus intervals were considerably increased in obese mares compared to non-obese animals (mean intervals 35.5 days and 24 days, resp.). Also, dioestrus, which lasts under normal conditions15 days on average, was doubled in the obese mares to a mean of 32.2 days.

CONCLUSIONS

Our results indicate that adequate nutrition, feeding regimen, exercise and management of horses are essential for the correct functioning of the reproductive apparatus of mares. The results allow us to conclude that the prevention of obesity contributes toward a strategy against the development of other diseases, such as insulin resistance, hyperinsulinaemia and laminitis. Veterinarians are an essential factor in this strategy, as they are able to explain to the farmers the importance of the relevant preventive measures and give them advice on optimum management of their animals.

ACKNOWLEDGEMENTS

The present study was supported by the project VEGA No. 1/0498/12.

REFERENCES

1. Bamford, N. J., Potter, S. J., Harris, P. A., Bailey, S. R., 2013: Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body conditions core. *Domestic Anima lEndocrinology*, 47, 101—107.

2. Frank, N., 2009: Equine metabolic syndrome. *Journal of Equine Veterinary Science*, 29, 259—267.

3. Kienzle, E., Schramme, S., 2004: Body condition scoring and prediction of bodyweight in adult warm-blooded horses. *Pferdeheilkunde*, 20, 517—524.

4. Lacombe, V. A., 2011: Glucose metabolism in insulin-sensitive tissue: from health to disease and glucose transport in adipose tissue: novel insights into the pathogenesis of insulin resistance. *Journal of Equine Veterinary Science*, 10, 578—581.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section II, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 101—103, 2014

IMPACT OF ELECTROMAGNETIC RADIATION ON THE TESTES OF RATS

Berík, M., Almášiová, V.

Department of Anatomy, Histology and Physiology
University of Veterinary Medicine and Pharmacy, Košice
The Slovak Republic

milan.berik@student.uvlf.sk

ABSTRACT

This study investigated the potential pathological changes in the structure of the testes of 48-day old male Wistar albino rats. Forty rats were divided into two groups, control (n = 20) and experimental (n = 20). The experimental rats were exposed to electromagnetic waves of frequency 2.45 GHz and density of 2.8 mW. cm^{-2}, for 3 hours per day during 3 weeks. After sacrificing the rats 3 hours after their last irradiation, the testes were removed and processed histologically. The examination by light microscopy of semi-thin sections stained with toluidine blue revealed signs of diffuse degenerative changes. Our results demonstrated a high sensitivity of the testes to electromagnetic radiation (EMR). This is important, in view of the potential health risks associated with the everyday use of devices emitting electromagnetic radiation, such as cell phones or microwave ovens.

Key words: electromagnetic radiation; rats; testes

INTRODUCTION

Presently we are exposed almost everywhere to apparatuses emitting electromagnetic radiation. The issue of the potential harmfulness of electromagnetic waves has become the subject of interest of many studies on a worldwide scale, including scientific institutions such as the World Health Organisation (WHO) and the International Commission on Non-Ionizing Radiation Protection (ICNIRP). Many of these studies have shown negative impact of these waves on human health [1]. The scientific and technical developments have produced many devices that have become inevitable for our life. Cell phones, radars or microwave ovens are sources of non-ionizing radiation of frequencies between 30 kHz and 300 GHz. Microwave ovens operate at wavelengths between 1 mm and 1 m which corresponds to frequencies ranging from 300 MHz up to 300 GHz. At these frequencies, molecules in solids and liquids start to vibrate and subsequently thermal effects are produced [2].

An important factor for the action of electromagnetic radiation (EMR) is the permeability of tissues which is almost identical with the permeability of free space and because of that, magnetic losses are almost negligible [3]. According to dielectric properties of biological tissues, they can be divided into two groups: tissues with high water content, such as skin and muscles, and tissues with low water content, such as bones. In addition to the thermal effect, EMR shows also non-thermal effects; the mechanisms of which have not yet been fully explained. It is conditional on electric properties of tissues and involves changes in action potential of cell membranes [4].

Our research focused on the action of EMR on testes, the organ responsible for spermatogenesis. Their normal function is dependent upon the optimum temperature of testicular tissue, therefore we assumed that besides the direct non-thermal effect of EMR, also the thermal effect may play an important negative role.

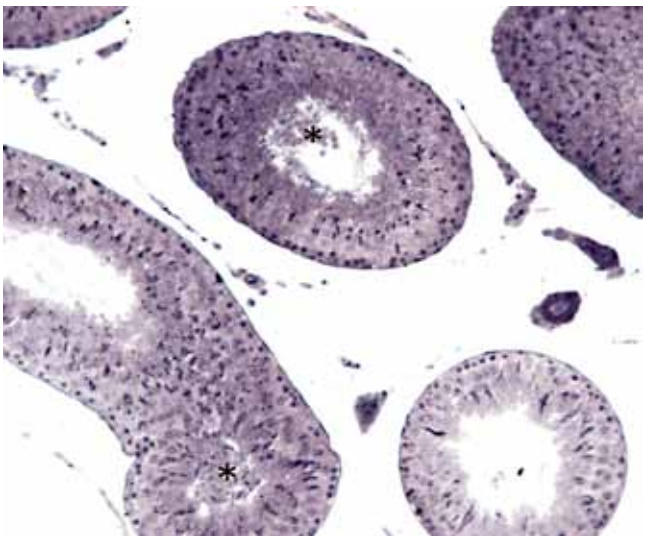


Fig. 1. Seminiferous tubules of irregular shape after 3-week exposure to EMR; semi-thin section, toluidine blue. Magn. ×100
* — desquamated cells of seminiferous epithelium

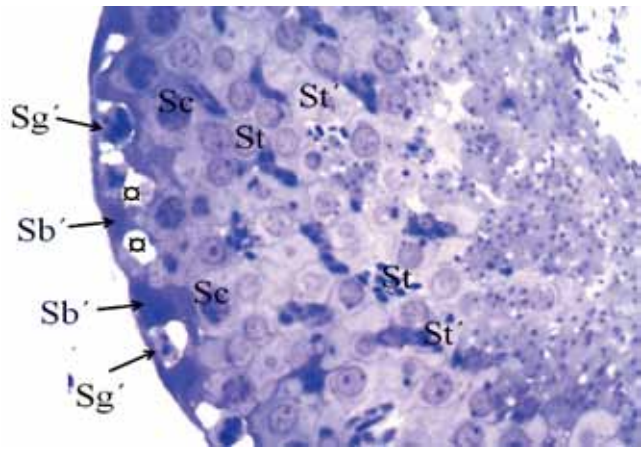


Fig. 2. Seminiferous epithelium with degenerative changes following 3-week exposure to EMR; semi-thin section, toluidine blue. Magn. × 400
Sb´ — damaged Sertoli cells; Sg´ — damaged spermatogonia; Sc — spermatocytes; St — round (early) spermatids and elongated (late) spermatids; St´ — damaged spermatids

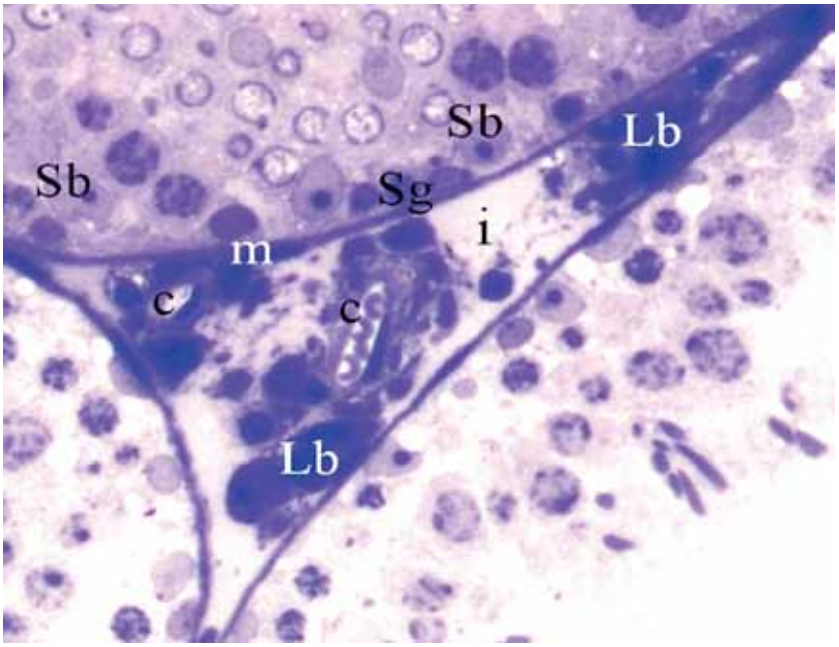


Fig. 3. Interstitial space and parts of seminiferous tubules after 3-week exposure to EMR; semi-thin section, toluidine blue, Magn. × 400
Lb — Leydig cells; m — myoid cells; c — blood vessels; i — interstitium; Sb — normal Sertoli cells; Sg — normal spermatogonia

MATERIALS AND METHODS

Our study was carried out on 40 sexually mature 48-day old male Wistar albino rats divided to two groups, control and experimental, with 20 rats in group. They were kept in cages at an optimum ambient temperature (21 °C ± 1 °C), 12-hour light regimen and had *ad libitum* access to water and feed. Experimental rats were exposed daily for 3 hours to electromagnetic waves of frequency 2.45 GHz and density 2.8 mW.cm^{-2} for a period of 3 weeks. The uniformity of EMR was checked by a spectral analyser. Three hours after the last irradiation, the control and experimental rats were euthanized i. p. by administration of xylazine in combination with

ketamine. Subsequently, perfusion with 4 % paraformaldehyde solution in a phosphate buffer (0.1 M, pH 7.3) was performed. Then 1 mm^3 samples of testes were obtained by excision, fixed with 3 % glutaraldehyde and 1 % OsO_4, dehydrated in acetone, cleared in propylene oxide and embedded in Durcupan ACM (Fluka). Semithin sections were prepared using an ultramicrotome LKB, stained with toluidine blue, observed under a light microscope Zeiss Axio LAB A1 and photographed by Axio Cam ERc 5.

RESULTS AND DISCUSSION

Observations of the testicular parenchyma from experimental animals by light microscopy showed diffuse degenerative changes. The shapes of seminiferous tubules (*tubuli seminiferi*) were irregular and the seminiferous epithelium contained many vacant spaces (Fig. 1 and 2). Desquamated cells were observed in the lumen of the tubules (Fig. 1). Testicular germ cells showed degenerative changes of varying intensity. Besides relatively preserved spermatogonia (Fig. 3), also necrotising spermatogonia with dark shrivelled cytoplasm and changes in the nucleus could be observed in some tubular sections (Fig. 2). No marked morphological changes were observed in spermatocytes. Besides normal spermatids, there were also spermatids with altered shape and slightly vacuolated cytoplasm. The structure of the late elongated spermatids was preserved but their number was reduced in some of the tubules. Vacant spaces in the seminiferous epithelium developed due to desquamation of immature germ cells (Fig. 2). In addition to normal Sertoli cells (Fig. 3), we observed also considerably shrivelled necrotic Sertoli cells in some of the tubule sections (Fig. 2). The histological structure of Leydig and myoid cells and interstitial vessels was preserved (Fig. 3). We ascribe the changes observed in the testes of irradiated rats to the thermal and non-thermal effects of EMR on testicular tissue of these animals

CONCLUSION

Our results demonstrate that whole body irradiation of rats with electromagnetic waves of frequency 2.45 GHz and density 2.8 $mW.cm^{-2}$ (for 3 hours daily during 3 weeks) caused diffuse degenerative changes in their testes. The changes involved the shape of the seminiferous tubules and vacant spaces in seminiferous epithelium caused by desquamation of germ cells in various stages which resulted in the accumulation of immature cells in the lumen of the tubules. Degeneration of spermatogonia was observed in many places. Spermatocytes and spermatids were damaged to a smaller extent. The number of elongated spermatids was reduced. No morphological damage to the interstitium and its components was observed. The changes described were probably caused by thermal and non-thermal effects of electromagnetic radiation.

ACKNOWLEDGEMENT

My thanks belong to the members of staff of the Slovak Academy of Sciences for their collaboration in irradiation of rats and to my tutor MVDr. Viera Almášiová, PhD. for professional help in carrying the experiments and evaluation of results.

REFERENCES

1. Khurana, V. G., Teo, C., Kundi, M., 2009: Cell phones and brain tumours: a review including the long-term epidemiologic data. *Surg. Neurol.,* 72, 205—215.

2. Hyland, G. J., 2000: Physics and biology of mobile telephony. *The Lancet,* 356, 1—8.

3. Cleveland, R. F. S., David, M. U., Jerrz, L., 1997: Evaluating compliance with FCC guidelines for human exposure to radiofrequency electromagnetic fields, *OST Bull.,* 65, 97—101.

4. Ferreri, F., Curcio, G., Pasqualetti, P., De Gennaro, L., Fini, R., Rossini, P. M., 2006: Mobile phone emissions and human brain excitability. *Ann. Neurol.* 60, 188—196.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section III, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 104—107, 2014

THE INFLUENCE OF CADMIUM ON THE STRUCTURE OF THE SMALL INTESTINE IN TURKEYS

Fagová, Z., Almášiová, V.

Department of Anatomy, Histology and Physiology
University of Veterinary Medicine and Pharmacy, Košice
The Slovak Republic

viera.almasiova@uvlf.sk

ABSTRACT

In this study we investigated and described the histological structure and morphological changes in the jejunal tissue of turkeys which were administered cadmium per os at a dose of 5 mg Cd.day^{-1}.head^{-1} for 71 days. The examination by light and transmission electron microscopy showed a negative influence of Cd on the small intestinal mucosa. We observed necrosis of enterocytes; they had shrivelled nuclei, dark cytoplasm and considerably damaged brush border. The goblets cells showed no signs of damage. Observation by transmission electron microscopy showed that the necrotizing enterocytes had markedly electron dense cytoplasm, which contained disintegrated mitochondria and cisternae of the endoplasmic reticulum; their nuclear membrane was undulated. The microvilli of the cells were damaged. Intercellular spaces among enterocytes were extended and the tight intercellular junctions were markedly damaged. The morphological changes observed in our study indicated that the chronic peroral intake of cadmium had a negative effect on the structure of the enterocytes. It loosened the tight intercellular junctions between epithelial cells, penetrated to the cells, impaired their biological membranes and caused their necrosis and exfoliation.

Key words: cadmium; jejunum; structure; turkeys; ultrastructure

INTRODUCTION

Most of the heavy metal, cadmium, enters the body via food. The mouth cavity and stomach play only a small role in its absorption [5]. The principal site of its absorption is the small intestine, particularly the duodenum [4], [13[. The amount of absorbed cadmium and its impact on animal bodies depends upon several factors, especially on the method of its intake, dose, exposure time and other feed components [11], [13], [14].

One of the mechanisms responsible for Cd intestinal absorption involves divalent metal transporter 1 (DMT1) present in the cytoplasmic membrane. It is a protein capable of transporting various divalent metals, especially Fe, but also other metals, including Cd. Its expression increases at Fe depletion. It occurs particularly in the proximal duodenum and its occurrence decreases in a caudal direction [11]. Another potential mechanism of transfer of Cd from the small intestinal lumen into the enterocytes, involves transporters of zinc [13], [14].

The gradual accumulation of Cd in cells results in various types of interactions. It causes, for example, the induction of the transcription of genes encoding metallothionein, a low-molecular intracellular protein with high content of cystein and high affinity for ions of divalent metals. Some cadmium ions bind to this molecule, but when the intake of Cd exceeds the capacity of protective mechanisms, the cell is subsequently exposed to oxidation stress, peroxidation of plasma membrane lipids and other changes in cellular metabolism. This can eventually lead to apoptosis or necrosis of these cells. Another factor leading to the increased Cd absorp-

tion, involves impairment of the intercellular junctions between individual enterocytes *(zonula occludens, zonula adherens, macula adherens).* These junctions affect permeability and normal transport of substances across the epithelium [2], [3], [10].

Through the blood circulation, Cd can penetrate to various organs of the live intact bodies [6], [7]. A number of studies have shown the ability of Cd to accumulate in the body, particularly in the liver, kidneys [8] and reproductive organs [9].

The aim of our study was to investigate and describe changes in the jejunal gross structure and ultrastructure in turkeys after individual long-term (71 days) administration of cadmium at a dose of 5 mg $Cd.day^{-1}.head^{-1}$, by light microscopy (semi-thin sections prepared from material processed for electron microscopy) and transmission electron microscopy.

MATERIALS AND METHODS

Experimental design and animal handling

The experiments were carried out on 48 turkey birds (35 days old) BIG-6, with a mean body weight of 1.66 kg. After 30 days of acclimatization, they were divided into two groups of 24 birds each, with equal number of males and females. Birds in the first (experimental) group were administered *per os* water solution of cadmium chloride ($CdCl_2$) individually, at a dose of 5 mg $Cd.day^{-1}.head^{-1}$, for 71 days. The second group served as a control and received no cadmium. The birds were fed *ad libitum*, initially with mixed feed HYD 14, from 9th week of age with HYD 15, and 2 weeks before the end of the experiments with HYD 16. They had unlimited access to water.

The experiments were approved by the local ethical commission and the State Veterinary and Food Agency (ŠVPS SR Č. k. Ro-7879/04-220/3). After the experiments, they were euthanized with ether, decapitated and samples were taken from the jejunum for light and electron microscopic examinations.

The excised samples for light microscopy (LM) and transmission electron microscopy (TEM) were fixed by immersion in 3 % glutaraldehyde for 3 hours and subsequently postfixed in 1 % OsO_4 in phosphate buffer (pH 7.4), dehydrated in acetone, cleared in propylene oxide and embedded in Durcupan ACM (Fluka).

The semi-thin sections for LM were stained with toluidine blue and observed with an Axiolab A1 light microscope. The ultrathin sections for TEM were contrasted with uranyl acetate and lead citrate and examined with a transmission electron microscope Tesla BS 500.

RESULTS

Light microscopic observations

The small intestinal epithelium of the control turkeys consisted of intestinal cells, enterocytes, the surface of which was covered with a continuous brush border with sporadic interspersed goblet cells (Fig. 1). Intraepithelial lymphocytes were observed among the epithelial cells.

The epithelium of the small intestine of experimental turkeys showed signs of marked morphological damage. In particular, the apical portion of the intestinal villi contained necrotizing enterocytes, usually forming groups of 5 or more cells (Fig. 2). They had dark coloured cytoplasm and a nucleus of irregular shape. The brush border on the apical surface of cells was discontinuous or completely absent in some places. Some intestinal villi lacked epithelium completely as the necrotic epithelial cells had sloughed off and the thin connective tissue, *lamina propria mucosae,* was exposed and contained inflammatory infiltrates. The structure of the goblet cells showed no significant damage.

Electron microscopic observations

The normal submicroscopic structures were observed in the individual intestinal epithelial cells of the control birds (Fig. 3). The enterocytes appeared as long slender cells with numerous regular microvilli on their free surface. They had a regular oval nucleus, located mostly close to the basal membrane and a conspicuous nucleolus of reticular type. The cytoplasm contained numerous oval mitochondria, tubules of rough and smooth endoplasmic reticulum, free ribosomes and lysosomes. There were tight intercellular junctions between the enterocytes. The individual goblet cells were interspersed among enterocytes; they had typically widened upper portion, filled with mucinogenous granules, and their lower portion, which contained the nucleus and sporadic organelles, rested on a continuous basal membrane.

The presence of necrotising enterocytes were observed in the experimental birds (Fig. 4). They were as necrotised individuals or in groups. They had electron dense cytoplasm, shrivelled nuclei of irregular shape with condensed chromatin, and contained damaged disintegrating cellular organelles and vacuoles of varying sizes. The intercellular junctions were impaired and widened intercellular spaces were frequently observed among the enterocytes. Numerous lymphocytes were seen in the epithelium and *lamina propria* of the mucosa.

DISCUSSION AND CONCLUSIONS

Cadmium enters the body mostly via food and its ions are absorbed through the gastrointestinal mocosa [5]. It has been recognised that the small intestine is the main site of the passage of cadmium into the blood circulation. Cells of the small intestinal epithelium and tight intercellular junctions between enterocytes are the main barrier against the passage of cadmium into the blood [2], [3], [10]. Niewenhuis et al. [10] and Prozialeck [12] ascribed the impairment of intercellular junctions, mainly to the interaction of cadmium with E-cadherin molecules, the main component of intercellular junctions responsible for interconnections of actin filaments of the cellular cytoskeleton. The impairment and subsequent disintegration of these junctions results in complete separation of the epithelial cells and resulted in the increased permeability of the intestinal epithelium. This conclusion was confirmed also in our study. We observed damage to tight intercellular junctions of the type of *zonula adhaerens* and *zonula occludens* between the enterocytes. The loosening of these intercellular junctions resulted in the

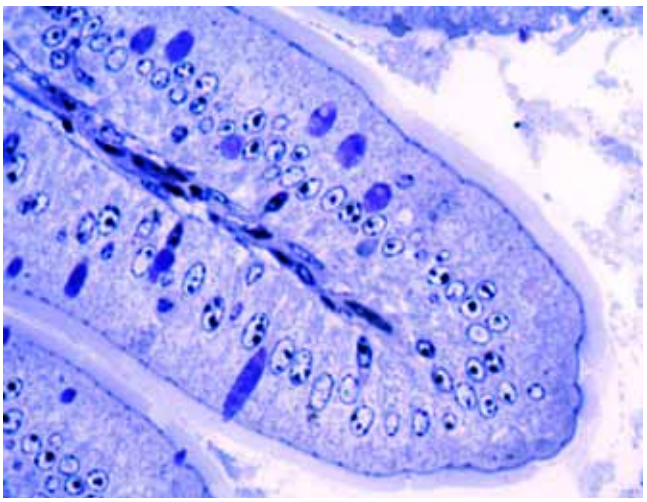


Fig. 1. Portion of intestinal villus — control; semi-thin section stained with toluidine blue. Magn. × 100

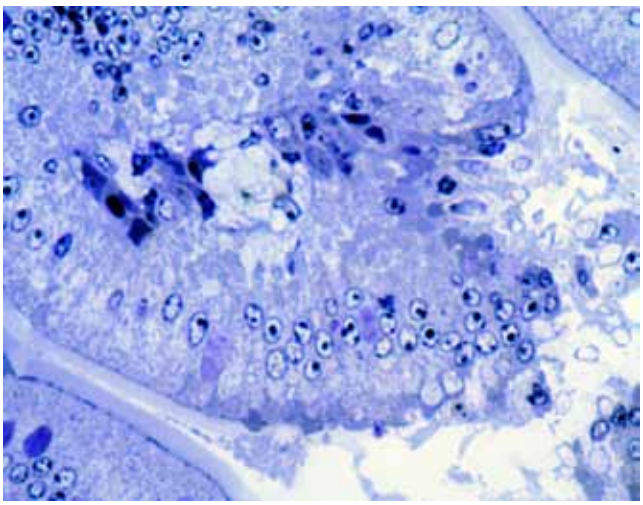


Fig. 2. Portion of intestinal villi with significantly damaged even absent epithelium — experimental bird; semi-thin section stained with toluidine blue. Magn. × 100.

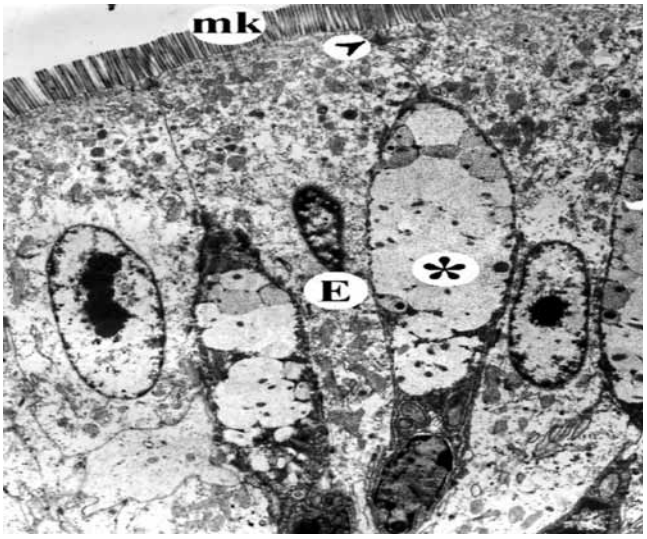


Fig. 3. Jejunal epithelium — control (electron micrograph). E — enterocyte; mk — microvilli; arrow — intercellular junction; asterisk — goblet cell. Magn. × 4 000

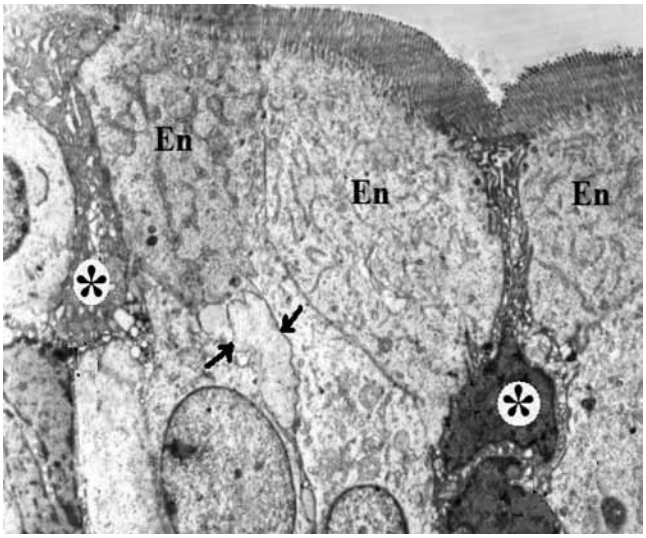


Fig. 4. Jejunal epithelium — experimental bird (electron micrograph). En — enterocytes; asterisks — necrotising enterocytes; arrows — widened space between enterocytes. Magn.× 4 000.

passage of cadmium into the enterocytes and subsequent damage to individual cell organelles and the gradual necrotization and exfoliation. Similar morphological changes in the intestinal epithelium after long-term administration of cadmium have been observed in cocks [1], Japanese quails [2] and crabs [15]. Contrary to the significant changes in the enterocytes, the structure of the goblet cells and blood and lymph capillaries were maintained.

REFERENCES

1. Berzina, N., Markovs, J., Isajevs, S., Apsite, M., Smirnova, G., 2007: Cadmium-induced enteropathy in domestic cocks: A biochemical and histological study after subchronic exposure. *Basic and Clinical Pharmacology and Toxicology,* 101, 29—34.

2. Cigánková, V., Almášiová, V., Holovská, K., 2010: Morphological changes in Japanese quail duodenal epithelium after chronic cadmium exposure. *Polish Journal of Environmental Studies,* 19, 275—282.

3. Duizer, E., Gilde, A. J., Versantvoort, C. H. M., Groten, J. P., 1999: Effects of cadmium chloride on the paracellular barrier function of intestinal epithelial cell lines. *Toxicol. Apl. Pharmacol.,* 155, 117–126.

4. Elsenhans, B., Strugala, G. J., Schafer, S. G., 1997: Small intestinal absorption of cadmium and the significance of mucosal mettalothionein. *Hum. Exp. Toxicol. 16*, 429.

5. Ewers, U., Schlipköter, H. W., 1991: Chronic toxicity of metals and metal compounds. In **Merian, E.:** *Metals and Their Compounds in the Environment.* Weinheim, VCH, 591–603.

6. Giari, L., Manera, M., Simoni, E., Dufuli, B. S., 2007: Cellular alterations in different organs of European sea bass *Dicenttratchus labrax* (L.) exposed to cadmium. *Chemosphere,* 67, 1171–1181.

7. Kim, M., Park, K., Park, J., Park, J., Kwak, I. S., 2013: Heavy metal contamination and metallothionein mRNA in blood and feathers of Black-tailed gulls (*Larus crassiristris*) from South Korea. *Environmental Monitoring and Assessment,* 185, 2221–2230.

8. Kramarova, M., Massanyi, P., Slamecka, J., Tataruch, F., Jancova, A., Gasparik, J., et al., 2005: Distribution of cadmium and lead in liver and kidney of some wild animals in Slovakia. *J. Environ. Sci. Health*, Part A, 40, 593–600.

9. Massanyi, P., Toman, R., Valent, M., Cupka, P., 1995: Evaluation of selected parametres of metabolic profile and levels of cadmium in reproductive organs of rabbits after an experimental administration. *Acta Physiol. Hung.,* 267–273.

10. Niewenhuis, R. J., Dimitriu, C., Prozialeck, W. C., 1997: Ultrastructural characterization of the early changes in intercellular junctions in response to cadmium (Cd^{2+}) exposure in LLC-PK_1 cells. *Toxicol. Appl. Pharmacol.,* 142, 1–12.

11. Park, J. D., Cherrington, N. J., Klaasen, C. D., 2002: Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. *Toxicol. Sci.,* 68, 288.

12. Prozialeck, W. C., 2000: Evidence That E-Cadherin May Be a Target for Cadmium Toxicity in Epithelial Cells. *Toxicol. Appl. Pharmacol.,* 164, 231–249.

13. Reeves, P. G., Chaney, R. L., Simmons, R. W., Cherian, M. G., 2005: Metallothionein induction is not involved in cadmium accumulation in the duodenum of mice and rats fed diets containing high-cadmium rice or sunflower kernels and marginal supply of zinc, iron, and calcium. *J. Nutr.,* 135, 99–108.

14. Smith, M. V., Shenoy, K. B., Debnam, E. S., Dashwood, M. R., Churchill, L. J., Srai, S. K., 2002: Divalent metal inhibition of non-haem iron uptake across the rat duodenal brush border membrane. *Br. J. Nutr.,* 88, 284.

15. Wu, H., Xuan, R. J., Li, Y. J., Zhang, X. M., Jing, W. X., Wang, L., 2014: Biochemical, histological and ultrastructural alterations of the alimentary system in the freshwater crab *Sinopotamon henanense* chronically exposed to cadmium. *Ecotoxicol.,* 23, 65–75.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section III, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 108—112, 2014

THE IMPACT OF PRODUCTION CHANGES IN THE PLANT KOVOHUTY IN KROMPACHY ON THE LEVEL OF SELECTED HEAVY METALS IN THE BLOOD SERA OF EWES ON SLATVINA FARM IN THE KROMPACHY AREA OF SLOVAKIA

Kurimská, E., Húska, M.

**Clinic for pigs, University of Veterinary Medicine and Pharmacy,
Komenského 73, 041 81 Košice
The Slovak Republic**

ekurimska@gmail.com

ABSTRACT

The aim of this study was to assess the influence of the production changes in the plant Kovohuty, JSC, in Krompachy, on the level of selected heavy metals (Cd, Pb, Zn, Cu) in the blood sera of ewes on Slatvina farm in the Krompachy area of Slovakia. The plant recovers copper from secondary scrap materials and restores them into the production cycle. The company is constantly upgrading the technologies used, to maximize the effectiveness of the input material recovery and minimize the impacts on the environment. In 2013, we examined the blood of sheep from Slatvina farm and also in control farms in Kišovce and Žakovce, and compared our results with those obtained by investigations carried out in 1983/1984 and 2005/2006 on Slatvina farm and a control farm in Vikartovce. The control farms were located in the sub-Tatra area which was considered an area with low environmental contamination. No copper poisoning of the sheep resulted from the copper accumulation in their bodies (as detected by our examination in 2013), although the level of copper in the sheep blood sera on Slatvina farm was slightly increased compared to the normal physiological range. Lead has becomes the main subject of concern, not only on the Slatvina farm, but also on the control farms as well.

Key words: cadmium; copper, heavy metals; lead; sheep; zinc

INTRODUCTION

The Spiš region of Slovakia has been known for its mineral deposits. For centuries, ores were mined and processed in a number of places in this area. In 1804 an ironworks with high furnace and refining smithery was built in the town Krompachy. The mining and processing activities, particularly emissions from the processing plants, have affected the environment and their impact on plants, animal and human health has persisted up to this day [1]. The awareness of these impacts enforced some improvements, for example, the installation of new modern filtration facilities for cleaning off-gases and dust separation.

The company, Kovohuty, JSC, placed in the town of Krompachy, was established in the year 2000, building on a long-lasting metallurgy tradition in this region. The main mission of the company is to effectively recover the copper from the secondary scrap materials with their restoration to the production cycle. Kovohuty, JSC, is constantly upgrading the technologies used in accordance with fulfilment of the vision, to minimize the adverse impact on the environment and maximize the effectiveness of the input material recovery. Modernization of the shaft furnace in 2005, lowered the copper losses and the undesirable environmental impacts. In 2010, the production of copper wire by the company increased by 71 %.

Due to geographic conditions, the animal most frequently kept in this area is sheep. Sheep health was unfortunately affected by grazing on contaminated grass at the foot of the surrounding hills [3].

The aim of this study was to assess changes in the impact of the emissions of selected heavy metals on the sheep on Slatvina farm, located close to the Kovohuty plant, compared to the situation in the previous years of 1983/1984 and 2005/2006.

MATERIALS AND METHODS

In the autumn of 2013, we investigated the levels of Cd, Pb, Cu, and Zn, and some parameters of the internal environment in the blood sera of 10 sheep of the improved Valachian breed, 2 to 3 years old, from Slatvina farm, located at a distance of about 8 km from the copper producing plant (Kovohuty a. s. in Krompachy). Control samples were obtained from control sheep of the same breed from Kišovce and Žakovce farms, located at the foot of the High Tatras mountains.

The blood for laboratory examination was collected from the *vena jugularis*. The blood samples were processed in the laboratories of the Clinic for ruminants and pigs of the University of Veterinary Medicine and Pharmacy in Košice. This paper deals only with the levels of selected heavy metals.

The serum was obtained by centrifugation. The Cu and Zn were determined by flame atomic absorption spectrometry using AAnalyst 100 (Perkin Elmer) and Pb and Cd by emission spectrometer Optima 2100DV (Perkin Elmer).

RESULTS

The results of the examination of selected heavy metals in the blood sera of sheep from Slatvina farm and control farms in Kišovce and Žakovce are presented in Tables 1 to 4, together with the results obtained during the previous investigations in the years of 1983/1984 and 2005/2006, when sheep from a farm in Vikartovce was used as the control.

The level of lead (0.577 μmol.l^{-1}) in 2013 was about 10-fold higher compared to the level measured in 1983/1984 (0.052 μmol.l^{-1}) and almost 20-fold higher compared to the level of lead in 2006/2006 (0.038 μmol.l^{-1}). The level measured in 2013 was 0.577 μmol.l^{-1}. Although the contamination of the control farms in Žakovce and Kišovce were considered relatively low, the serum levels of lead in all of the control sheep on these farms exceeded the higher standard limit.

The highest mean level of cadmium in the sheep blood was recorded in 1983/1984 (0.380 μmol.l^{-1}), and the lowest in 2005/2006 (0.107 μmol.l^{-1}). In 2013 the mean cadmium level reached 0.210 μmol.l^{-1} and exceeded the upper standard limit of 0.172 μmol.l^{-1}).

The level of Zn in sheep sera was below the lower physiological limit in all sheep from Slatvina examined in 1983/1984 and in 2005/2006, while in 2013 they were in the physiological range in most of the examined sheep. Most of the control sheep examined in 2005/2006 showed a deficit of zinc. The level of zinc in the control sheep in 2013 was slightly higher, but the variations were considerable and the mean level of zinc was below the lower physiological limits.

The mean blood level of copper was the highest in 1983/1984, when it reached 17.79 μmol.l^{-1}. It decreased by 2005/2006 to 10.62 μmol.l^{-1} and reached the mean level of 12.40 μmol.l^{-1} in 2013.

Overall, the results from the years of 2005/2006 and 2013, were better in comparison with those obtained in 1983/1984. The biggest differences were observed in the levels of Cd and Pb.

DISCUSSION

It has been reported that trace elements are of equal importance as hormones and vitamins. Various systems are affected by a deficiency or excess of trace elements, such as zinc, copper and iron, or by the negative effects of elements that contribute to metal pollution poisoning, such as lead and cadmium. The presence of excessive levels of Pb and Cd negatively affects primarily the homeopoietic system, but also the urogenital system, the gastrointestinal system and the neurologic system, as well as the absorption of other trace elements [4].

Cadmium is a soft, white, lustrous metal. In nature it occurs, together with zinc, at ratios of 1 : 100 or 1 : 1000. Chronic intoxication of animals with cadmium presents clinically as digestive problems, body wasting, and anaemia. Cadmium accumulates slowly in the body, particularly in the kidneys, liver, lungs and hair. Its concentration increases progressively. Even small amount of cadmium can act as a tissue poison. It binds to thiol (-SH) groups of cysteine residues in proteins, inhibiting the activity of enzymes containing this particular group. It also inhibits the activity of enzymes which easily bind zinc. Cadmium affects the production of insulin and thus interferes with the metabolism of sugars. Prolonged exposure to cadmium frequently results in increased levels of proteins in the urine. The yellow discoloration of teeth has been reported in cadmium-affected individuals. The blood level of Cd in 2013 reached 0.210 μmol.l^{-1} and was almost doubled in comparison with 2005/2006. The mean blood level of cadmium in the control sheep in 2013 was higher (0.226 μmol.l^{-1}) than on the Slatvina farm.

Lead is a gray-blue, shiny metal. It is not a noble metal. Lead reduces the life span of red blood cells which is gradually reflected in changes in the blood picture The symptoms of chronic lead poisoning are ambiguous and frequently involve equilibrium disturbances. Lead affects the; digestive system, kidneys and endocrine glands. An increased level of heavy metals in the body can exacerbate the course of primary diseases and weaken the body. The mean level of Pb measured in 1983/1984 was 0.052 μmol.l^{-1}, while in 2005/2006, the Pb level was decreased to 0.038 μmol.l^{-1}. Measurements in 2013 showed a 10-fold increase on Slatvina farm (0.577 μmol.l^{-1}) and more than 25-fold increase on farms in Žakovce and Kišovce (1.305 μmol.l^{-1}) compared to the level in 1984 in the Krompachy area.

Zinc deficiency manifests as: worsened eyesight; difficult healing of wounds; retarded growth; skin diseases; loss of wool; claw and hoof disorders; impaired reproductive abilities; delayed maturation; susceptibility to infections; atherosclerosis; impaired blood circulation; fatigue; and inappe-

Table 1. Concentration of Pb [µmol.l^{-1}] in the sera of sheep from contaminated areas (Slatvina; n = 10) and control sheep (n = 10)

Standard range Pb 0.025—0.88 µmol.l^{-1}		Slatvina	Control
Years 1983/1984	min—max	0.034—0.09	
	x	0.052	
	± SD	0.0016	
Years 2005/2006	min—max	0.015—0.072	0.027—0.088
	x	0.03-8	0.062
	± SD	0.017	0.017
Autumn 2013	min—max	0.449—0.921	0.93—1.61
	x	0.577	1.305
	± SD	0.14	0.244

x — mean level; SD — standard deviation

Table 2. Concentration of Cd [µmol.l^{-1}] in the sera of sheep from contaminated areas (Slatvina; n = 10) and control sheep (n = 10)

Standard range Cd 0.067—0.172 µmol.l^{-1}		Slatvina	Control
Years 1983/1984	min—max	0.17—0.72	
	x	0.380	
	± SD	0.211	
Years 2005/2006	min—max	0.067—.15	0.055—0.148
	x	0.107	0.094
	± SD	0.031	0.024
Autumn 2013	min—max	0.088—0.306	0.13—0.302
	x	0.21	0.226
	± SD	0.069	0.052

x — mean level; SD — standard deviation

Table 3. Concentration of Zn [µmol.l^{-1}] in the sera of sheep from contaminated areas (Slatvina; n = 10) and control sheep (n = 10)

Physiological range Zn 12.5—24.0 µmol.l^{-1}		Slatvina	Control
Years 1983/1984	min—max	4.17—12.2	
	x	9.12	
	± SD	2.59	
Years 2005/2006	min—max	7.64—10.7	9.17—15.29
	x	8.94	11.72
	± SD	1.023	1.406
Autumn 2013	min—max	11.3—15.95	8.66—18.34
	x	13.292	11.82
	± SD	1.46	2.53

x — mean level; SD — standard deviation

Table 4. Concentration of Cu [µmol.l^{-1}] in the sera of sheep from contaminated areas (Slatvina; n = 10) and control sheep (n = 10)

Physiological range Cu 9.00—12.0 µmol.l^{-1}		Slatvina	Control
Years 1983/1984	min—max	7.08—24.4	
	x	17.79	
	± SD	5.89	
Years 2005/2006	min—max	9.44—11.8	9.44—15.74
	x	10.62	11.64
	± SD	0.848	1.79
Autumn 2013	min—max	10.18—14.81	10.18—15.74
	x	12.40	12.542
	± SD	1.256	1.478

x — mean level; SD — standard deviation

tence. The levels of zinc on Slatvina farm determined in 2013 were within the physiological range in most of the examined sheep, while the mean level of zinc in the control sheep was below the lower physiological limit. All sheep from Slatvina farm examined in 1983/1984 and 2005/2006 showed a deficit of zinc.

Copper is necessary for the production of myelin; it supports the action of oxido-reductases. All copper compounds are toxic. More serious cases of intoxication manifest as haemolysis and subsequent jaundice, impairment of kidneys, spleen and liver damage. Copper accumulates mostly in the liver. The external symptoms include dark red rings at dental necks and greenish discolouration of hair and wool after prolonged exposures. In 2005/2006, the copper levels were within the physiological range (9—12 $\mu mol.l^{-1}$), while in 2013, a slight increase was recorded as the mean copper levels reached 12.4 $\mu mol.l^{-1}$ on Slatvina farm and 12.54 $\mu moll^{-1}$ on the farms in Žakovce and Kišovce. The highest levels of copper were recorded in 1984, when signs of poisoning due to the accumulation of copper were observed.

The monitoring carried out in 2013 showed that changes in production in the plant Kovohuty in Krompachy and the installation of filters considerably decreased the environmental pollution in the affected region. However, with regard to environmental pollution, one should consider the fact that pollutants transmitted by air recognise no borders, which may explain the high level of Pb in the blood sera of sheep on the control farms in 2013.

CONCLUSIONS

Copper poisoning was observed on sheep farms in the Krompachy area 30 years ago. No signs of copper poisoning resulting from copper accumulation in sheep were observed after the changes in Kovohuty a. s., which evidently reduced the environmental pollution. Although the level of copper in the sheep blood sera on Slatvina farm was slightly increased, lead has become presently the main subject of concern not only on Slatvina farm, but also on relatively uncontaminated control farms in Žakovce and Kišovce. Our results indicate the need for regular examination of sheep's blood, to detect the presence of potential contaminants that may affect the health of animals, but also for monitoring environmental pollution and looking for its potential sources.

ACKNOWLEDGEMENTS

The study was supported by the projects VEGA 1/0537/12 and 1/0467/14, and KEGA 007 UVLF-4/2012.

REFERENCES

1. Dietzová, Z., Labancová, J., 2007: The influence of emissions from TKO incineration plant (KOSIT, as Košice) on the quality of air, water and health of inhabitants of the adjacent villages (In Slovak). In *Proceedings of the XVIth Scientific Symposium on Ecology in Selected Agglomerations of Jelšava-Lubeník and Central Spiš*, Grafotlač Prešov, 50 pp.

2. Húska, M., Bíreš, J., Dudríková, E., Michaeli, E., 2006: The influence of experimental intoxication with zinc and copper caused by industrial emissions in the Central Spiš region on the health of sheep (In Slovak). *Folia Geographica*, 45, 194—198.

3. Kováč, G. et al., 2001: *Diseases of Cattle* (In Slovak). M & M Prešov, 874 pp.

4. Or, M. E., Kayar, A., Kiziler, A. R., Parkan, Ç., Gönül, B. Barutçu, B., Dodurka, H. -T., 2005: Determination of levels of some essential (iron, copper, zinc) and toxic (lead, cadmium) metals in the blood of sheep and in samples of water, plants and soil in Northwest Turkey. *Veterinarski Arhiv*, 75, 359—368.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section III, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 113—115, 2014

EVALUATION OF PHYSICO-CHEMICAL CHANGES DURING MATURATION OF THE MEAT OF THE COMMON PHEASANT

Januška, M., Koréneková, B., Brenesselová, M., Sopoliga, I.*, Mačanga J.

Department of Food Hygiene and Technology, Institute of Meat Hygiene and Technology
***Specialised establishment for rearing and diseases of game, fish and bees in Rozhanovce**
University of Veterinary Medicine and Pharmacy, Košice
The Slovak Republic

januska.martin@gmail.com

ABSTRACT

The aim of this study was to observe the maturation process of the meat of the common pheasant (*Phasianus colchicus*). In the first experiment, we observed changes in the pheasant meat from hunted down birds (n = 6) and in the second one we focused on the influence of transportation (5 hours, 22 °C) the on maturation processes of the pheasants (n = 6) before their slaughter. Breast and thigh muscles collected from pheasants were stored in a refrigerator (4 °C) for 24 hours after slaughter/killing. In the shot pheasants, the level of phosphates and lactic acid increased in both breast and thigh muscles up to day 7 of the experiments. The pH of the breast muscles showed a decreasing trend up to day 7, in comparison with day 14, while in the thigh muscles, the pH increased up to day 14. In the slaughtered and bled birds, the lactic acid continued to decrease throughout the experiment in both the breast and thigh muscles. The level of phosphates decreased up to day 7 and then increased in both muscle types. The pH level increased up to day 14 in breast and thigh muscles.

Key words: common pheasant; lactic acid; maturation of meat; pH

INTRODUCTION

The common pheasant *(Phasianus colchicus)* is the most geographical widespread bird in the pheasant family (*Phasianidae*). Pheasants are propagated by gamekeepers to satisfy hunting interests, but also they are farmed intensively or extensively [4]. Pheasant meat: has a unique taste and aroma; is highly nutritious; contains high level of vitamins and proteins; and its digestibility is high due to its low fat content [2]. After killing, the pH of pheasant meat is neutral or mildly alkaline. *Rigor mortis* occurs after several hours and the muscles change to meat which, however, lacks suitable culinary properties. During maturation, the enzymatic anaerobic processes convert glycogen to lactic acid. The meat becomes tender and acquires flavour and aromatic substances. The maturation period depends on the storage temperature, season and the handling after killing/slaughter [8]. The aim of the maturation process is to obtain tender, hygienically safe and savoury meat with characteristic taste and aromas [1].

The aim of this study was to observe the levels of lactic acid, phosphates and pH, during the maturation of pheasant meat (breast and thigh muscles) originating from shot pheasants and pheasants slaughtered after 5 hours of transportation.

MATERIALS AND METHODS

The first experiment was carried out on 6 common pheasants *(Phasianus colchicus)*, 8—9 months old, shot on the hunting area of Makovica. We collected samples of breast and thigh muscles from shot pheasants and stored them in a refrigerator at 4 °C and then analysed on days 1, 7, and 14 after shooting.

The second experiment, focused on the influence of transportation stress before slaughter, 6 common pheasants, 9—10 months

Table 1. Mean levels (g.100g^{-1} muscle) of lactic acid and phosphates in breast and thigh muscles of pheasants during 14-day maturation process

Parameter	Muscle type		Day 1		Day 7		Day 14	
			Shot	Slaughtered	Shot	Slaughtered	Shot	Slaughtered
Phosphates	Thigh	X	0.639	0.795	0.818	0.645	0.577***	0.787
		SD	0.098	0.132	0.040	0.076	0.057	0.096
	Breast	X	0.77	1.025	0.818^{+}	0.653	0.611*$^{++}$	1.041**$^{+++}$
		SD	0.094	0.113	0.052	0.045	0.07	0.253
Lactic acid	Thigh	X	0.704	1.227	1.001	0.703	0.992	0.454
		SD	0.187	0.73	0.17	0.324	0.356	0.226
	Breast	X	1.826	2.395	1.965	1.542***	1.489	0.760**$^{+++}$
		SD	0.376	0.209	0.258	0.359	0.161	0.369

Statistical significance compared to day1 level: * — P ≤ 0.05; ** — P ≤ 0.01; *** — P ≤ 0.001
Statistical significance compared to day 7 level: $^{+}$ — P ≤ 0.05; $^{++}$ — P ≤ 0.01
X — mean; SD — standard deviation

Table 2. The levels of pH in breast and thigh muscles of pheasants during 14-day maturation process

Muscle type		Day 1		Day 7		Day14	
		Shot	Slaughtered	Shot	Slaughtered	Shot	Slaughtered
Breast	X	6.158	5.972	5.396***	6.182***	5.706***$^{+++}$	6.932***$^{+++}$
	SD	0.27	0.161	0.126	0.297	0.179	0.323
Thigh	X	5.402	6.98	6.176***	7.092***	6.784***$^{+++}$	7.348*
	SD	0.249	0.076	0.188	0.173	0.23	0.131

Statistical significance compared to day 1 level: * — P ≤ 0.05; *** — P ≤ 0.001
Statistical significance compared to day 7 level: $^{+++}$ — P ≤ 0.01
X — mean; SD — standard deviation

old, were transported before slaughter for 5 hours at 22 °C in boxes of dimensions 50 × 30 × 30 cm. After slaughter, the pheasants were bled and samples of the breast and thigh muscles collected from them were handled in the same way as those from the shot birds.

The samples of the breast and thigh muscles were analysed by an electrophoretic apparatus EA 102 (Villa Labeco s. r. o., SR) equipped with a conductivity detector. The leading electrolyte was 10 mM HCL, β-alanine and 0.1 % mHEC, and the terminating electrolyte was 5 mM capronic acid and 5 mM TRIS. The electrophoretogram was evaluated by software ITPPpro 32. The level of the pH was determined by a pH meter InoLab WTW 720.

Our results were evaluated statistically by means of Microsoft Excel 2013 software, using Student *t*-test and a correlation coefficient.

RESULTS

The shot pheasants showed an increase in the level of lactic acid in the breast muscles on day 7 compared to day 1 of the experiment (Table 1). The highest level of lactic acid (2.333 g.100 g^{-1}) was measured on day 7. In the thigh muscles

lactic acid reached the highest level also on day 7 of the experiment. The level of phosphates in both types of muscles showed similar dynamics. A significantly higher level of phosphates ($P \leq 0.05$) in the breast muscles was observed on days 7 and 14 compared to day 1 (Table 1). In the thigh muscles, the level of phosphates was decreased significantly ($P \leq 0.001$) on day 14 compared to day 1.

The slaughtered pheasants showed significantly decreased levels of lactic acid in breast muscles ($P \leq 0.001$ and $P \leq 0.01$) on days 7 and 14 of the experiment, respectively, compared to day 1 (Table 1). Similar dynamics of lactic acid was observed in the thigh muscles in these pheasants with the lowest levels on day 14 of maturation, but the difference compared to day 1 was insignificant. The highest level of lactic acid in the thigh muscles was measured on day 1 of the experiment (2.518 g.100 g^{-1}).The dynamics of phosphate levels in both muscle types was similar. In the thigh muscles, phosphates decreased insignificantly by day 7 and then increased by day 14, but were still lower in comparison with day 1. The highest level of phosphates in the thigh muscles was measured on day 1 (0.949). In the breast muscles, the lowest levels of phosphates were measured on day 7 of the experiment. They increased again significantly ($P \leq 0.01$) by day 14.

The level of the pH in the breast muscles of the shot pheasants decreased significantly ($P \leq 0.001$) by day 7 of the experiment compared to day 1 and then increased by day 14, when they reached a level that differed significantly ($P \leq 0.001$) from day 1. The correlation coefficient was 0.93. The thigh muscle pH levels increased by day 7 to 6.176 and by day 14 to 6. 784. Both levels differed significantly ($P \leq 0.001$) from the pH measured on day 1 (Table 2).

The level of the pH in the breast muscles of the slaughtered pheasants was significantly higher ($P \leq 0.001$) on days 7 and 14 compared to day 1. The highest pH was measured on day 14 (7.310). The thigh muscle pH increased significantly during maturation ($P \leq 0.001$ and $P \leq 0.05$ on days 7 and 14, resp.), in comparison with day 1. The highest pH was measured on day 14 (7.510).

DISCUSSION

The level of the pH in the thigh muscles in the shot pheasants, increased during the maturation period. Our results agree with those of Kottferová [5]. In the breast muscles, the pH decreased by day 7 of maturation in comparison with day 1. On day 14 we observed an additional increase in the pH in comparison with day 7. The lactic acid level showed a decrease by day 14. This agreed with the results of Mačanga et al. [6]. The lactic acid level was ascribed to the gradual decomposition of the depot glycogen. This can be explained by the short action of stress factors at hunting [3]. The level of phosphates in these birds increased up to day 7 of maturation and then decreased by day 14 in both muscle types. This is in agreement with the results of Mačanga et al. [6].

In the slaughtered pheasants, the changes in phosphate levels showed a similar trend in both breast and thigh muscles. We observed a decrease in the phosphate level by day 7 followed by an increase observed on day 14. The pH levels also showed a similar course in both muscle types. Our results agreed with those obtained by Mačanga et al. [7]. The levels of lactic acid decreased from day 1 to day 14 in both muscle types, probably due to previous transport stress.

CONCLUSION

Our results indicate that situations inducing stress before death of the pheasants affected the quality of their meat. The intravital factors, such as transport or increased physical activity of birds before they were shot, affected glycogen stores in their muscles. Our results also indicate that the negative effect of transport-induced stress on the parameters investigated in our study was higher than the hunting-induced stress.

REFERENCES

1. Haščík, P., Bobko, M., Weiss, J., Čuboň, J., 2012: *Processing of Poultry and Minor Animal Products* (In Slovak). 1st edn., SPU Nitra, 157 pp.

2. Haščík, P., Kulíšek, V., Kačániová, M., Čuboň, J., Vavrišinová, K., 2010: *Meat Yield and Quality of Meat of Selected Species of Small Feather Game* (In Slovak). SPU Nitra, 187 pp.

3. Henckel, P., Karlsson, A., Jensen, M.T., Oksbjerg, N., Petersen, J.S., 2002: Metabolic condition in porcine longissimus muscle immediately pre-slaughter and its influence on peri- and post-mortem energy metabolism. *Meat Science*, 62, 145—155.

4. Komárek, V., Kočiš, J., Bartoš, L., Cimbal, D., Hell, P., Lazar, P., et al., 1991: *Biological Basis of Game* (In Slovak). 1st edn., Príroda Bratislava, 200 pp.

5. Kottferová, L., 2011: *Evaluation of Post-mortem Changes in Wild Duck Muscles in Relation to Duck Meat Quality* (In Slovak). Diploma thesis, UVMP in Košice, 66 pp.

6. Mačanga, J., Koréneková, B., Nagy, J., 2009a: Development of ripening process of hunted, eviscerated pheasants (*Phasianus colchicus*), *Folia Veterinaria*, 53, 144—145.

7. Mačanga, J., Koréneková, B., Nagy, J., 2009b: Physicochemical changes in meat of the common pheasant during maturation (In Slovak). In *Scientific Papers of PhD. Students*, UVMP in Košice, 59—62.

8. Nagy, J. et al., 2009: *Hygiene of Game* (In Slovak). 1st edn., UVMP in Košice, 121 pp.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section III, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 116—118, 2014

SUPERFICIAL MICROBIAL CONTAMINATION OF BROILER CHICKEN CARCASSES AFTER THEIR SLAUGHTER

Hučková, M., Pipová, M., Jevinová, P.

University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

monika.pipova@uvlf.sk

ABSTRACT

Four series of swabs from the skin of broiler chicken carcasses were taken at a poultry slaughterhouse from June to September, 2012. Six chickens were tested on each sampling date. Swabs were taken from the same carcasses before and after chilling. Forty eight swabs from the surface area of 100 cm² were inspected for the total plate count (TPC), the count of *Enterobacteriaceae* (EBC) and of staphylococci. Twenty-four skin samples from the neck of broilers were inspected for the presence of *Salmonella* species. The comparison of results before and after chilling showed that the combined chilling caused an increase in both TPC ($P < 0.05$) and EBC ($P > 0.05$). On the other hand, the counts of staphylococci (including those coagulase-positive) were reduced after chilling. However, this decrease was insignificant ($P > 0.05$). The presence of *Salmonella* spp. was determined in one out of 24 samples inspected.

Keywords: chilling; *Enterobacteriaceae*; poultry; *Salmonella*; staphylococci

INTRODUCTION

Currently, the production of slaughtered poultry is one of the fastest growing segments of animal husbandry. The consumption of poultry meat is generally accepted in most cultures. The meat of broilers is healthy and cheap. Thus, its consumption increases year by year. On the other hand, raw poultry carcasses are excellent substrates for the growth of various micro-organisms, including food pathogens, among them, *Salmonella* spp. and *Campylobacter* spp. being the most important [10]. Therefore, measures must be taken to reduce the counts of undesirable micro-organisms during the whole process of slaughtering, evisceration and chilling of broiler chickens. Chilling of poultry is essential to ensure food safety [1]. As required by the Regulation (EC) No. 853/2004 [3], poultry carcasses must be chilled to a maximum temperature of 4 °C as soon as possible after the slaughter.

MATERIALS AND METHODS

Swabs from chicken carcasses were taken four times within three months (from June to September, 2012); six broilers being tested during each sampling. A destructive sampling method was used for the detection of *Salmonella* spp., while samples for the determination of TPC, EBC and the count of staphylococci were taken with the help of a non-destructive sampling method [8]. Swabs from each carcass were taken before as well as immediately after its combined chilling. The microbiological examinations were performed in accordance with the current European standards [4], [5], [6], [7].

Table 1. Total plate counts (TPCs), the counts of *Enterobacteriaceae* (EBCs), staphylococci (STAs) and coagulase-positive staphylococci (CPSs) in swabs taken from chicken carcasses before (A) and after chilling (B)

Sample No.	TPCs [CFU.100 cm^{-2}]		EBCs [CFU.100 cm^{-2}]		STAs [CFU.100 cm^{-2}]		CPSs [CFU.100 cm^{-2}]	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
1	8.0×10^3	1.9×10^4	7.2×10^2	6.0×10^2	2.5×10^3	3.6×10^3	0	0
2	7.0×10^3	2.3×10^4	1.3×10^3	1.3×10^3	6.0×10^3	5.4×10^3	1.2×10^3	1.1×10^3
3	1.0×10^4	2.5×10^4	6.9×10^2	1.1×10^3	1.3×10^4	6.2×10^3	2.6×10^3	1.2×10^3
4	1.5×10^4	2.0×10^4	1.3×10^3	1.9×10^3	2.4×10^4	3.8×10^3	4.8×10^3	0
5	1.8×10^4	1.2×10^4	6.2×10^3	7.0×10^2	8.8×10^3	4.4×10^3	1.8×10^3	0
6	6.0×10^3	1.9×10^4	8.7×10^2	5.2×10^2	5.6×10^3	2.6×10^3	1.1×10^3	0
7	6.4×10^3	2.4×10^4	1.1×10^3	2.5×10^3	7.6×10^3	6.3×10^3	1.5×10^3	1.3×10^3
8	1.9×10^3	3.6×10^3	1.4×10^2	3.6×10^2	1.6×10^3	3.5×10^3	0	0
9	3.7×10^3	1.7×10^4	3.0×10^2	4.4×10^3	3.8×10^3	5.7×10^3	0	1.1×10^3
10	2.8×10^3	9.2×10^3	4.1×10^2	4.0×10^2	2.6×10^3	1.1×10^3	0	0
11	5.5×10^3	9.7×10^3	1.6×10^3	1.9×10^3	3.6×10^3	5.3×10^3	0	1.0×10^3
12	1.9×10^3	2.5×10^4	2.4×10^2	5.8×10^2	3.4×10^3	2.8×10^3	0	0
13	5.4×10^3	1.6×10^4	1.6×10^3	7.7×10^3	1.2×10^4	4.3×10^3	2.4×10^3	8.6×10^2
14	2.6×10^4	1.1×10^5	3.6×10^3	2.0×10^4	1.1×10^4	4.2×10^3	2.2×10^3	8.4×10^2
15	5.3×10^3	1.5×10^4	5.2×10^3	6.6×10^3	6.8×10^3	1.4×10^4	1.4×10^3	2.8×10^3
16	1.7×10^4	3.0×10^4	7.5×10^3	7.2×10^3	1.4×10^4	6.4×10^3	2.8×10^3	1.3×10^3
17	1.8×10^4	2.1×10^4	1.3×10^4	2.6×10^4	2.0×10^4	5.6×10^3	4.0×10^3	1.1×10^3
18	3.2×10^3	2.0×10^4	2.8×10^3	6.0×10^3	1.0×10^4	1.0×10^4	2.0×10^3	2.0×10^3
19	2.3×10^3	1.6×10^4	5.3×10^2	1.3×10^3	4.5×10^3	3.8×10^3	9.0×10^2	0
20	2.5×10^3	1.5×10^4	1.0×10^3	1.8×10^3	3.3×10^3	5.4×10^3	0	1.1×10^3
21	1.3×10^3	2.9×10^4	7.0×10^2	3.6×10^3	5.5×10^3	7.8×10^3	1.1×10^4	1.6×10^3
22	1.3×10^3	2.8×10^4	1.4×10^3	1.6×10^3	3.1×10^3	5.8×10^3	0	1.2×10^3
23	2.3×10^3	1.5×10^4	8.9×10^2	1.4×10^3	6.0×10^3	1.1×10^4	1.2×10^3	2.2×10^3
24	2.9×10^3	2.5×10^4	1.5×10^3	2.0×10^3	6.8×10^3	1.3×10^3	1.4×10^3	0
Mean	**7.2×10^3**	**2.3×10^4**	**2.3×10^3**	**3.5×10^3**	**7.7×10^3**	**5.4×10^3**	**1.8×10^3**	**8.6×10^2**

RESULTS AND DISCUSSION

The results of the quantitative microbiological examinations before (A) and after chilling (B) are shown in Table 1. As seen in the table, the combined chilling resulted in a significant increase in TPC ($P < 0.05$) and the insignificant increase in EBC ($P > 0.05$). On the contrary, the counts of staphylococci (including those coagulase-positive) were reduced after chilling. However, this reduction was insignificant ($P > 0.05$). The presence of *Salmonella* spp. was confirmed in one out of the 24 samples inspected. In accordance with the Commission Regulation (EC) No. 1441/2007 [2], this finding was evaluated as acceptable.

If the chilling of poultry carcasses is performed properly, microbial populations on the skin should be reduced. As reported, water immersion chilling usually causes cross-contamination and water absorption. These problems can be avoided by the air chilling of poultry. However, the surface of air chilled carcasses frequently shows higher microbial loads due to the absence of the „washing effect". Moreover, this method does not eliminate cross-contamination, as the bacteria are able to circulate in aerosols [10]. Combined chilling of poultry has been developed in order to take advantage of both water and air chilling methods. However, spraying of aerosol into cold air has led to a significant increase in counts of bacteria, especially pseudomonades [9]. As follows from the results of our study, the method of combined chilling was less effective as expected, because it resulted in the increase of both TPC and EBC on the surface of broiler carcasses.

REFERENCES

1. Carroll, C. D., Alvarado, C. Z., 2008: Comparison of air and immersion chilling on meat quality and shelf life of marinated broiler breast fillets. *Poultry Sci.*, 87, 368—372.

2. Commission Regulation (EC) No. 1441/2007 of 5th December 2007 amending Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, L 322, Dec. 7, 2007, 12—29.

3. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29th April 2004, laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union*, *L139, April 30, 2004, 55—205.*

4. STN EN ISO 4833, 2004: *Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Enumeration of Microorganisms. Colony-count Technique at 30 °C* (In Slovak), SÚTN Bratislava, SR.

5. STN EN ISO 6579/AC, 2006: *Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Detection of Salmonella spp.* (In Slovak), SÚTN Bratislava, SR.

6. STN EN ISO 6888-1/A1, 2004: *Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Enumeration of Coagulase-positive Staphylococci (Staphylococcus aureus* and other species). Part 1: *Technique Using Baird-Parker Agar Medium.* Amendment 1: *Inclusion of Precision Data* (In Slovak), SÚTN Bratislava, SR.

7. STN EN ISO 21528-2, 2007: *Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Detection and Enumeration of Enterobacteriaceae.* Part 2: *Colony-count Method* (In Slovak), SÚTN Bratislava, SR.

8. STN ISO 17604, 2007: *Microbiology of Food and Animal Feeding Stuffs. Carcass Sampling for Microbiological Analysis* (In Slovak), SÚTN Bratislava. SR.

9. Tuncer, B., Sireli, U. T., 2008: Microbial growth on broiler carcasses stored at different temperatures after air- or water-chilling. *Poultry Sci.*, 87, 793—799.

10. Wojtón, B., Rózycki, M., 2006: Bacterial poultry carcasses contamination. In *Proceedings Hygiena Alimentorum XXVII*, Štrbské pleso, the High Tatras, May 18—20, 33—37.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section III, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 119—121, 2014

THE USE OF PREMI® TEST IN SCREENING OF COCCIDIOST AT RESIDUES IN POULTRY MEAT

Pániková, A., Kožárová, I., Nagy, J.

University of Veterinary Medicine and Pharmacy in Košice
Komenského 73, 041 81 Košice
The Slovak Republic

andreapanikova@gmail.com

ABSTRACT

The aim of our study was to determine the presence of coccidiostat residues in the tissues and blood sera of broiler chicks during the legislation-specified withdrawal period by means of an official Premi® Test method. Broilers of slaughter weight were obtained from a farm registered in Slovakia and for the duration of study were housed in accredited premises of the Clinic for birds, exotic and free-living animals at the University of Veterinary Medicine and Pharmacy (UVMP) in Košice. During the first two days they were fed commercial feed BR3 containing sodium salinomycinate, using the same feeding regimen as that on the farm of their origin and on the following 3 days they were fed a mixed feed BR4 without coccidiostats. Our screening showed that some samples were positive for coccidiostat residues even after the elapse of the 1-day withdrawal period specified for salinomycin.

Key words: coccidiostats; Premi® Test; residues; screening

INTRODUCTION

Coccidiosis is a parasitic disease caused by protozoa from the family *Eimeriidae.* They are strictly host-, age- and topically-specific unicellular organisms. The infection spreads rapidly among individuals by the oral-faecal route. Because of considerable economic losses, preventive measures are taken in poultry flocks to prevent its spreading.

One of the preventive measures is the administration of coccidiostats in the mixed feed provided to poultry. Currently, there are 11 cocciodiostats approved for poultry [5]. Because cocciodiostats are veterinary medicines, they are subject to: the requirements set by the Act of the National Council of the Slovak Republic No. 39/2007 Coll. on veterinary care, as amended [4]; and obligatory monitoring of their residues according to the Government Decree of the Slovak Republic No. 320/2003 Coll. on the monitoring of certain substances and their residues in live animals and in animal products [2].

Premi® Test is a broad-spectrum microbiological method officially approved for primary screening of residues [3]. This vial method serves for the determination of residues of inhibitory substances in meat juices (muscle, kidney, liver), fish, eggs and urine of animals treated with antibiotics. It combines the principle of agar diffusion tests with colour changes of indicators caused by the active metabolism of the test micro-organism in the absence of inhibitors. The test sample is delivered into vials filled with agar nutrient medium containing the test strain *Bacillus stearothermophilus* var. *calidolactis*. The normal growth of the test strain during the incubation of the vials causes a pH indicator to change its colour from blue-violet to yellow. Inhibition of the growth of the test strain prevents the colour change. Due to the sensitivity of the test strain to a broad spectrum of antimicrobials (β-lactams, cephalosporins, macrolides, tetracyclins, sulphonamides, aminoglycosides), this test is ideal for the rapid screening of residues, as it can detect residues at or below the maximum residual limits (MRL) set for individual substances by Commission Regulation (EU) No. 37/2010, as amended [1].

Premi® Test declares that it has sensitivity to some ionophore coccidiostats. Due to the antibiotic character of all ionophore coccidiostats, we focused on the determination of the presence of salinomycin in the tissues of broiler chickens during and after the specified 1-day withdrawal period.

MATERIALS AND METHODS

Experimental animals: broiler chickens (COBB 500) were fed a mixed feed BR3 containing 70 mg sodium salinomycinate per kg of complete feed (De Heus a. s., Czech Republic and mixed feed BR4 free of coccidiostat (BarbaraSp. Z. o. o., Poland).

Microbiological method: Premi®Test (R-26, 2013), purchased from R-Biopharm AG (Germany).

Preparation of samples: samples (thigh/breast muscles, liver, stomach, heart, skin/fat, kidney, serum) were obtained from 3 broilers killed by bleeding during the withdrawal period. Blood serum was obtained by centrifugation of the blood after bleeding. The samples were wrapped, marked and stored at –18 °C until analysis. Matrices negative for residues were used as the control. Before examination, the samples were defrosted in a microwave oven (3 min at Defrost).

Examination procedure: 100 µl aliquots of tissue fluid and serum were transferred to test vials that were covered with a foil. The samples of the organs were inactivated by exposing them to increased temperatures (80 °C) for 10 min. Vials were incubated in a thermoblock at 64 ± 0.5 °C for 3 hours.

Evaluation of results: after incubation, we evaluated the colour of the lower two thirds of the agar. The yellow colour of the solid medium indicated the absence of antibiotics, while the violet colour indicated their presence. Yellow/violet colour indicated the presence of antibiotics in an amount that corresponded to the level of the test of detectability.

RESULTS AND DISCUSSION

The results of the determination of salinomycin residues in the tissues and sera of broiler chicks by the Premi® Test are presented in Table 1.

Table 1 show that on day 0 (the last day of feeding BR3), four samples were positive and 3 were dubious. On day 1 of the withdrawal period, none of the samples were unambiguously positive. However, six of the tested samples provided dubious result. This means that the levels of salinomycin detected by the Premi®Testom were close to the detection limit of the method. On day 2 (day after the official 1-day withdrawal period) the dubious results were obtained for 3 (liver, kidney and serum) of the 8 tested samples.

CONCLUSIONS

The detection sensitivity of the Premi®Test to salinomycin declared by the test producer is 1000 µg.kg^{-1}. The MRL in the respective animal matrices is 5 µg salinomycin per kg of any tissue (original dry matter (5)). Our results allowed us to state that the 1-day withdrawal period set for salinomycin is insufficient due to the potential presence of residues exceeding the levels acceptable according to legislative provisions.

ACKNOWLEDGEMENTS

This study was supported by VEGA Grant No. 1/0939/12.

REFERENCES

1. Comission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union,* L 15, 1—76.

2. Government Decree of the Slovak Republic No. 320/2003 Coll. on the monitoring of certain substances and their residues in live animals and in animal products. *Collection of laws*, No. 320/2003, 145, 2541—2549.

3. R-26: Determination of residues of inhibitory substances in meat by Premi® Test method. Official method of laboratory diagnosis. Available on internet: //www.svps.sk/dokumenty/zakladne_info/Diagnostika/R%2026%20PREMi%20test.pdf

Table 1. Results of the determination of salinomycin residues by the Premi® Test

Day of withdrawal period	Thigh muscles	Breast muscles	Kidney	Liver	Skin and fat	Stomach	Heart	Serum
0	±	+	±	+	+	–	+	±
1	±	±	±	±	±	–	–	±
2	–	–	±	±	–	–	–	±
Control	–	–	–	–	–	–	–	

+ — positive result; ± — dubious result; – — negative result

4. Act of the National Council of the Slovak Republic No. 39/2007 Coll. on veterinary care. *Collection of Laws*, No. 39/2007, 28, 162—208.

5. List of feed supplements, 2013: Category 5: Coccidiostats and antihistaminics. *Bulletin of the Ministry of Agriculture and Rural Development of SR*, 23, 316—319.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section IV, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014-

FOLIA VETERINARIA, 58, 2: 122—126, 2014

THE INFLUENCE OF PROBIOTIC LACTOBACILLI AND FLAXSEED ON THE IMMUNE RESPONSE OF WEANED PIGLETS EXPERIMENTALLY INFECTED WITH ENTEROTOXIGENIC *E. COLI*

Kubašová, I., Mudroňová, D.

Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy
Komenskeho 73, 041 81, Košice
The Slovak Republic

dagmar.mudronova@uvlf.sk

ABSTRACT

The weaning period of piglets is associated with nutritional, psychical, environmental and immunological stress which has a negative effect on their health and the growth of these animals and frequently results in the so-called post-weaning syndrome. This study investigated the influence of probiotic lactobacilli in the form of cheese and flaxseed as a source of omega-3 polyunsaturated fatty acids (PUFA) on the clinical state and immunological responses of weaned piglets experimentally infected with enterotoxigenic *E. coli*. The experiments were carried out on 48 clinically healthy piglets allocated into two groups, i.e., control (C) and experimental (LFA). The piglets from the LFA group were offered probiotic lactobacilli-containing cheese and were fed rations containing 10% crushed flaxseed starting 10 days before weaning, up to 14 days post-weaning. On day 28 of age (day of weaning), animals in both groups were infected with a single dose of enterotoxigenic *E. coli* and their clinical state was checked daily. The influence of the feed additives on the immune response of piglets was evaluated by the determination of their phagocytic activity and the percentages of the proportion of lymphocyte subpopulations in the peripheral blood and jejunal Peyer's patches (CD3+, CD21+, CD4+, CD8+, CD4+CD8+). We recorded a positive influence of the combination of administered immunomodulators on the clinical state of the piglets in terms of the occurrence and duration of diarrhoea and the faecal score. Throughout the experiments we observed a significantly lower total phagocytic activity in LFA group which was related to the better clinical state of the experimental animals. In this group, we observed a significant increase in CD21+ lymphocytes in the peripheral blood on the day of weaning and 3 days post-weaning ($P < 0.01$). A significantly lower proportion of CD4+ lymphocytes in peripheral blood of the LFA piglets at weaning and 14 days post-weaning ($P < 0.01$) was accompanied by a significant increase in the CD8+ subpopulation ($P < 0.05 - P < 0.01$). Also the proportion of B lymphocytes in the blood and Peyer's patches was higher in the LFA group compared to the C group piglets. Our observations of the positive effects of immunomodulators on the clinical state of the piglets allowed us to conclude that a combination of probiotic lactobacilli and flaxseed can be effectively used in prevention of the post-weaning syndrome of piglets.

Key words: flaxseed; immunity; lactobacilli; piglets; probiotic cheese; weaning

INTRODUCTION

The post-weaning syndrome in piglets results in considerable economic losses. The losses are associated with decreased intake of feed, reduced weight gains, body wasting, occasional diarrhoea, morbidity and death. Since the use of antibiotics as growth stimulators was banned in 2006, the performance and health of these animals has been reduced. Various natural substances have been tested as a replacement of feed antibiotics, with the aim of improving piglet health and their immune response, quality of slaughter products

and thus also indirectly to the health of humans. Recently, probiotics, prebiotics, various plant extracts, fatty acids and other feed supplements have appeared as prospective positive agents. Polyunsaturated fatty acids (PUFA) increase the adherence of lactobacilli to the intestinal wall and thus potentiate the immunomodulatory effect of probiotics [1], [3].

The aim of our study was to observe the influence of probiotic lactobacilli in the form of cheese and crushed flaxseed as a source of omega-3 (PUFA) on the clinical state and total cellular and local intestinal immune responses of piglets experimentally infected with enterotoxigenic *E. coli.*

MATERIALS AND METHODS

The experiments were carried out on 48 piglets (Slovak large white × Landraces) at the age of 28—42 days, divided into two groups, control (C) and experimental (LFA), 24 piglets in each group. Starting 10 days before weaning, up to 14 days post-weaning, the piglets from the LFA group were offered probiotic cheeses L81 and 2I3 containing *Lactobacillus plantarum* and *Lactobacillus fermentum* and were fed rations containing 10 % crushed flaxseed. The control piglets were offered cheese without probiotic lactobacilli. At weaning (day 28 of age), all piglets were infected with *E. coli* O8:K88ab:H9 ENT$^+$ (2 ml per animal; 10^5 CFU.ml^{-1}). Blood samples from *plexus venosus suborbitalis* and jejunal Peyer's patches were collected on the day of weaning and on days 3, 7 and 14 post-weaning (6 animals were sacrificed at each sampling). The influence of additives on the immune response of piglets was evaluated by determination of the phagocytic activity (PHAGOTEST®) and percentage proportion of lymphocyte subpopulations in peripheral blood and Peyer's patches (FACS). The clinical state of the piglets was checked daily. We observed changes in the overall state and recorded mortality, occurrence and mean duration of diarrhoea, faecal score (evaluation based on consistence of faeces: 1 — solid; 2 — paste-like; 3 — thin; 4 — watery; 5 — presence of mucus or blood) and changes in body temperature.

RESULTS AND DISCUSSION

The clinical state of LFA piglets was positively affected which was reflected in lower occurrence of diarrhoea in this group (11 in LFA group, 18 in C group) and its shorter duration and in the lower faecal scores compared to the control group (Table 1).

The body temperatures of the piglets from the LFA group were within the standard range, while hypothermia was observed in 13 control piglets.

Throughout the experiments we recorded lower phagocytic activities in the group of piglets supplemented with probiotic cheese and flaxseed (Fig. 1). This was ascribed to their better clinical state and the effect of omega-3 PUFA. The CD21+ subpopulation of lymphocytes in the peripheral blood was increased significantly on the day of weaning and 3 days post-weaning (Fig. 2), probably due to the immunostimulative effect of lactobacilli. The influence of lactobacilli eliminated the antiproliferative effect of PUFA which should have caused a decrease in the production of the lymphocytes, as reported by Chytilová et al. [1] and Firmentová et al. [2]. We recorded an increase in the subpopulation of CD4+ lymphocytes after weaning and a subsequent decrease on day 14 post-weaning when the health of the LFA piglets improved contrary to group C, which showed an increase also on day 14, related to the poor health and a high incidence of diarrhoea (Fig. 4). An opposite tendency could be observed in the subpopulation of CD8+ lymphocytes (Fig. 5).

Table 1. Faecal scores of piglets during the experiment

Faecal score	Group C (n = 6)	Group LFA (n = 6)	*t*-test
Weaning	1 ± 0	1 ± 0	Not tested
Day 3	1.71 ± 0.82	1 ± 0	P = 0.0001
Day 7	2.42 ± 0.96	1.52 ± 0.77	P < 0.0001
Day 14	2.47 ± 0.98	1.82 ± 0.74	P < 0.0001
Total	2.29 ± 1.00	1.59 ± 0.74	P < 0.0001

Supplementation of probiotic cheese and flaxseed significantly affected also the local intestinal immune response. We recorded significantly higher proportion of B lymphocytes in Peyer's patches in the LFA group compared to the C group (Fig. 6). The most pronounced differences in subpopulations of CD3+, CD4+ and CD8+ were observed on day 7 post-weaning when the animals suffered from diarrhoea and there was an increase in the observed subpopulations (Figures 7, 8 and 9).

The results obtained in this study confirmed the immunomodulatory effects of probiotic lactobacilli and flaxseed on the clinical state of animals.

ACKNOWLEDGEMENT

The study was supported by the Competence Centre for Biomodulators and Nutrition Additives – Probiotech, č. 26220220152 and the project VEGA 1/0435/11: „Modulation of intestinal biochemism, intestinal microflora and immune response of pigs by means of probiotic micro-organisms and flaxseed as a source of n-3 PUFA and fibres".

REFERENCES

1. Chytilová, M., Mudroňová, D., Nemcová, R., Gancarčíková, S., Buleca, V., Koščová, J., Tkáčiková, Ľ., 2013: Anti-inflammatory and immunoregulatory effects of flax-seed oil and *Lactobacillus plantarum* – Biocenol™ LP96 in gnotobiotic pigs challenged with enterotoxigenic *Escherichia coli. Research in Veterinary Science*, 95, 103—109.

2. Firmentová, S., Mudroňová, D., Nemcová R., Gancarčíková, S., 2012: The influence of supplementation of probiotic

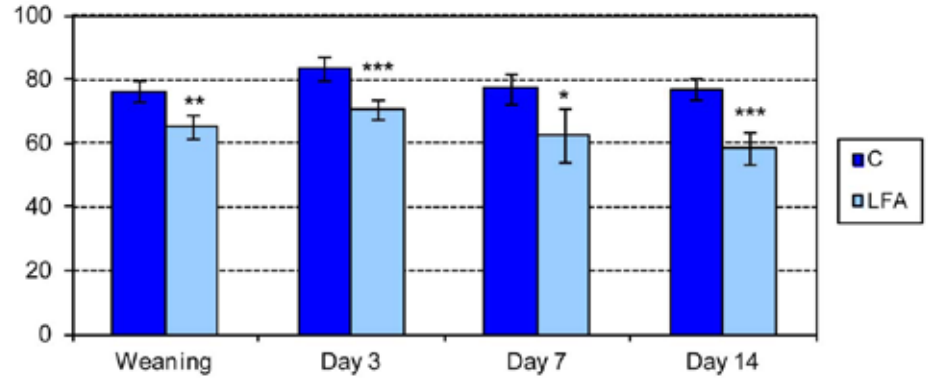


Fig. 1. Total phagocytic activity in experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

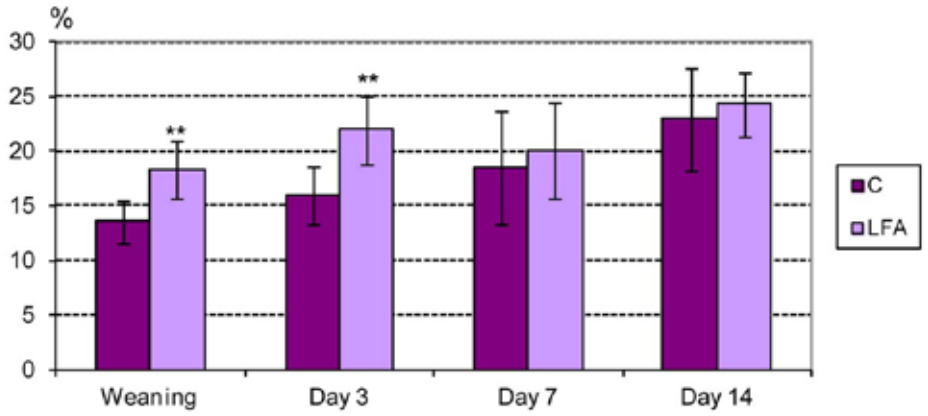


Fig. 2. Proportion (%) of CD21+ lymphocytes in peripheral blood of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

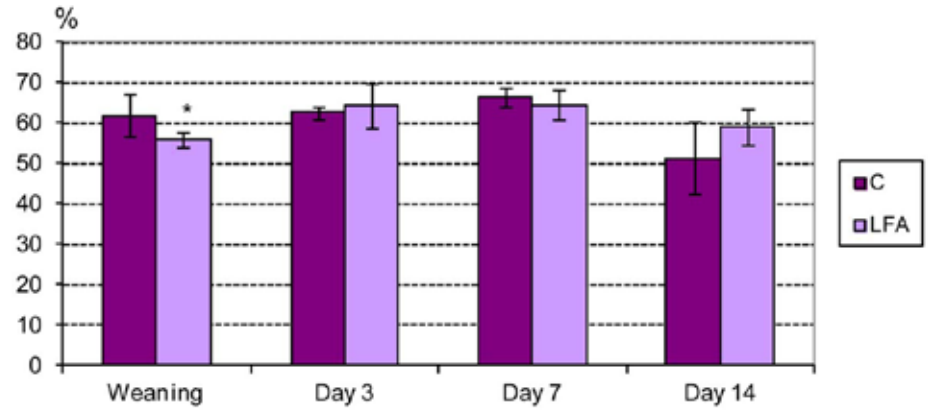


Fig. 3. Proportion (%) of CD3+ lymphocytes in peripheral blood of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

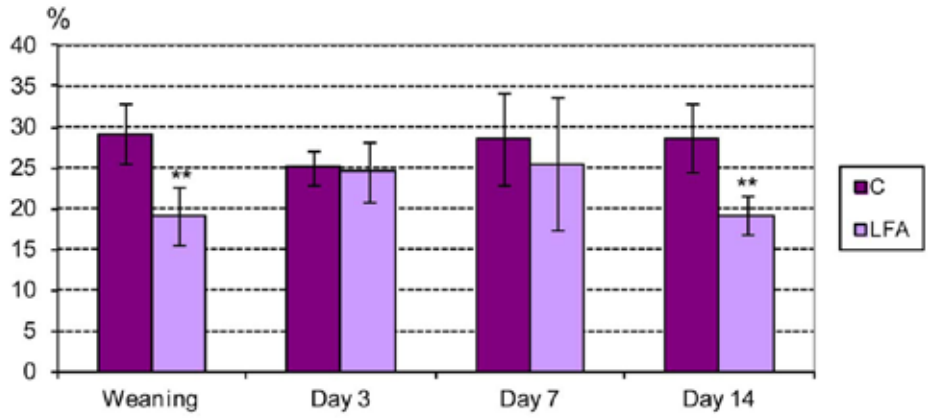


Fig. 4. Proportion (%) of CD4+ lymphocytes in peripheral blood of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

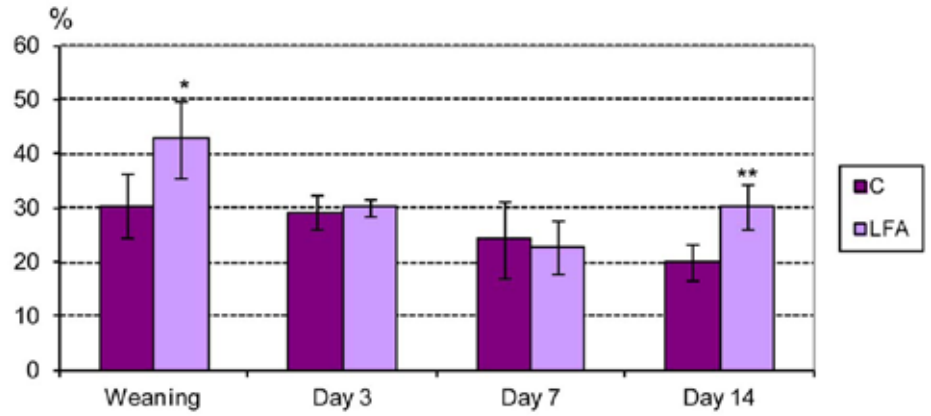


Fig. 5. Proportion (%) of CD8+ lymphocytes in peripheral blood of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

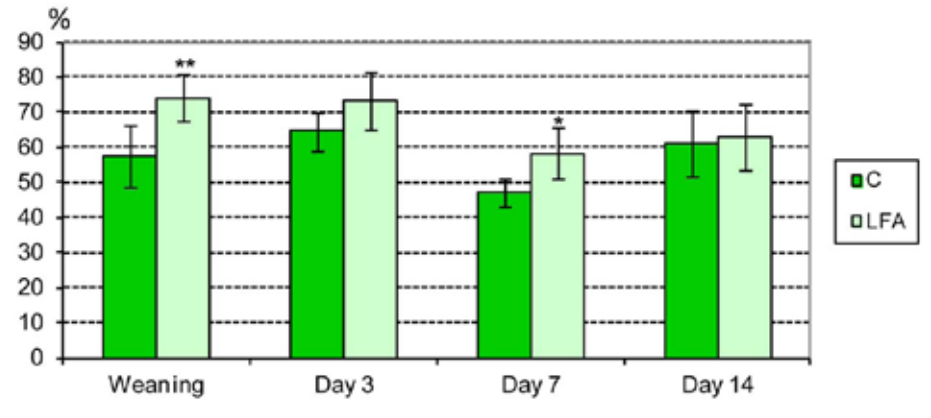


Fig. 6. Proportion (%) of CD21+ lymphocytes in Peyer's patches of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

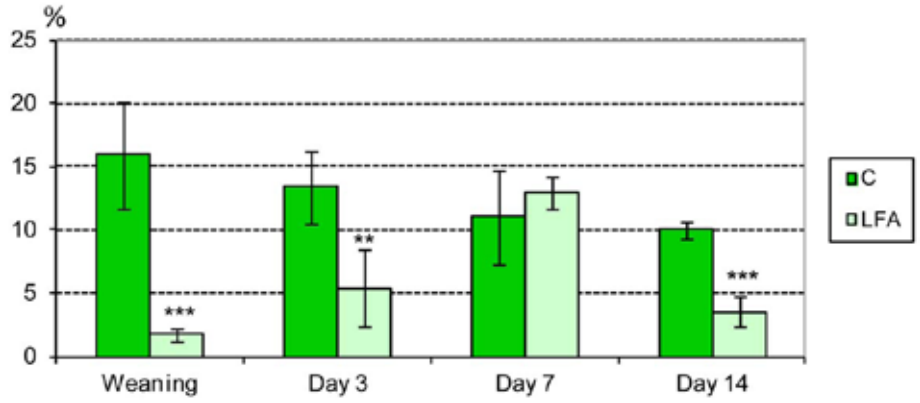


Fig. 7. Proportion (%) of CD3+ lymphocytes in Peyer's patches of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

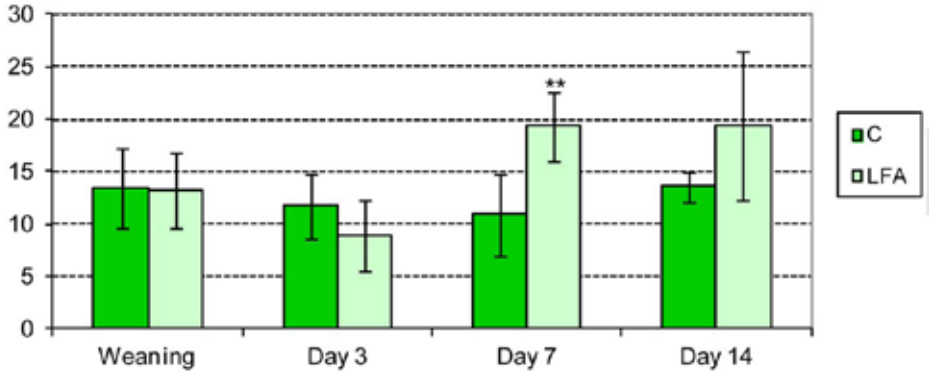


Fig. 8. Proportion (%) of CD4+ lymphocytes in Peyer's patches of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

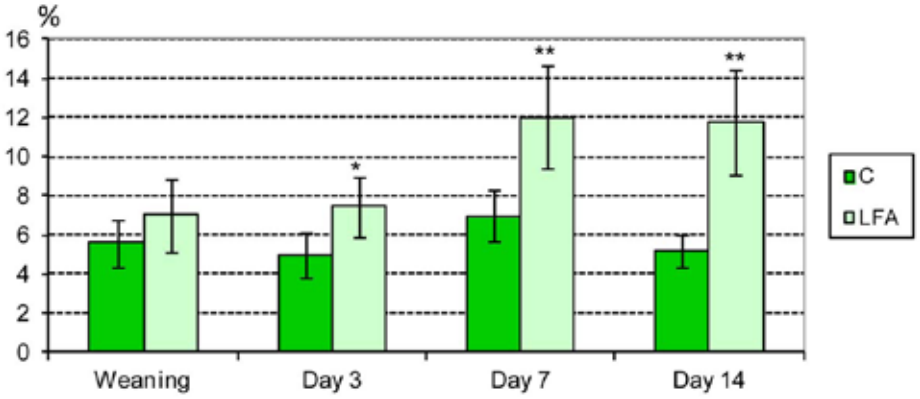


Fig. 9. Proportion (%) of CD8+ lymphocytes in Peyer's patches of experimental (n = 6) and control piglets (n = 6)
* — P < 0.05; ** — P < 0.01; *** — P < 0.001

cheese and flaxseed on immune response of piglets. *Folia Veterinaria*, 56, 10—12.

3. Nemcová, R., Borovská, D., Koščová, J., Gancarčíková, S., Mudroňová, D., Buleca, V., Pistl, J., 2012: The effect of supplementation of flax-seed oil on interaction of *Lactobacillus plantarum* – Biocenol™ LP96 and *Escherichia coli* O8:K88ab:H9 in the gut of germ-free piglets. *Research in Veterinary Science*, 93, 39—41.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section IV, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 127—129, 2014

HISTAMINE INTOLERANCE AND HISTAMINE IN FOOD

Nezbedová, Z., Dičáková, Z., Dudriková, E.

Department of Food Hygiene and Technology, University of Veterinary Medicine and Pharmacy
Komenského 73, 041 81 Košice
The Slovak Republic

zuzana.dicakova@uvlf.sk

ABSTRACT

Histamine (HIS) was determined by thin layer chromatography (TLC) in 48 samples of various foodstuffs. Histamine levels ranged from undetectable (less than 0.6 mg.kg^{-1}) up to 80 mg.kg^{-1}. The lowest levels were determined in fresh raw vegetables, mushrooms, citrus juices, coffee, some seafood, carp, poultry meat, and in mozzarella, where histamine was undetectable by the method used. On the contrary, high levels of HIS were determined in Malokarpatská salami, green and black olives, and in a ripened soft cheese "Olomoucké syrečky", where the histamine level was the highest (80 mg.kg^{-1}).

Key words: food; histamine; histamine intolerance; thin layer chromatography

INTRODUCTION

Histamine is one of the biogenic amines that commonly occur in the tissues of live organisms. In humans, histamine is present in endogenous forms and some is taken up in exogenous forms with food. It can occur in higher concentrations (above 100 mg.kg^{-1}) predominantly in marine fish, and at such concentrations, can have toxic effects [2], [8]. However, foods containing histamine even at low levels can cause problems in some individuals. This is called histamine intolerance (HIT). In patients with histamine intolerance, health problems may occur even at minimal levels (5—30 ng) [7]. Histamine intolerance results from a disequilibrium of the accumulated histamine or the capacity for histamine degradation. Symptoms of HIT are the same as in allergies, but they develop without the direct involvement of the immune system. In food allergies, the production of specific antibodies plays an important role. HIT is therefore not considered an allergy, instead, we can refer to it as a pseudoallergy [5], [6]. The body can be overloaded by high doses of histamine in several ways: food rich in histamine; liberators of histamine; other biogenic amines; inhibition of enzyme diamine oxidase (DAO); inherent enzymatic disorders; and infections of the digestive tract [3]. Symptoms of HIT are multiple: headache; vertigo; nausea; vomitus; hypotension; tachycardia; arhythmia, pruritus, rash, urticaria; dyspnoea; running nose; dysmenorrhoea; diarrhoea; stomach ache; and bloating. The treatment of HIT is based on a low-histamine diet [1], [7].

The aim of this study was to determine the content of histamine in various foods commonly consumed in our region and prepare a list of foods unsuitable for patients to consume with HIT.

MATERIALS AND METHODS

Histamine was determined in 48 samples of food from domestic sources or foods purchased in chain stores in Slovakia during the period of January—August, 2013. The samples were extracted with trichloroacetic acid, derivatized with dansyl chloride and analysed by thin-layer chromatography (TLC), by applying respective aliquots to thin-layer plates and employing a double-development

Table 1. Histamine content in vegetables and fruit

Commodity	Histamine [mg.kg^{-1}]	Commodity	Histamine [mg.kg^{-1}]
Tomato	2	Lemon, whole	0.6
Broccoli	ND	Plums	3
Cucumber, fresh	ND	Blackberry	3
Carrot	ND	Banana	ND
Cabbage, raw	ND	Olives, black	15
Orange, whole	1.5	Olives, green	40

ND — not-detectable by TLC (< 0.6 mg.kg^{-1})

Table 2. Histamine content in cheeses, meat and meat products

Commodity	Histamine [mg.kg^{-1}]	Commodity	Histamine [mg.kg^{-1}]
Hermelin	8	Pork	2
Gouda	10	Chicken breasts	ND
Olomoucké syrečky	80	Malokarpatská salami	20
Grana padano	10	Frankfurters, fine	1.5
Mozzarella	ND	Spiš frankfurters	1.2

ND — not-detectable by TLC (< 0.6 mg.kg^{-1})

Table 3. Histamine content in seafood, fish and mushrooms

Commodity	Histamine [mg.kg^{-1}]	Commodity	Histamine [mg.kg^{-1}]
Octopus	0.6	Button mushrooms, packaged	1.5
Mussels	0.6	(*Agaricus bisphorus*) Button mushrooms, loose	1.5
Shrimps	ND	Button mushrooms, canned	3
Calmars	ND	Stump mushrooms	1
Tunafish	10	Oyster mushrooms	2,5
Mackerel	5	Summer Bolete mushrooms	ND
Carp	ND	Dried mushrooms, mix	1

ND — not-detectable by TLC (< 0.6 mg.kg^{-1})

Table 4. Histamine content in teas, juices and beverages

Commodity	Histamine [mg.l^{-1}]	Commodity	Histamine [mg.l^{-1}]
Pigi tea	15	Wine, red	6
Instant tea	1.5	Wine, white	2
Green tea	1.5	Beer	5
Nettle tea	4.5	Coffee	ND
Orange juice	ND	Sauerkraut juice (from shop)	6
Lemon juice	ND	Sauerkraut juice (home-made)	ND

ND — not-detectable by TLC (< 0.6 mg.kg^{-1})

procedure [4]. The following mobile phases were used: phase I — chloroform : benzene : triethylamine (6 : 4 : 1); and phase II — benzene : acetone : triethylamine (10 : 2 : 1). After the separation on TLC plates, the spots obtained from individual samples were compared with the spots of histamine standards under a UV lamp at 365 nm, and the histamine levels were calculated for individual foods. Determinations were carried out in duplicate.

RESULTS AND DISCUSSION

The results obtained by TLC analysis are presented in Tables 1 to 4, grouped according to individual commodities. The histamine content ranged from undetectable by the method used (< 0.6 mg.kg^{-1}) up to the maximum of 80 mg.kg^{-1}. We observed that juice from sauerkraut prepared at home contained less histamine that similar juice from sauerkraut purchased in a store. The level of histamine in nettle tea was 3-fold higher than the levels in all other tested teas.

CONCLUSIONS

Histamine was determined in various foods by thin layer chromatography in order to identify foods suitable for patients with HIT. Long-fermenting products, such as cheeses Olomoucké syrečky and Gouda, Malokarpatská salami and black and green olives appear unsuitable, as the level of histamine in them ranges between 10 and 80 mg.kg^{-1}. Commodities suitable for patients with HIT include; vegetables (carrot, broccoli), some mushrooms, freshwater fish, and fresh and steamed cheeses. They may also drink coffee, black and green tea or fresh juices.

ACKNOWLEDGEMENTS

The study was carried out within the projects APVV SK-CN-0028-12 and Kega No. 011UVLF4/2012.

REFERENCES

1. Hrubiško, M., 2011: *Histamine Intolerance* (In Slovak). 3–22. [cit. 2012.03.06.] http://www.daosin.sk/Daosin-brozura-350DPI.pdf

2. Katzung Bertram, G., 1995: *Basic and Clinical Pharmacology* (In Czech). Jinočany, H & H, 240—244.

3. Maintz, L., Novak, N., 2007: Histamine and histamine intolerance. *Am. J. Clin. Nutr.*, 85, 1185—1196.

4. Shalaby, A. R., 1999: Simple, rapid valid thin layer chromatographic method for determining of biogenic amines in foods. *Food Chem.*, 65, 117—121.

5. Ortolani, C., Pastorello, E. A., 2006: Food allergies and food intolerances. *Best Practice and Research Clinical Gastroenterology*, 20, 467—483.

6. Eaton, K. K., Howard, M., Hunnisett, A., 1998: Urinary histidine excretion in patients with classical allergy (type A allergy), food intolerance (type B allergy), and fungal — type dysbiosis. *J. Nutr. Biochem.*, 9, 586—590.

7. Schleip, T., 2009: *Histamine Intolerance* (In Czech). Prague, Galén, 120 pp.

8. Křížek, M., Kalač, M., 1998: Review. Biogenic amines and their role in nutrition. *Czech J. Food Sci.*, 16, 151—159.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section IV, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 130—133, 2014

POTENTIAL DUAL EFFECT OF GREATER CELANDINE (*CHELIDONIUM MAJUS*) ON TUMOUR CELL LINES *IN VITRO*

Tkáčová, V.[1], Tomečková, V.[2], Mojžiš, J.[3]
Šutorová, M.[4], Urban, P.[2]

[1]University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
[2]Department of Medical and Clinical Biochemistry
[3]Department of Pharmacology
Pavol Jozef Šafárik University in Košice, Faculty of Medicine, Trieda SNP 1, 040 66 Košice
[4]Green pharmacy, Komenského 61, 040 01 Košice
The Slovak Republic

vladimira.tomeckova@upjs.sk

ABSTRACT

Water and ether extracts of greater celandine (*Chelidonium majus*) were applied to the following tumour cell lines: *cervix uteri* (HeLa), mammary gland adenocarcinoma (MCF-7) and acute lymphoblastic leukemia (CEM) under *in vitro* conditions. The dual antioxidative and antiproliferative effects of water and ether extracts of greater celandine on in vitro viability of cells were determined by MTT tests. The results obtained confirmed the cytotoxic effects of water and ether extracts of greater celandine on individual cell lines. The water extract was most effective on CEM cells, less on MCF-7 and least on HeLa cells. The ether extract was most effective on CEM and MCF-7 cells, at all tested concentrations, but its effect on HeLa cells was significant only at the highest concentrations. The antiproliferative effect of the ether extract of the greater celandine on all three cell lines tested was higher compared to the water extract. This could be explained by the higher content of lipophilic substances in this extract which can pass across cell membranes, bind to various proteins and change their biological activity.

Key words: cytotoxic effect; greater celandine extracts; MTT test

INTRODUCTION

The advantages of medicinal plants include their occurrence in nature, low costs, good availability and lower occurrence of side effects. Greater celandine (*Chelidonium majus*) contains a mixture of various hydrophilic and lipophilic biologically active compounds. It contains alkaloids as its major constituent. The results presented in the available scientific literature allow one to assume dual effects of greater celandine extracts *in vitro* and *in vivo* [5] situations.

Over many decades, scientists have indicated a potential hepatotoxic effect of greater celandine when used in large quantities [7]. As a medicine, its short term use at smaller doses is popular, especially for its cytoprotective, cholagogic and spasmolytic effects [4], but also for some anti-inflammatory, antitumorous and antibacterial effects [12]. Many studies have confirmed the cytotoxic effect of greater celandine in the treatment of cancer [8]. Presently, a semi-synthetic medicine, Ukrain™ (NSC-631570), containing alkaloids from greater celandine, is used in some countries, such as Ukraine and Mexico [3]. The high antiproliferative activity of greater celandine has been ascribed to the content of substances with lipophilic character which can pass easily across cellular membranes and preferentially bind to various proteins and change their biological activity [11].

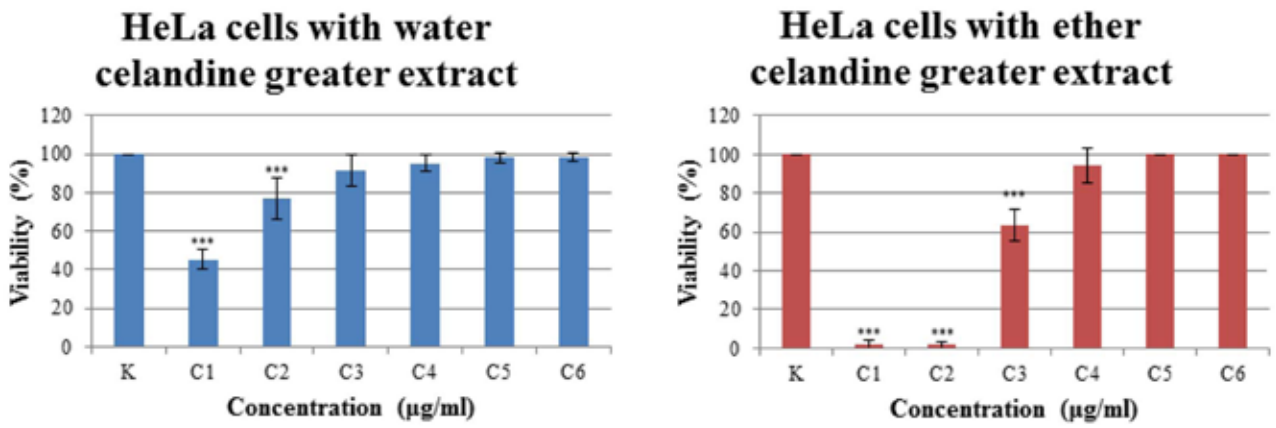


Fig. 1. Comparison of the effect of celandine greater extracts on viability of HeLa cells

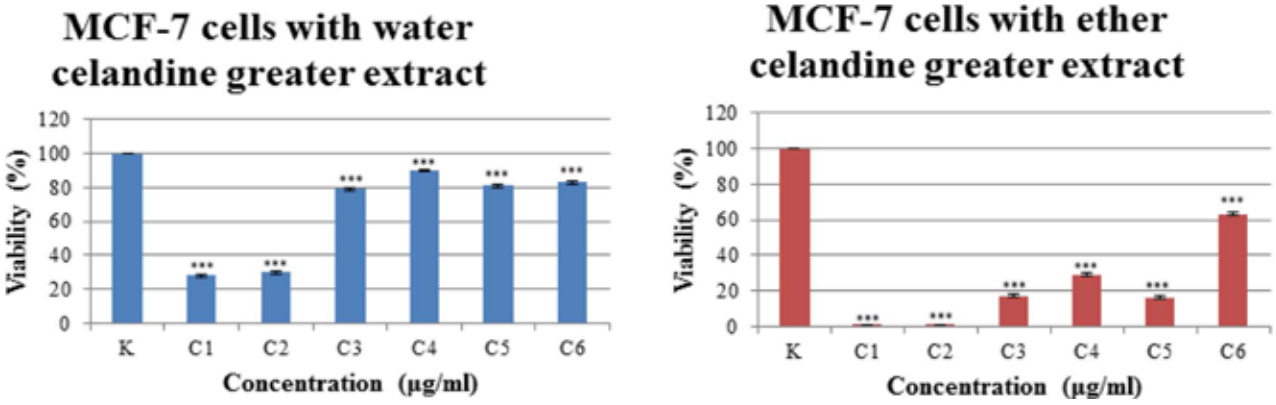


Fig. 2. Comparison of the effect of celandine greater extracts on viability of MCF-7 cells

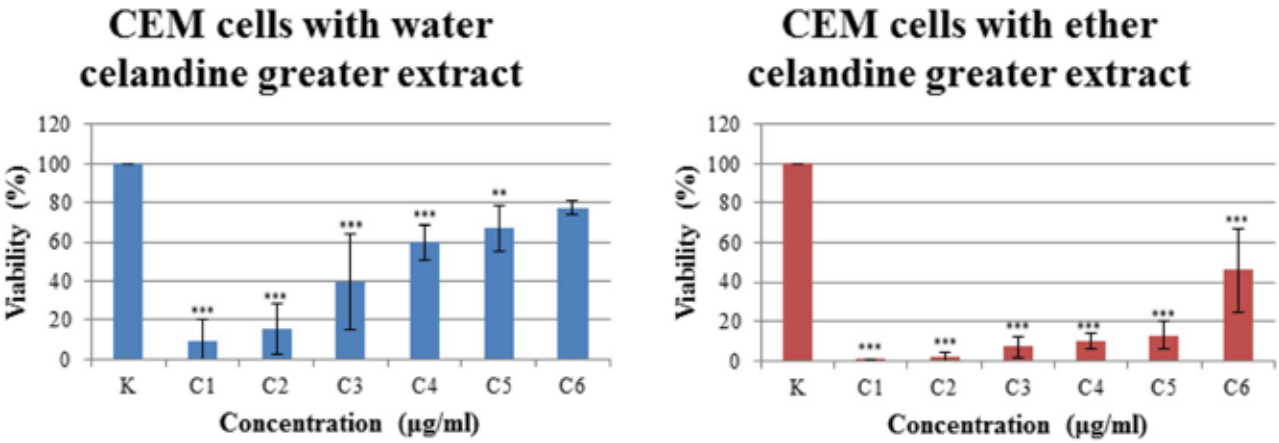


Fig. 3. Comparison of the effect of celandine greater extracts on viability of CEM cells

This study focused on the investigation of the potential dual (cytoprotective and cytotoxic) effects of greater celandine on human tumour lines of types; HeLa, MCF-7 and CEM, using a MTT colorimetric test.

MATERIALS AND METHODS

Water and ether extracts were prepared from dried aboveground parts of the drug *Chelidonii herba*. Water extract was diluted with a calculated volume of culturing medium to obtain the highest concentration c_1, which was gradually diluted down to the lowest concentration c_6. Ether extract was first dissolved in dimethyl sulphoxide and then diluted with the culturing medium. Cell lines of types HeLa and CEM were cultured in RPMI 1640 medium containing calf serum and antibiotics (penicillin 10 000 U.ml^{-1} and streptomycin 10 000 µg.ml^{-1}); MCF-7 cells were incubated in DMEM medium containing GlutaMAX, foetal calf serum and antibiotics. Passaging and incubation of the control (without plant extracts) and experimental cells (with water/ether extracts of greater celandine) were carried out in a thermostat (SANYO CO_2 incubator, model MCO-19AIC, Japan) at 37 °C, in an atmosphere containing 5 % CO_2 on a 96-well culture plate for 72 hours. The cytotoxic effects of the greater celandine extracts on the viability of the tumour cell lines *in vitro* by means of a spectrophotometric MTT test [10] using a spectrophotometer ELISA reader PowerWave HT Microplate Spectrophotometer (BioTek®, USA) at a wavelength of $\lambda = 540$ nm were carried out. The results obtained were processed by software GEN5™. Individual results of absorbances allowed us to determine the viability of experimental cells (x %) in comparison with control cells (100 %). The significant differences between the mean viability of experimental and control groups of cells were determined by software GraphPad InSTAT, using Tukey-Kramer test. All of the data obtained were processed by one-way ANOVA. To minimise the variability of the biological material studied, detection of changes in each biological sample was performed in triplicate.

RESULTS AND DISCUSSION

The results obtained in this study showed that the cytotoxic effects on individual tumour cell lines of both water and ether extracts of greater celandine was concentration-dependent. The cytotoxic effect of the ether extract on HeLa cells was more intensive compared to that of the water extract (Fig. 1). The water extract of greater celandine was most effective on the CEM cells (Fig. 3) and the MCF-7 cells (Fig. 2) and least effective on the HeLa cells (Fig. 1). The effect of ether extract on the viability of the CEM cells (Fig. 3) and the MCF-7 cells (Fig. 2) was significant ($P < 0.001$) at all tested concentrations , while the viability of the HeLa cells (Fig. 1) was affected by this extract only at the highest concentrations.

The majority of *in vitro* studies have ascribed the antitumour activity of the greater celandine extracts to the constituents in the extracts. The structure and action of these constituents are affected by the cell environment, e. g. pH and polarity of the solvent. Benzofenantridine alkaloids, as the major constituents in the greater celandine extracts, pass across cellular membranes as lipophilic tertiary structures and inside the cell convert to hydrophilic quaternary structures. During the interaction with molecular DNA, they intercalate in the quaternary structures. Sanguinarine is the strongest anti-tumour alkaloid and intercalator of DNA [13], [14], while chelerytrine, berberine and chelidonine exhibit lower anti-tumour activity *in vitro* [2] and *in vivo* [14]. Chelidonine induces apoptosis of tumour cells through the inhibition of the polymerisation of tubuline, telomerase of tumour cells and cell mitosis [2], [6]. The structure of alkaloids in the greater celandine is responsible for the diversity of binding of these alkaloids to the DNA molecule [1]. When transported by blood, alkaloids form weak bonds with albumin in tertiary structures, but generally prefer covalent bonds with proteins in tertiary structures, which is responsible, for example, for the antibacterial action of alkaloids [9].

CONCLUSIONS

The results of our study indicated a more intensive antiproliferative activity of the ether extract of greater celandine on all tested tumour cell lines compared to the water extract. The water extract of greater celandine contains polar constituents, while the ether extract contains lipophilic compounds. The high antiproliferative activity of greater celandine, most likely, results due to the content of substances with lipophilic character, which pass easily across cellular membranes and preferentially bind to various proteins and change their biological activity.

REFERENCES

1. Bashmakova, N. et al., 2008: The DNA influence on spectral properties of the berberine, chelidonine and sanguinarine alkaloids. *WDS'08 Proceedings of Contributed Papers, Part III*, 163—167.

2. Biswas, S. J., 2013: *Chelidonium majus* L. — a review on pharmacological activities and clinical effects. *Global Journal of Research on Medicinal Plants and Indigenous Medicine*, 2, 238—245.

3. Boehm, K., Edzard, E., 2013: *Ukrain* [online dokument]. CAM — Cancer Consortium, 2013.[cited 25.2.2014]. Available on internet: <http://cam-cancer.org/CAM-Summaries/Herbal-products/Ukrain>.

4. Crijns, A. P., de Smet, P. A., van den Heuvel, M., Schot, B. W., Haaqsma, E. B., 2002: Acute hepatitis after use of a herbal preparation with greater celandine (*Chelidonium majus*). *Ned Tijdschr Geneeskd*., 146, 100—102.

5. Dostál, J., Slavík, J., 2000: Later knowledge about sanguinarine and related alkaloids. *Chem. Lett*., 94, 15—20.

6. Gausage, F., Ramadani, M., Gausage, S., 2001: Cytotoxic effects of the alkaloid chelidonine from *Chelidonium majus* on pancreatic cancer cells. An old highly potent anticancer drug. *Gastroenterology,* 120, A617–A618.

7. Greving, I., Meister, V., Monnerjahn, C., Müller, K. M., May, B., 1998: *Chelidonium majus*: a rare reason for severe hepatotoxic reaction. *Pharmacoepidemiology and Drug Safety*, 7, S66—S69.

8. Lanvers-Kaminsky, C., Nolting, D.M., Koster, J., Schroder, A., Sandkotter, J., Boos, J., 2006: *In vitro* toxicity of Ukrain against human Ewing tumor cell lines. *Anti-Cancer Drugs*, 17, 1025–1030.

9. Maliková, J., Zdarilová, A., Hlobilková, A., 2006: Effects of sanguinarine and chelerythrine on the cell cycle and apoptosis. *Biomedical Papers of the Medical Faculty of the University Palacký,* Olomouc, Czech Republic, 150, 5—12.

10. Mosmann, T., 1983: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55—63.

11. Motilal, M., Gopinatha, S.K., 2007: Molecular aspects on the interaction of protoberberine, benzophenanthridine, and aristolochia group of alkaloids with nucleic acid structures and biological perspectives. *Med. Res. Rev.*, 27, 649—695.

12. Tong, J., Liu, Y., Chen, G., Liu, X., Chen, S., Meng, Y., 2001: A study on relieving cough and asthma effects of total alkaloid from Chelidonium majus. *Journal of Chengde Medical College*, 18, 277—279.

13. Vavrečková, C., Ulrichová, J., 1994: Biological activity of quarternary benzo[c]phenanthridine alkaloids of sanguinarine and chelerytrine. *Chem. Lett.,* 88, 238.

14. www.ema.europa.eu. [cited 5.3.2014]. Available on internet <http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_HMPC_assessment_report/2012/01/WC500120711.pdf>.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section IV, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 134—138, 2014

SYNTHESIS OF PENTACYCLIC ANALOGUES OF ASCIDIDEMIN

Zima, J., Ungvarská Maľučká, L.

Department of Chemistry, Biochemistry and Biophysics
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

lucia.ungvarska.malucka@uvlf.sk

ABSTRACT

Alkaloids are a group of naturally occurring chemical compounds with a wide range of biological activity. Recently many N-alkaloids with antiviral, antibacterial, anti-inflammatory, antimalaric, antioxidative and anti-tumour activities have been isolated from marine organisms. Pyridoacridines are coloured natural products of marine organisms with a polycyclic planar heteroaromatic 11*H*-pyrido[4,3,2-*mn*] acridine system. The main representative of pentacyclic alkaloids isolated from marine organisms is ascididemin. The aim of this study was to prepare modified analogues of ascididemin that have not been prepared before, and determine their physic-chemical properties (logP — partition coefficient). The experimental part of this study focused on the synthesis of selected modified aromatic amines, the intermediates of 4-stage synthesis of analogues of ascididemin. It also dealt with spectroscopic characterization of ascididemin analogues using 1D and 2D NMR methods, and with the quantum-chemical calculations of logP.

Key words: ascididemin; cytotoxicity; log P; pyridoacridines; synthesis

INTRODUCTION

Presently, there has been a keen interest in marine-derived pyridoacridines due to their interesting biological properties. Almost all of them show marked cytotoxicity. Tetracyclic heteroaromaticpyrido[4,3,2-*mn*]acridine (Fig. 1) forms the basic chemical structure of pyridoacridine alkaloids. The majority of pyridoacridines are planar heterocycles. According to the number of rings, they are divided to tetracyclic, pentacyclic, hexacyclic, heptacyclic and octacyclic. Pentacyclic pyridoacridines are divided into two structural type-based groups, according to the fusion of an additional ring to pyridoacridine. This can occur with a linear ring fusion at C-8 and C-9 of the pyridoacridine ring, or an angular fusion at C-9 and C-10 of the pyridoacridine ring [4]. One of the most important pentacyclicpyridoacridines is ascididemin, 9*H*-quino[4,3,2-*de*][1,10]phenanthroline-9-one (Fig. 1), denoted as ASC 2 hereafter.

ASC 2 was isolated from two marine species, initially in 1988 from an Okinawan *Didemnum* sp. [3] and in 1995 from *Cystodytes dellechiajei,* which is found in the world's oceans [1]. Both *Cystodytes dellechiajei* and *Didemnum* are included in the class of *Ascidiacea*. The chemical structure of ASC 2 was determined on the basis of 1D and 2D NMR spectra [4]. The cytotoxic effect of ASC 2 involves the cleavage of DNA by human topoisomerases I and II. In the tests with the superhelix DNA, ASC 2 stimulated the cleavage of both of the DNA strands by topoisomerase II and was observed with the stabilisation of the complexes DNA-topoisomerase [2]. For this reason, there has been an increasing interest in the synthesis of modified analogues of ASC2 which is aimed at the improvement of the pharmacodynamic and pharmacokinetic properties of this alkaloid.

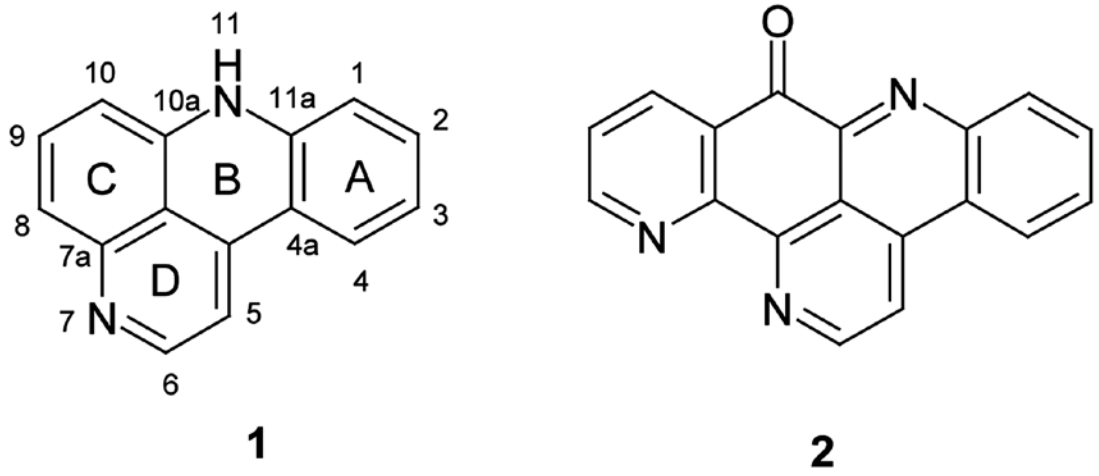
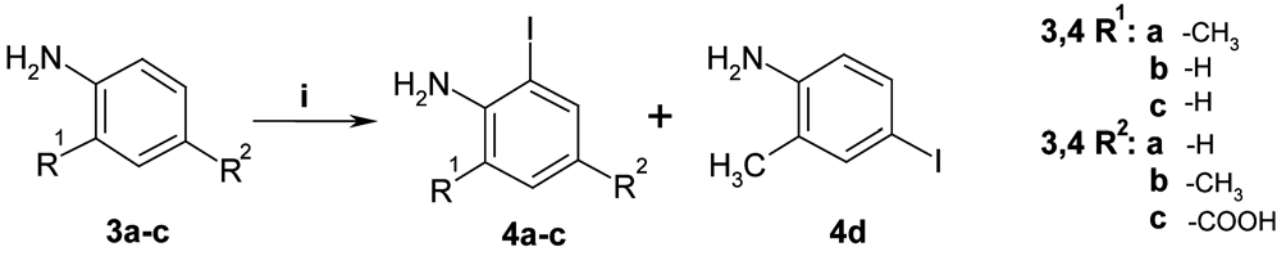
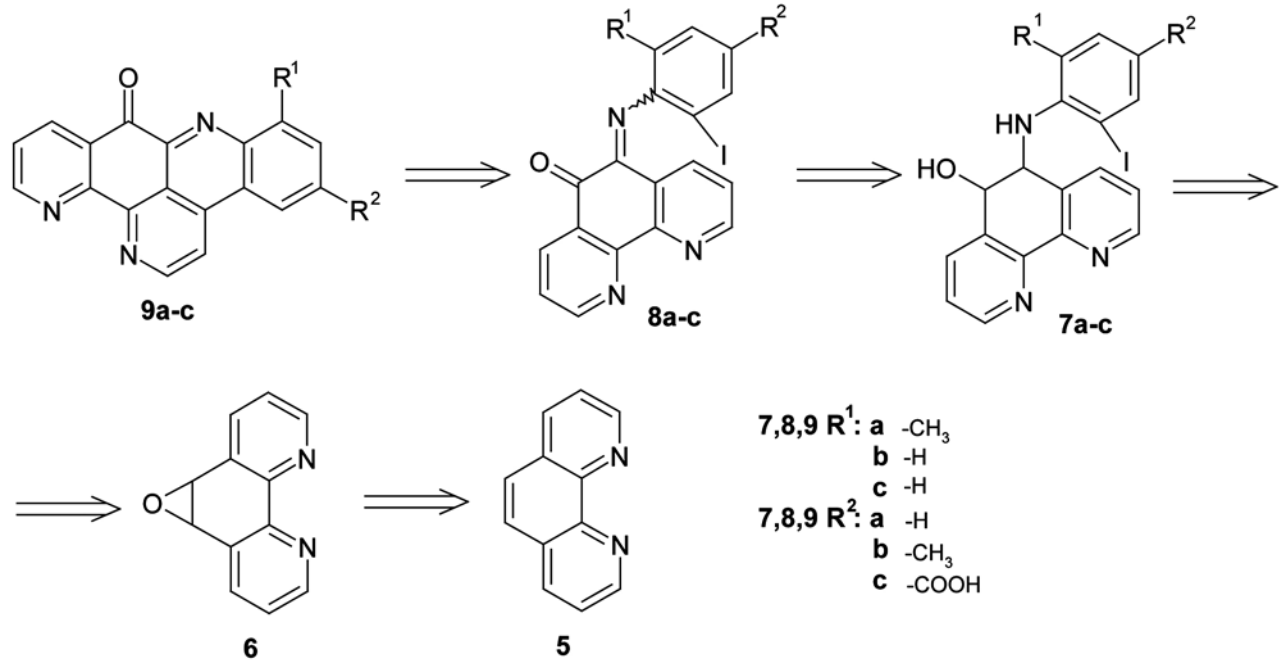


Fig. 1. Structure of pyridoacridine alkaloids:
1 — heteroaromatic pyrido[4,3,2-mn]acridine; 2 — ascididemin 9H-quino[4,3,2-de][1,10]phenanthroline-9-one.



Reaction conditions: i — Ag_2SO_4/EtOH; I2, 17 h, room temp.; yield 4a — 0 %; 4b – 76 %; 4c —35 %; 4d — 62 %

Scheme 1. Synthesis of aromatic analogues of aniline 4a–d



Scheme 2. Retrosynthesis of the proposed analogues of ASC 9a–c

MATERIALS AND METHODS

The ^{1}H, ^{13}C NMR spectra (ppm) were measured on NMR spectrometers Varian Mercury Plus (400,13 MHz) and Varian VNMRS (599,87 MHz) at laboratory temperature in deuterated chloroform ($CDCl_3$-d_1) and dimethyl sulfoxide (DMSO-d_6), using tetramethylsilane as an internal standard (δ = 0.00 ppm). The assignment of proton and carbon was carried out on the basis of the analysis: COSY, HSQC, HMBC, NOESY and TOCSY spectra. The UV/VIS spectra were measured by a spectrophotometer Libra S12 in 1 cm cells at laboratory temperature in methanol. Chemicals were used in the commercially available state without further purification (Sigma-Aldrich, MikroChem). The calculations of the partition coefficients (logP) were performed using the freely available softwares; ChemSketch (ACDLabs/LogP), MarvinSketch, and HyperChem.

RESULTS AND DISCUSSION

The intermediates 4a-c was prepared by electrophilic substitution reactions using the reaction conditions shown in Scheme 1. The derivative 4a was prepared by substitution with iodine in the *p*- position, opposite the -NH_2 group, and thus the derivative 4d was obtained.

The prepared iodine derivatives 4a-c can enter into the reaction with epoxide 6 and result in the products 7a-c (Scheme 2). By selection of the oxidative reagent, it is possible to oxidize amines 7a-c to imines 8a-c, that can result in three basic structures 9a-c by photochemical cyclization. Following a sequence of reaction steps, these structures can be transformed to alcohol, carboxamide, oxime, imine, hydrazide, and others.

To determine the structure of the prepared derivatives 4b-d, we used 1 D and 2 D NMR measurements (Tables 1, 2).

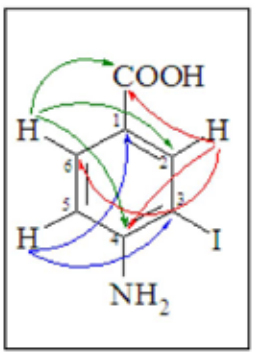
Table 1. **^{1}H NMR chemical shifts in the prepared intermediates 4b-d**

δ ^{1}H (ppm)/derivative	**H-2**	**H-3**	**H-5**	**H-6**	**NH_2**	**CH_3**
4b (R^1 = H, R^2 = CH_3)	–	7.47	6.94	6.66	3.92	2.21
4c (R^1 = H, R^2 = COOH)	8.25	–	6.77	7.74	4.25	–
4d	–	7.33	7.28	6.44	3.56	2.10

Table 2. **^{13}C NMR chemical shifts in the prepared intermediates 4b-d**

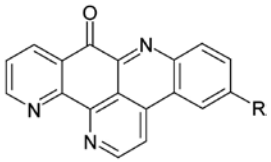
δ ^{13}C (ppm)/derivative	C-1	C-2	C-3	C-4	**C5**	**C–6**	**CH_3**	**COOH**
4b (R^1 = H, R^2 = CH_3)	144.4	84.5	139.2	129.7	130.2	114.8	19.9	–
4c (R^1 = H, R^2 = COOH)	120.7	140.9	81.6	151.5	113.0	131.0	–	166.9
4d	144.3	125.0	138.6	79.5	135.5	116.9	17.4	–

Table 3. Determination of the structure of molecule 4c based on HMBC spectra (→)



δ ^{1}H (ppm)	**Correlation**	**δ ^{13}C (ppm)**
H-2	C=O (^{3}J)	166.9
8.25	C-4 (^{3}J)	151.5
H-5	C-3 (^{3}J)	81.6
6.77	C-1 (^{3}J)	120.7
H-6	C-2 (^{3}J)	140.9
7.74	C=O (^{3}J)	166.9
	C-4 (^{3}J)	151.5

Table 4. Calculated logP of ASC 2 analogues 10a–i

 10a-i		logP		
		MarvinSketch	HyperChem*	ACD/Labs
R³	–H, ASC **2**	2.98	–0.68	0.29
a	$-CH_3$	3.49	–0.53	0.75
b	$-CH_2Br$	3.75	–0.32	0.99
c	–CH=O	2.69	–1.71	–0.28
d	$-CH_2OH$	2.21	–1.53	–0.89
e	–COOH	2.63	–1.30	0.02
f	$-CONH_2$	1.83	–2.16	–1.13
g	$-CONHNH_2$	1.53	–2.17	–1.44
h	–CH=NH	2.70	–1.54	–0.83
ch	$-CH=N-NH_2$	2.40	–1.55	–0.51
i	–CH=N–OH	2.70	–1.05	–0.01

* — Quantum-chemical calculations were used to calculate log P.
Geometric structure of all proposed analogues 10a-i were optimized on the semi-empirical level using AM1 method

In a COSY experiment, a spin interaction between protons H–5 and H–6 was observed in a molecule of carboxylic acid 4c. The HSQC spectrum allowed us to identify signals belonging to three proton–containing carbon atoms (C-2, 5, 6). In the HMBC spectrum of the derivative 4c, the signals of quaternary carbons atoms (C-1, 3, 4) were assigned indirectly on the basis of the magnetization transfer from protons to carbon atoms along three bonds (^{3}J, Table 3). The structure of 4b,d compounds were determined by a similar analysis of ^{1}H, ^{13}C, COSY, HSQC and HMBC spectra.

One of the pharmacokinetic parameters is logP, which indicates the value of the properties of the drug; its accumulation or rapid release from the body. Contributions of hydrophobic fragments of various functional groups affect considerably the total value of logP. In order to modify chemical structure of a known model structure and thus obtain new analogues of ASC 10a-i with a lower logP, we decided to use the available software to calculate the respective logP values. Table 4 presents the summary of the results obtained by three different softwares.

From the selected derivatives, the lowest logP (introduction of the highest polarity into an ASC 2 molecule) was calculated for the hydrazide group $-CONHNH_2$ 10g, followed by amide $-CONH_2$ 10f, alcohol $-CH_2OH$ 10d and carboxylic acid group -COOH 10e. On the contrary, the highest logP (dependent on the hydrophobic contribution of the respective functional groups) was calculated for the derivatives with bromomethyl 10b and methyl groups 10a (derivatives with these substituents had the highest lipophility). A similar trend (with small deviations) was observed after the theoretical calculations using the three freely available software.

CONCLUSION

Intermediates 4b,c,d was prepared in one reaction step from the respective aromatic amines 3a–c. Their structures were determined by modern 1D and 2D NMR methods. Aniline derivatives 4a–c can be used to synthesize analogues 9a–c. According to the theoretical calculations of logP, modification of derivatives 9a–c, the pharmacokinetic properties can be improved by additional reactions (introduction of carboxamide10f, hydrazide10g or carbaldehyde group 10c). On the contrary, the presence of bromomethyl group 10b in the structure of ASC 2 can result in the accumulation of a hypothetic drug in the body.

ACKNOWLEDGEMENT

Our thanks belong to Assoc. Prof. RNDr. Ján Imrich, CSc. from the University of Pavol Jozef Šafárik in Košice (Nuclear magnetic resonance laboratory) for the measurement of the NMR spectra.

REFERENCES

1.Bonnard, I., Bontemps, N., Lahmy, S., Banaigs, B., Combaut, G., Francisco, C. et al., 1995: Binding to DNA and cytotoxic evaluation of ascididemin, the major alkaloid from the Mediterranean ascidian *Cystodytes dellechiajei*. *Anti-cancer Drug Design,* 10, 333—346.

2. Dassonneville, L., Wattez, N., Baldeyrou, B., Mahieu, Ch., Lansiaux, A., Banaigs, B. et al., 2000: Inhibition of topoisomerase II by the marine alkaloid ascididemin and induction of apoptosis in leukemia cells. *Biochem.Pharmacol.,* 60, 527—537.

3. Kobayashi, J., Cheng, J., Nakamura, H., Ohizumi, Y., Hirata, Y., Sasaki, T. et al., 1988: Ascididemin, a novel pentacyclic aromatic alkaloid with potential leukemic activity from the Okinawan tunicate *Didemnum* sp.. *Tetrahedron Letters,* 29, 1177—1180.

4. Molinski, T. F., 1993: Marine pyridoacridine alkaloids: structure, synthesis and biological chemistry. *Chemical Reviews*, 93, 1825—1838.

*

Selected papers from the 57th STUDENT SCIENTIFIC CONFERENCE, Section IV, held at the University of Veterinary Medicine and Pharmacy in Košice on April 9, 2014.

FOLIA VETERINARIA, 58, 2: 139—142, 2014

HEPATOTOXIC EFFECT OF SELECTED NUTRITIONAL SUBSTANCES AND ANABOLICS USED TO ENHANCE SPORTS PERFORMANCES

Matejovič, A., Marcinčáková, D., Csank, T.

University of Veterinary Medicine and Pharmacy, Komenského 73, 04181 Košice The Slovak Republic

dana.marcincakova@uvlf.sk

ABSTRACT

This study investigated the effects of various concentrations of creatine, Branched Chain Amino Acids (BCAA) preparations and the anabolics, testosterone and nandrolone (hormonal preparations), on hepatocytes. Real time cell analysis (RTCA) was carried out during the action of the tested substances (24 h) and the changes in the state of cells expressed as cell index (CI) values were recorded and the respective curves were constructed using an xCELLigence system. At the 24th hour, we measured the viability of the cells by a colorimetric Methyl Thiazole Tetrazolium (MTT) test. The most pronounced cytotoxic effect was recorded for nandrolone decanoate and the lowest for BCAA. Monitoring by the RTCA system showed marked changes in CI at all tested concentrations of testosterone and nandrolone. Creatine and BCAA caused significant differences only at higher concentrations, while differences at the remaining concentrations were insignificant in comparison with untreated control cells. The MTT showed a significant effect of the test substances on the viability of cells at all concentrations of testosterone, nandrolone, creatine and at 3 concentrations of BCAA.

Key words: BCCA; creatine; hepatotoxicity; MTT; nandrolone; RTCA; testosterone

INTRODUCTION

Nutritional supplements intended for increased physical performance or body building have become available worldwide products. The largest group that use them in the long term were athletes and sportsmen, but in the recent 15 years they have been increasingly used by physically active individuals of all age categories. Moreover, the use of banned substances, such as pro-hormones and anabolics, has been increasing as well.

Despite the general belief about their desired effects, many cases have been recorded where the use of these supplements have presented health risks, due to insufficient information about their interactions with other supplements, freely sold drugs or prescription medicines. Evidence has accumulated about the contamination or excessive concentration of substances not declared on the label in such supplements. The use of some supplements has been associated with hepatotoxicity or renal failure. Because the existing legislative provisions characterise nutritional supplements as foods, there is no obligation to test them clinically or to determine their toxicological profiles. The risks related to the concentrations of individual substances, composition, contamination or interactions have become an important public health issue [2]. Presently, the majority of relevant studies have focused on the effectiveness of supplements and almost no attention has been paid to their toxicological-pharmacological profile.

Because of the increasing use of supplements by the wider public and the poor legislative controls and protection of consumers, we have made an effort to point to their potential health risk by investigating; hepatotoxicity of creatine, amino acid preparation BCAA (Branched Chain Amino Acids), and the anabolics, androlone and testosterone, at the recommended and excessive doses.

MATERIALS AND METHODS

Before the testing, the test substances were diluted with 1 % dimethyl sulphoxide (DMSO) to concentrations corresponding to the recommended doses and to concentrations exceeding these doses 2—8-fold in order to investigate their hepatotoxicity. Hepa1c1c7-murine hepatoma cells were used as a model line. For testing, we used 24-hour cell cultures exposed to the tested substances in respective concentrations for 24 hours (Table 1).

Table 1. Tested concentrations of investigated substances

Substance tested	Concentration [mg.l^{-1}]			
	RDI*	2x	4x	8x
Creatine	0.43	0.86	1.72	3.44
BCAA	0.6	1.2	2.4	4.8
Testosterone	0.4	1.4	2.8	–
Nandrolone	3	4	6	9

RDI* — Recommended Daily Intake

System xCELLigence (RTCA)

The real time cell analyser (RTCA) is a new cell-response profiling technology which enables investigators to observe the cells (adherence, proliferation, morphology) throughout an experiment. With this method, cells are cultured on special E-plates with gold electrodes on the bottom which detect cellular impedance. The more cells adhere to the electrodes, the higher the cell index and vice versa. Changes in the state of the cells (detachment, morphology changes, proliferation) cause changes in the impedance [7]. The results are expressed as the cell index (CI) and recorded every hour throughout the experiment providing real-time curves.

MTT

In parallel with RTCA we carried out a colorimetric Methyl Thiazole Tetrazolium (MTT) test to investigate cell viability. Live cells reduce MTT 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to formazan because they possess active dehydrogenases of mitochondria and are stained by this products. The quantity of produced MTT-formazan is determined spectrophotometrically upon dissolving. MTT is a yellow water soluble tetrazolium stain which is reduced by live cells to a violet formazan product insoluble in water. The measured absorbance of formazan is directly proportional to the number of viable cells [3]. The levels of absorbance determined by this test as well as the levels of cell index are expressed in % using the following formula: % = sample value × 100/control value.

Statistical processing of results

Results were processed by software Graf Pad Prism 4.0 (2003). We used one-way Anova (Dunnet's comparison test) for comparison of the results (obtained in triplicate) with the control, with $P < 0.05$ considered as significant.

RESULTS

The effect of all tested substances on the proliferation of hepatocytes was monitored and recorded by the RTCA system. The cell index for each substance was standardised to CI = 1 before the treatment, 24 hours after mounting the cells. The difference in cell index between experimental and control cells were significant ($P < 0.0001$) with all tested concentrations of testosterone and nandrolone. This means that even commonly used doses of these compound can have negative effect on hepatocytes. The difference was most pronounced with nandrolone (9 mg.l^{-1}), as shown in Fig. 1 obtained by RTCA.

A significant difference in CI of treated cells compared to the control was caused also by the highest concentrations of creatine (3.4 mg.l^{-1}) and BCAA (4.8 mg.l^{-1}) (not presented).

The MTT test indicated an important influence of all tested substances on hepatocytes ($P < 0.0001$) at all concentrations, except for the lowest one for BCAA 0.6 mg.l^{-1} (Fig. 2). Despite the absence of changes in CI, the viability of the cells was markedly decreased. Fig. 2 shows changes in % viability and % proliferation compared to control for individual concentrations of the tested substances.

DISCUSSION

The side effects and potential toxicity of nutritional substances are always the "big unknown", because clinical testing and evaluation of toxicologic-pharmacologic profiles is obligatory only for medicines. This is the reason why we focused on the testing of cytotoxicity of selected nutritional supplements and anabolics used to enhance sports performance, namely on their effects on liver cells because their hepatotoxicity has been reported [6]. Our study showed the most pronounced hepatotoxic effect after exposure of cells to the anabolic nandrolone (3.4 mg.l^{-1}). Similar results were obtained by other authors [1], [4] who described functional disorders of the liver, hepatic cholestasis and increased incidence of cancer associated with nandrolone. The effects of testosterone was evident at all tested concentrations and the differences in CI were significant ($P < 0.05$) in comparison with the control. A considerable decrease in the metabolic activity was observed at all doses of all tested substances with the exception of BCAA at a concentration of 0.6 mg.l^{-1} (corresponds to the recommended daily dose). This observation is an important proof of negative effects of the investigated substances to hepatocytes. Although no marked changes

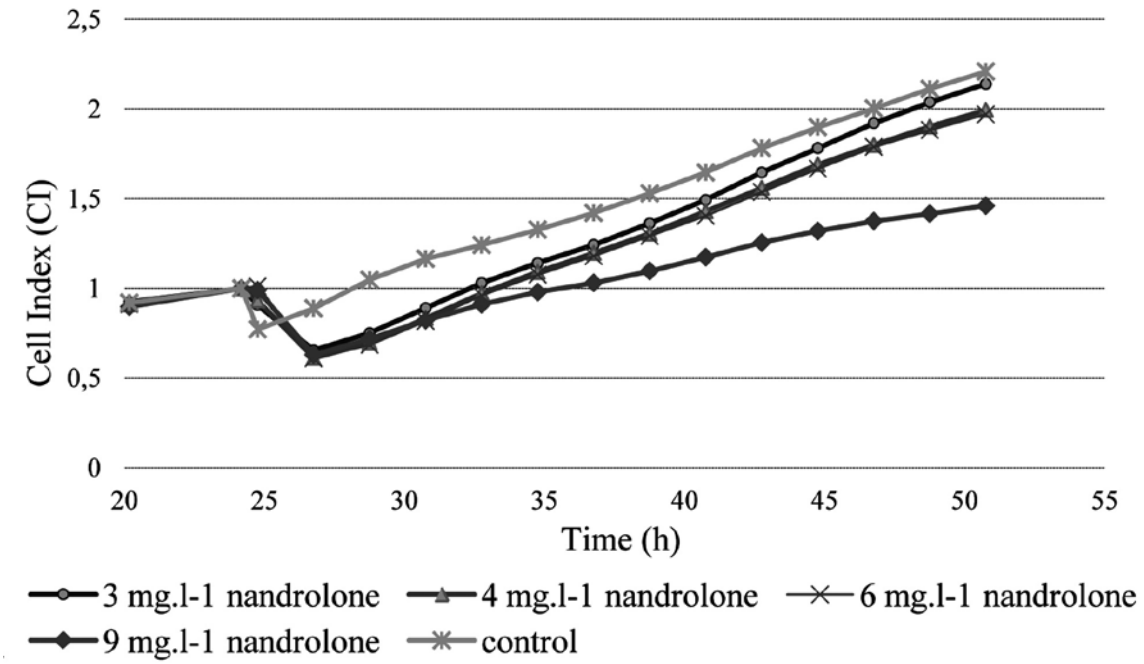


Fig. 1: Real time proliferation of cells 24 hours after treatment with nandrolone (the results obtained for concentrations 4 mg.l^{-1} and 6 mg.l^{-1} nandrolone were almost identical)

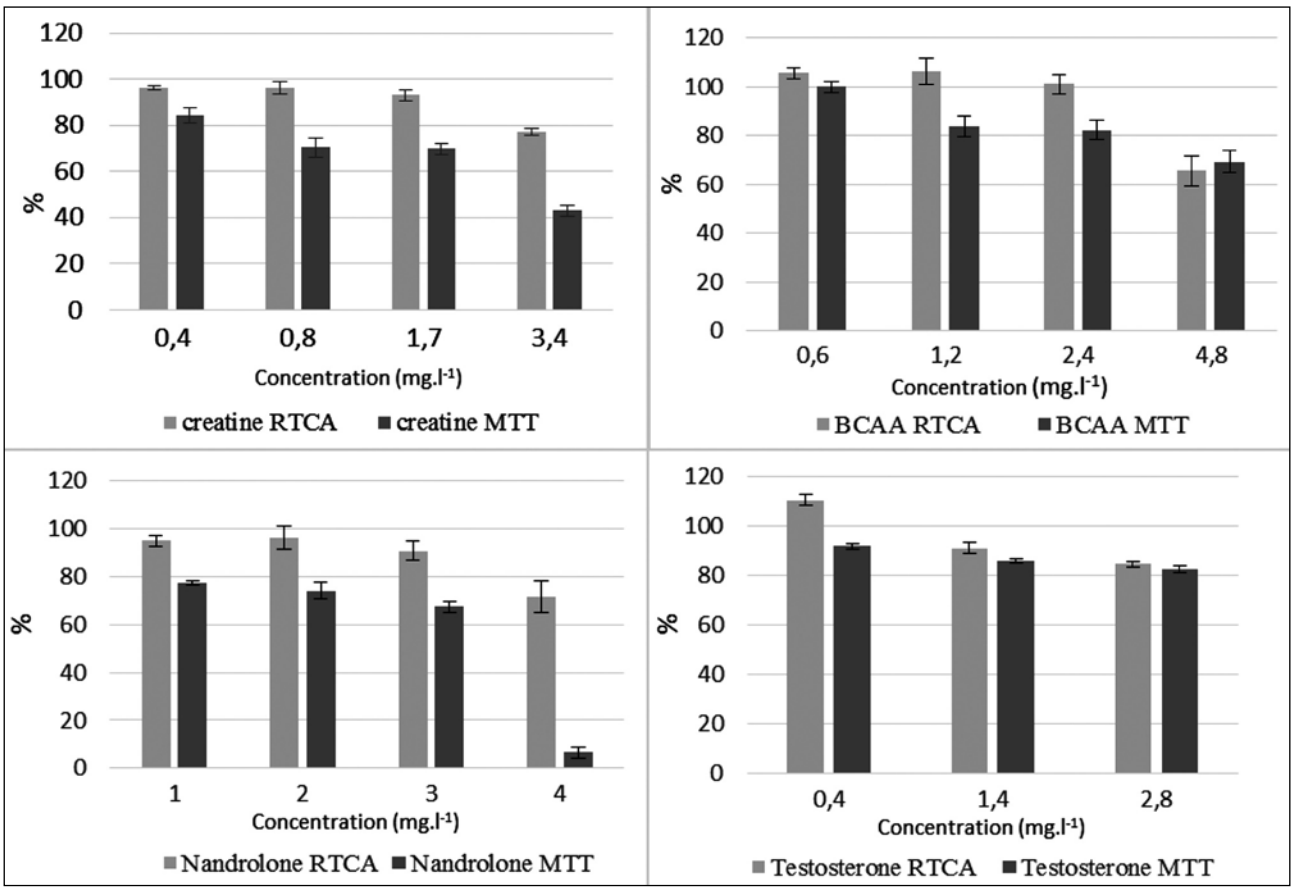


Fig. 2. Changes (%) in proliferation (RTCA) and viability (MTT) of cells treated with tested substances compared to control cells

were observed in growth curves, the ability to synthesize new DNA was impaired and the viability of the cells was affected. Similar results were reported by Okazaki et al. [5], who detected acute toxicity in mice and rats after the administration of BCAA. Tests on animals indicated the relative safety of amino acids administration provided that the ratios of the three amino acids (leucine, isoleucine, valine) resemble those in animal proteins.

CONCLUSIONS

Our results showed a dose-dependent cytotoxic effect of the tested substances on hepatocytes. The highest effect on these cells was observed after exposure to nandrolone; however, even with exposure to testosterone, creatine and BCAA, the activity of the cells was considerably decreased. The branched amino acids (BCAA) appeared to be the least toxic as they caused no proliferation of the cells or other detectable toxic effects at the recommended daily dose (0.6 mg.l^{-1}). However, at increased doses, they also affected the cell proliferation and the cell index.

ACKNOWLEDGEMENT

The present study was supported by the National Reference Laboratory for Pesticides of the University of Veterinary Medicine and Pharmacy in Košice.

REFERENCES

1. Bagatell, C. J., Bremner, W. J., 1996: Androgens in men — uses and abuses. *N. Engl. J. Med.*, 334, 707—714.

2. Baume, N., Mahler, N., Kmaber, M., Mangin, P., Saugy, M., 2006: Research of stimulants and anabolic steroids in dietary supplements. *Scand. J. Med. Sci.Sports*, 16, 41—48.

3. Fotakis, G., Timbrell, J. A., 2006: In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, 160, 171—177.

4. Melchert, R. B., Welder, A. A., 1995: Cardiovascular effects of androgenic–anabolic steroids. *Med. Sci. Sports Exerc.*, 27, 1252—1262.

5. Okazaki, S., Hatakeyama, K., Tamura, K., Tsufuhisa, S., Shiotani, S., 1989: Acute and subacute toxicity study of BCAA-G in rats. *Clin. Rep.*, 23, 1843—1862.

6. Stimac, D., Milic, S., Dintinjana, R. D., Kovac, D., Ristic, S., 2002: Androgenic/anabolic steroid-induced toxic hepatitis. *J. Clin. Gastroenterol.*, 35, 350—352.

7. Xing, J. Z., Zhu, L., Gabos, S., Xie, L., 2006: Microelectronic cell sensors assay for detection of cytotoxicity and prediction of acute toxicity. *Toxicology in vitro,* 20, 995—1004.