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EXPRESSION OF CYTOKERATIN 18 IN THE PERIPHERAL NERVES

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ABSTRACT

The perineurium constitutes the basis for the regulation of endoneurial fluid homeostasis. In the work presented here, cytokeratin 18, as an immunohistochemical marker for epithelial cells, was used to identify the perineurium in the peripheral nerves of two species. Two organs, rich in peripheral nerves, were used; the tongue of the bull and the *ductus deferens* of the male goat. Special attention was paid to one of the the nerve sheath cells — the perineurial cells of myelinated nerves in the skeletal muscle of the tongue and in the smooth muscle in the wall of the *ductus deferens*. A positive reaction to cytokeratin 18 was found in the perineurial cells of the perineurial sheath in the nerves of various sizes. No difference in the reactivity was observed between the peripheral nerves of the tongue and that of the *ductus deferens*.

Key words: bull; cytokeratin 18; *ductus deferens*; goat; peripheral nerves; tongue

INTRODUCTION

Histologically, the peripheral nerves consist of axons and coats — the endoneurium, perineurium, and epineurium. The endoneurium surrounds each nerve fibre, while the perineurium isolates individual nerve fascicles. The epineurium forms the outer surface of a peripheral nerve and consists of connective tissue. The perineurium forms a protective barrier around nerve fascicles. It is made of layers of perineurial cells surrounded by a basement membrane and collagen fibers, forming a concentrically laminated structure around a single nerve fascicle. Morphologically, perineurial cells resemble fibroblasts. The perineurial cells are characterized by distinct ultrastructural features which include: non-branching thin cytoplasmic processes coated by an external lamina and joined at their ends by tight junctions, few organelles, actin and vimentin filaments, and numerous pinocytotic vesicles. They are 3-dimensionally flattened, polygonal, or squamous cells, and form many concentric layers around the axons [15], [16]. Functionally, the perineurium modulates external stretching forces that could be potentially harmful for nerve fibers, and addition-

ally, along with endoneurial vessels, forms the blood-nerve barrier [17]. The perineurium constitutes the basis for the regulation of endoneurial fluid homeostasis and therefore is comparable to the blood-brain barrier of the CNS. It protects the Schwann cell axon units and the endoneurial connective tissue from ionic alterations, antigens, toxins and infectious agents.

Cytokeratins (CK) are considered markers of epithelial differentiation and the cytokeratins typical of simple epithelia, with the primary keratin pair 8/18 as a significant component, have been detected in neuroectodermal tissues [2],[6], [13]. In lower vertebrates, immunoreactivity for keratin 8 and 18 has been reported in nonepithelial cells; particularly in mesenchymal progenitor cells of regenerating complex body structures [3]. In contrast, cytokeratin intermediate filaments (IF) and desmosomes have not been detected in the glial cells of the brain and spinal cord, or in certain peripheral nerves [15].

Multiple pathologic conditions associated with the perineurium have been described, including certain non-epithelium-derived tumors. Perineurial-like cells were noted in three tumors and cells intermediate between perineurial and Schwann cells [10]. Cytokeratins have been reported to occur, occasionally and focally in certain human ependymomas, astrocytomas, and primitive neuroectodermal tumors [4], [8], [18]. To clarify the cellular composition of various peripheral nerve tumorous lesions, the expression of several markers specific to nerve sheath cells, were immunohistochemically investigated [11]. The aim of this study was to identify localized perineurial cells by the immunohistochemical marker cytokeratin 18 in the tongue and *ductus deferens* of the adult bull and goat (respectively).

MATERIALS AND METHODS

Tissue samples of the tongue of the bull and the *ductus deferens* of the goat were used in this study. Samples of the tissues were fixed in 10% neutral formalin in 0.2 mol phosphate buffer for 24 h and thereafter routinely embedded in paraffin. For the immunohistochemical study, the deparaffinized and rehydrated sections were rinsed in phosphate-buffered saline (PBS) and endogenous peroxidase was blocked by incubation with 3% v/v H₂O₂ for 20 minutes. After rinsing with PBS, the sections were incubated with 1% w/v Bovine Serum Albumin (BSA) in PBS at room tempera-

ture to prevent unspecific binding. The sections were then incubated in a humidified chamber overnight with primary antibodies. The washed sections were incubated overnight with monoclonal antibody against cytokeratin 18 (mouse IgG2b, clone 611 B-1, Sigma). After incubation, the sections were stained using the avidin-biotinylated-peroxidase complex detection system (ABC kit; Vector Laboratories, Burlingame, CA). The slides were then washed three times for 5 min with PBS. The specific binding was visualized with the peroxidase substrate 3,3'-diaminobenzidine (DAB). For negative controls, the first antibody was substituted by PBS or by normal rabbit serum. Sections were counterstained with Mayer's hematoxylin and methylene blue.

RESULTS

Perineurial cells of the perineurium found in both organs are elongated spindle shaped cells with eosinophilic cytoplasm with curved or wavy thin nuclei and thin, elongated cytoplasmic processes, arranged in lamellae. Microscopically, two components were found in the perineurium: fibrous perineurium, an outer layer of collagenous connective tissue and perineural epithelium, and an inner layer of squamous epithelial cells. Collagen fibrils are dispersed between the layers. Perineural epithelium forms a continuous sheet. The number of layers decrease in number as fascicles become smaller (Figures 1—2).

Positive reactions to CK 18 in both organs studied were found in the perineurial sheaths of the nerves of various sizes. The intensity of reactivity was equal in all perineurial cells and was equal in the cells body and in cytoplasmic processes which formed the long flattened lamellae. The endoneurium was not stained with CK 18 (Figures 1—3).

DISCUSSION

It is widely accepted that cytokeratins are usually restricted to epithelium-derived tissues. This has been elaborated in several mammals, notably: man, cow, rat and mouse (and also in birds). In the fetal neuroectodermal tissues studied, the cytokeratin pairs 8/18 are the first ones to be expressed during embryonic development [13]. Cytokeratins 8 and 18, occasionally accompanied by CK19, can be seen, often transiently, in certain non-epithelial tissues,



Fig. 1. Immunoreactivity for CK 18. The nerves in cross sections in the tongue muscle. Perineurium is positively stained for CK 18 (arrows)

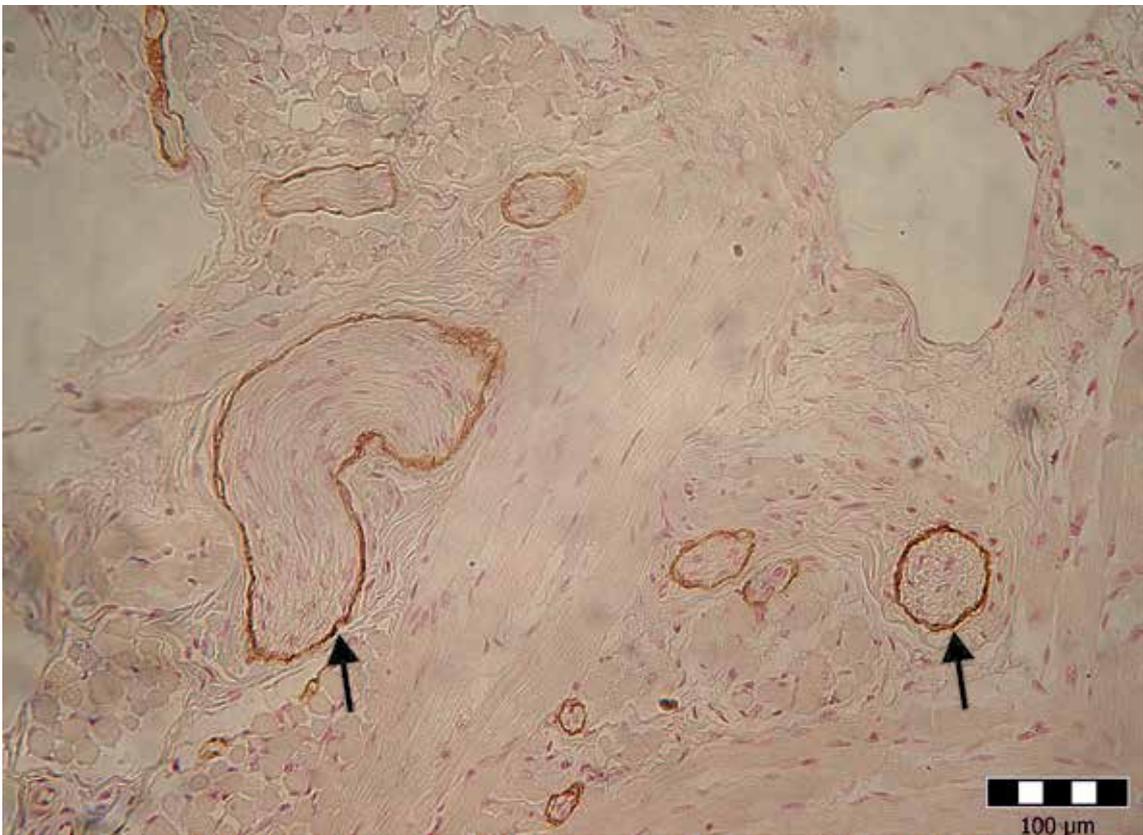


Fig. 2. Immunoreactivity for CK 18. The nerves of various sizes in cross and oblique sections in tongue muscle. Perineurium is positively stained for CK 18 (arrows)



Fig.3. Immunoreactivity for CK 18. The small nerves in cross sections in the connective tissue of the *ductus deferens*. Perineurium is positively stained for CK 18 (arrows)

especially embryonal ones, and in a rare subtype of human endothelial cells [6], [12]. A small amounts of cytokeratins 8, 18, and 19 (or their homologues) have been identified in certain amphibian and human smooth muscle tissues [12].

Numerous studies of the embryogenesis of the avian and mammalian nervous systems and of in vitro differentiation of neural cells have shown that differentiating glial and neuronal cells are characterized by the absence of cyto-

keratin [7]. In higher vertebrates, the cytoskeleton of glial cells are characterized by masses of intermediate filaments (IF) that contain the hallmark protein of glial differentiation (the glial filament protein) and also by the absence of cytokeratin IFs.

The single or multi-layered sheaths of epithelioid cells surrounding peripheral nerves (perineurial cells) or structures of the central nervous system, including the optic

nerve (arachnoid cells), show remarkable interspecies differences in their cytoskeletal complements [2]. In some amphibia, the perineurial cells of the sheaths of peripheral nerves, as well as the arachnoidal cells of the meninges, are interconnected by desmosomes and contain cytokeratin intermediate filaments; an occurrence that has also been reported for some mammals [1]. Among higher vertebrates, a similar situation is found in the bovine and chicken nervous systems; the perineurial cells of many nerves contain cytokeratin intermediate filaments. Intense staining for cytokeratins was observed, not only in the meningeal cells, but also in the glial elements of the nerve interior. In contrast, in rat perineurial cells, significant reactions have not been observed for cytokeratins or for desmosomal proteins [2]. In our study, CK 18 was observed in the peripheral nerves of both organs studied and thus, both animal species.

The cytogenesis of the perineurium remains disputable, with morphologic, immunohistochemical, and experimental evidence supporting an origin from the fibroblast, Schwann cell, and arachnoid cap cells [5]. It was found that all meningeal layers of the spinal cord, the perineurium and the endoneurium of peripheral nerves, as well as the capsular and inner space cells of Herbst sensory corpuscles, develop from the local mesenchymal cells, whereas Schwann cells and cells of the inner core of sensory corpuscles are of neural crest origin [9]. The selective mesenchymal expression of keratins includes the presence of keratins 7, 8 and 18 in some smooth muscle cells (myometrium, vascular and some parenchymal smooth muscle) and expression of keratins 7 and 18 in some vascular endothelial cells [14]. Moreover, small amounts of cytokeratins 8, 18, and 19 (or their homologues) have been recently identified in certain amphibian and human smooth muscle tissues [12]. Thus, the expression of cytokeratins may be characteristic of a certain type of astrocytes that remains in an embryonal proliferative state.

CONCLUSIONS

A positive reaction to cytokeratin 18 was found in the perineurial sheath of the nerves in the skeletal muscle of the tongue of the bull and in the smooth muscle of the wall of the *ductus deferens* in the goat. No difference in the reactivity was observed between the peripheral nerves of the organs studied. The morphological features common for

perineurial cells and described in the peripheral nerves were confirmed by immunostaining in the tongue and *ductus deferens* of the bull and goat.

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LABLAB PURPUREUS SEEDS DISRUPT HEPATIC AND RENAL ANTIOXIDANT STATUS IN MALE RATS

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ABSTRACT

Nutrition constitutes an essential aspect of health care in both humans and other animals. Despite the numerous studies conducted on *Lablab purpureus* seeds, there is a paucity of information on its effects on hepatic and renal antioxidant status. The present study investigated the influence of three varieties of *Lablab purpureus* seeds (Rongai Brown, Rongai White and Highworth Black) on hepatorenal antioxidant status in male Wistar rats. Group I (control) rats were fed with the standard rat chow for 14 days while Groups II, III and IV rats were separately fed with feed containing the Rongai Brown, Rongai White and Highworth Black for 14 days, respectively. *Lablab purpureus* caused a significant decrease in renal superoxide dismutase (SOD) but increased hepatic SOD activity along with increased catalase, glutathione-S-transferase and glutathione peroxidase activities in both liver and renal tissues when compared with the control. Moreover, there was a significant decrease in the hepatic glutathione (GSH) level with concomitant elevation in hepatic and renal hydrogen peroxide and

lipid peroxidation levels in all *Lablab purpureus*-fed rats. *Lablab purpureus*-fed rats demonstrated significant elevations in serum marker enzymes, aspartate aminotransferase and alkaline phosphatase along with increases in urea and creatinine levels. Histopathologically, kidney sections revealed normal renal architecture, whereas treatment-related lesions were identified in the liver of *Lablab purpureus*-fed rats. This study concluded that consumption of raw *Lablab purpureus* seeds induced hepatorenal toxicity in rats via the induction of oxidative stress.

Key words: antioxidant; hepatorenal toxicity; *Lablab purpureus*; oxidative stress; rats

INTRODUCTION

Legumes are a vital component of the diet for both human and other animals because they contain different bioactive compounds that may have beneficial effects [29]. *Lablab purpureus* is a drought-resistant legume which

remain green during the dry season. It is a domesticated legume specie widely cultivated to provide adequate nourishment for animal production and it constitutes the main leguminous fodder crop in several developing countries including Sudan and India [4], [19]. It is used in combination with natural pastures and crop residues to reduce weight losses in livestock during the dry season. The herbage is also applied to control erosion, used for green manure and feed supplement for cattle grazing in the dry season [12]. The seed and immature pods are used as human food and herbal medicines [6], [21].

The nutritive value of *Lablab purpureus* seeds has been reported [1], [13], [23], [32]. However, the concomitant occurrence of toxic anti-nutritional factors such as haemagglutinins, saponins, phytic acids, amylase inhibitors, trypsin inhibitors, tannis, lectins, alkaloids and cyanogens in lablab beans has been reported [1], [14], [30], [33]. The antioxidant status shows the dynamic balance between the cellular reactive oxygen species (ROS) production and the antioxidant defense capacity and it has been demonstrated to be a useful tool in estimating the risk of oxidative damage [22], [34]. Previous investigations from our laboratory have implicated *Lablab purpureus* in the impairment of testicular function of spermatogenesis in laboratory animals through the generation of elevated reactive oxygen species [31]. In spite of the several studies conducted on this important legume, there is a dearth of information in the literature on the effects of *Lablab purpureus* varieties namely the Rongai White, Rongai Brown and Highworth Black on the antioxidant statuses of the liver and kidney. The present investigation assessed the hepatic and renal antioxidant enzymes activities, lipid peroxidation levels along with the histology of the liver and kidney in rats following the consumption of the three varieties of *Lablab purpureus* for fourteen days.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), epinephrine, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grades and were obtained from the British Drug Houses (Poole, Dorset, UK).

Percentage feed composition

Three varieties of *Lablab purpureus*, namely Rongai Brown (P1 509114), Rongai White (NAPRI 4) and Highworth Black (GRIF 12293) obtained from the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria were used in this study. Three types of feed containing the three varieties of raw *Lablab purpureus* were separately prepared. All of the feeds were calculated and prepared to contain 20% crude protein and 3 kilocalories of energy (iso-proteinous and iso-caloric diet). The percentage feed composition of the control and test diets are presented in Table 1. The proximate analysis of the three varieties of raw *Lablab purpureus* seeds has been previously reported [32].

Table 1. Percentage feed composition of the control and test diets

Ingredients	Control	Rongai Brown	Rongai White	Highworth Black
Casein	20	–	–	–
Lablab bean meal	–	82.8	86.6	87.7
Corn starch	15	3.6	1.7	1.15
Sucrose	50	–	–	–
Corn oil	5.0	3.6	1.7	1.15
Cellulose	5.0	5.0	5.0	5.0
Methionine	0.3	0.3	0.3	0.3
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2	0.2

Vitamin A 10 000 000 IU.2.5 kg⁻¹; Vitamin D3 2 000 000 IU. 2.5 kg⁻¹; Vitamin E 12 000 mg.2.5 kg⁻¹; Vitamin K3 2000 mg. 2.5 kg⁻¹; Vitamin B1 1 500 mg.2.5 kg⁻¹; Vitamin B2 5000 mg. 2.5 kg⁻¹; Vitamin B6 500 mg.2.5 kg⁻¹; Vitamin B12 10 mg. 2.5 kg⁻¹; niacin 15 000 mg. 2.5 kg⁻¹; Calpan 5 000 mg.2.5 kg⁻¹; folic acid 600 mg.2.5 kg⁻¹; biotin 20 mg.2.5 kg⁻¹; choline chloride 150 000 mg.2.5 kg⁻¹; manganese 80 000 mg.2.5 kg⁻¹; iron 40 000 mg.2.5 kg⁻¹; zinc 60 000 mg.2.5 kg⁻¹; copper 8 000 mg. 2.5 kg⁻¹; iodine 1 000 mg.2.5 kg⁻¹; cobalt 25 mg.2.5 kg⁻¹. Source: [31].

Experimental protocol

Thirty-two adult male Wistar rats (195 ± 3g) obtained from the animal house of the Faculty of Veterinary Medi-

cine, University of Ibadan, Ibadan, were used for this study. The animals were housed in plastic cages placed in a well-ventilated rat house, provided rat chow and water *ad libitum* and subjected to natural photoperiod of 12-hr light: 12-hr dark. All the animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health. The ethic regulations have been followed in accordance with National and institutional guidelines for the protection of animal welfare during experiments [24].

The rats were randomly assigned into four groups of eight rats per group. Group I rats were fed with the standard rat chow for 14 consecutive days and served as the control. Rats in groups II, III and IV were fed with feed containing Rongai Brown, Rongai White and Highworth Black respectively for 14 consecutive days. Twenty-four hours after the 14th day, all the animals were killed by cervical dislocation under light ether anaesthesia. The livers and kidneys were quickly removed, weighed, and placed on an ice bath. The blood was allowed to clot and centrifuged at low speed 3000 g at room temperature for 15 minutes.

This study is in agreement with the standard on the use of laboratory animals as reported by the [35].

Plasma biochemistry

The biomarkers of liver and kidney function were assessed using commercially available kits from Randox Laboratory Limited (UK). Serum activities of aspartate aminotransferases (AST) and alkaline phosphatase (ALP) were measured according to the method described by [27]. Serum urea and creatinine levels were estimated according to the method of [15].

Determination of liver and kidney antioxidant statuses

The post-mitochondrial fraction of the liver and kidney of the rats were prepared by homogenizing in 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride. The homogenate was subsequently centrifuged at 12000 g for 15 min at 4°C and the supernatant was collected for biochemical parameters. Protein concentration was determined according to the method of [18]. Superoxide dismutase (SOD) activity was assayed according to the method described by [20]. Catalase (CAT) activity was assayed using hydrogen peroxide as a substrate according to the method described by [7]. Glutathione peroxidase

(GPx) activity was determined according to the method of [28]. Glutathione-S-transferase (GST) was assayed by the method of [11]. Reduced glutathione (GSH) level was determined at 412 nm using the method described by [16]. Hydrogen peroxide (H₂O₂) generation was determined according to the method described by [36]. Lipid peroxidation (LPO) was quantified as malondialdehyde (MDA) according to the established method [9] and the result expressed as micromoles of MDA per milligram protein.

Microscopic examination of the liver and kidney

Samples of liver and kidney from rats in each group were fixed in 10% formalin and processed for histology according to [5]. Briefly, liver and kidney specimens were fixed in 10% neutral-buffered formaldehyde solution. After dehydration procedures, the samples were embedded in paraffin. Sections of 4–5 μm were cut by a microtome and stained with hematoxylin and eosin (H&E). All slides were coded before examination under a light microscope (Olympus CH; Olympus, Tokyo, Japan) by pathologists who were blinded to control and treatment groups. Photomicrographs were taken with a Sony DSC-W 30 Cyber-shot (Sony, Tokyo, Japan).

Statistical Analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's test to identify significantly different groups (SPSS for Windows, version 17). $P < 0.05$ was considered to be statistically significance.

RESULTS

Hepatic and renal antioxidant statuses

Figures 1–3 show the effects of the consumption of Rongai Brown, Rongai White and Highworth Black on antioxidant systems and lipid peroxidation in the liver and kidney of the rats. The three varieties of *Lablab purpureus* significantly increased the activity of hepatic SOD whereas Rongai White and Highworth Black significantly decreased the renal SOD. The activities of CAT, GST and GPx were significantly increased in all the *Lablab purpureus*-fed rats when compared with the control. Hepatic GSH level was significantly decreased, whereas renal GSH levels remain unaffected in all the *Lablab purpureus*-fed rats

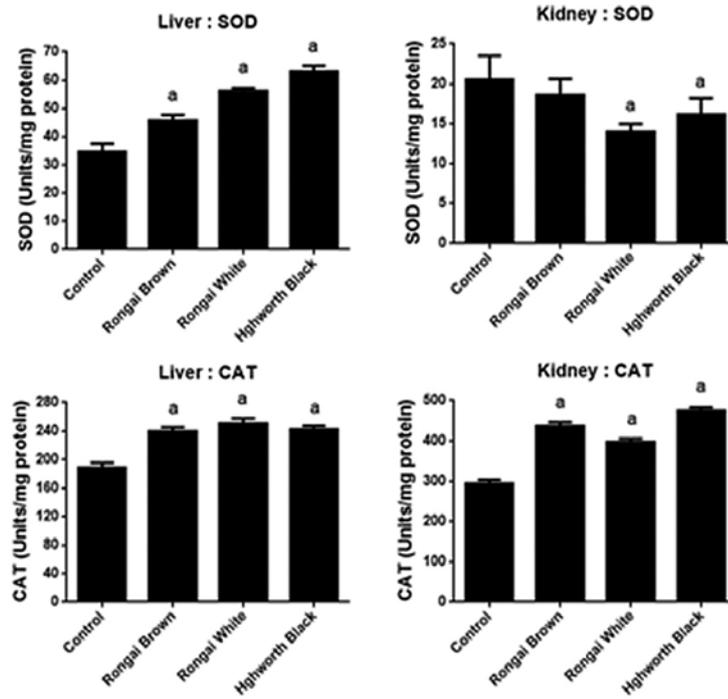


Fig. 1. Activities of SOD and CAT in the liver and kidney of rats following consumption of Rongai brown, Rongai white and Highworth black for 14 consecutive days. Values are expressed as mean \pm SD of eight rats. ^a — P < 0.05 versus control

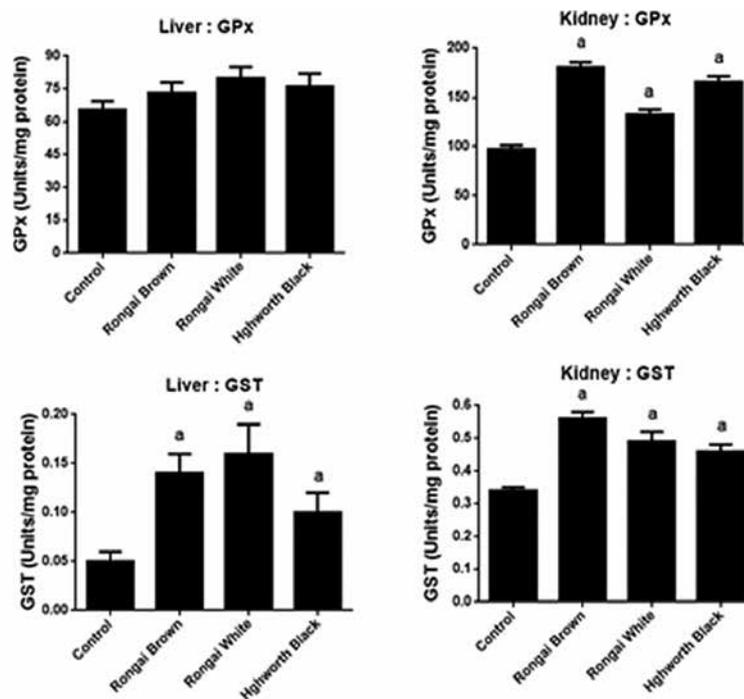


Fig. 2. Activities of GPx and GST in the liver and kidney of rats following consumption of Rongai brown, Rongai white and Highworth black for 14 consecutive days. Values are expressed as mean \pm SD of eight rats. ^a — P < 0.05 versus control

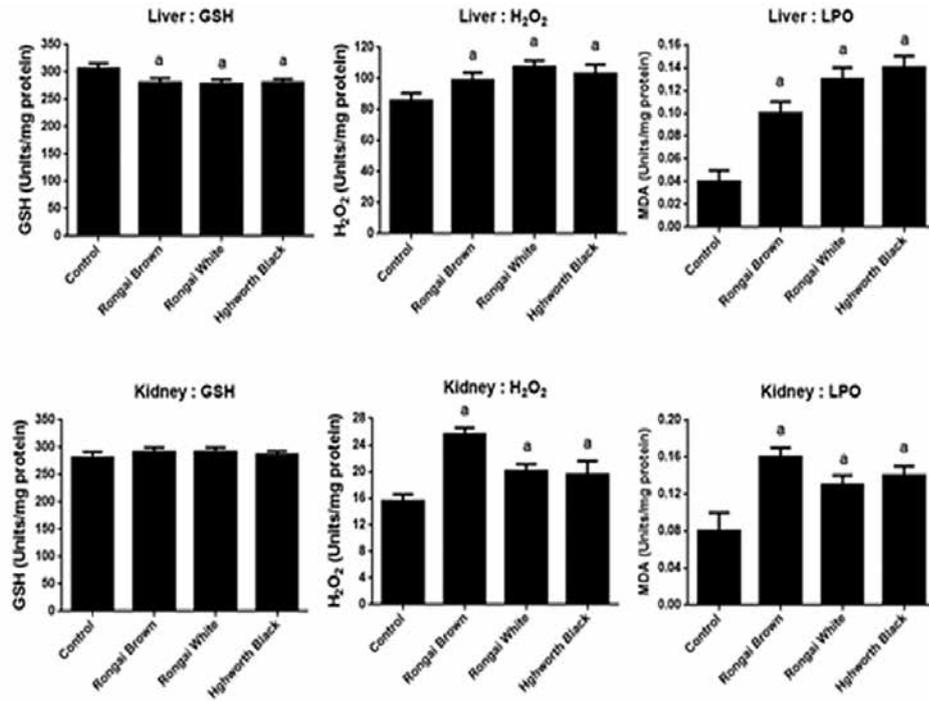


Fig. 3. Levels of GSH, H₂O₂ and LPO in the liver and kidney of rats following consumption of Rongai brown, Rongai white and Highworth black for 14 consecutive days. Values are expressed as mean ± SD of eight rats. a — P < 0.05 versus control

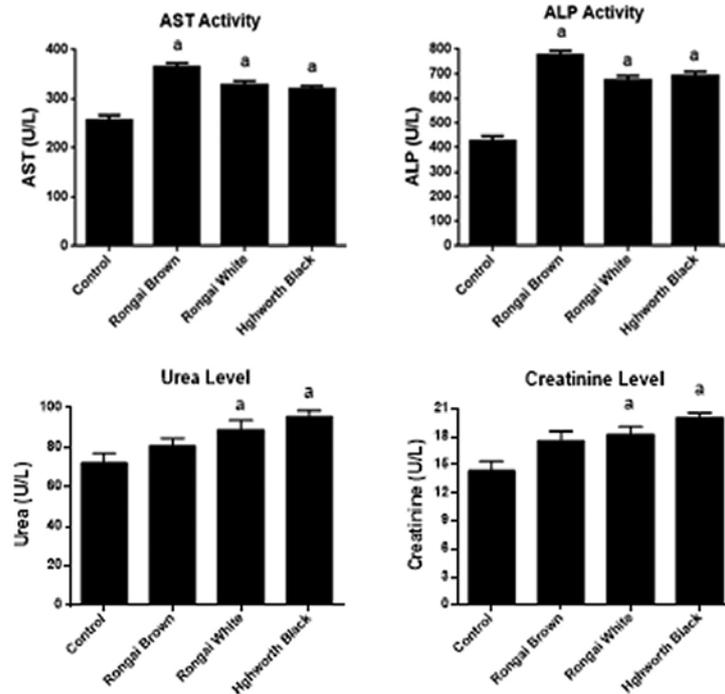


Fig. 4. Biomarkers of liver function enzymes (AST and ALP) and kidney function indices (urea and creatinine level) in rats following the consumption of the Rongai brown, Rongai white and Highworth black for 14 consecutive days. Values are expressed as mean ± SD of eight rats. a — P < 0.05 versus control

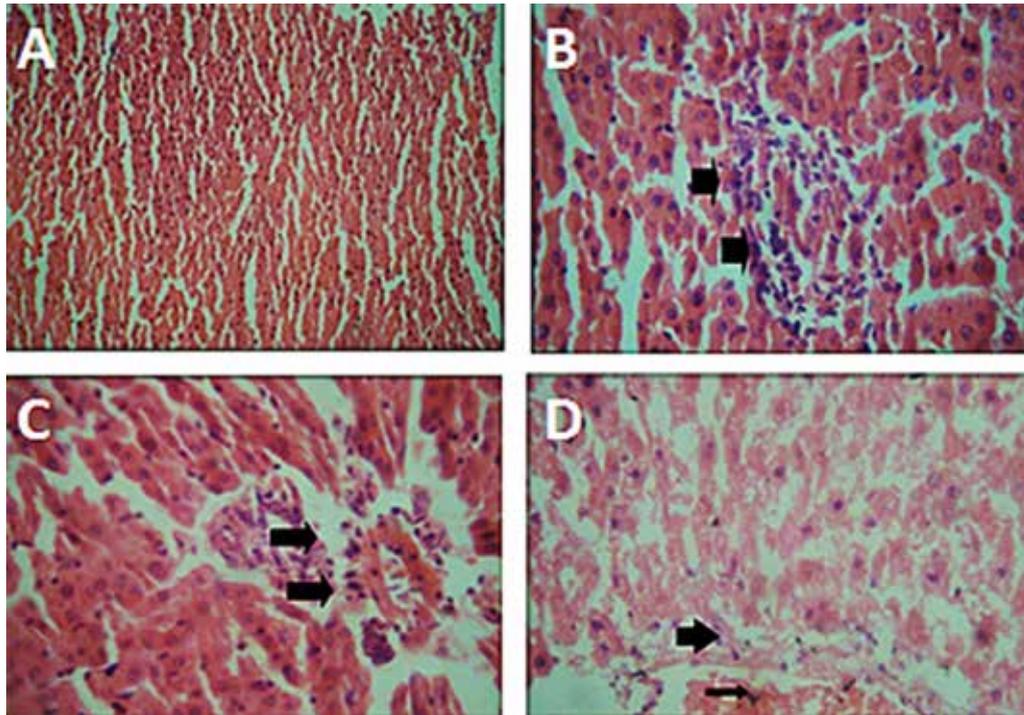


Fig. 5. Photomicrographs of the liver. Control rat showing normal liver architecture (A). Liver of rats fed with Rongai brown diet showed central venous and portal congestion with mild mononuclear cell infiltration (B). Mild periportal cellular infiltration was identified in the liver of Rongai white-fed rats (C). Few foci of cellular infiltration with hepatic necrosis were identified in the liver of Highworth black fed rats (D). Original magn. $\times 240$

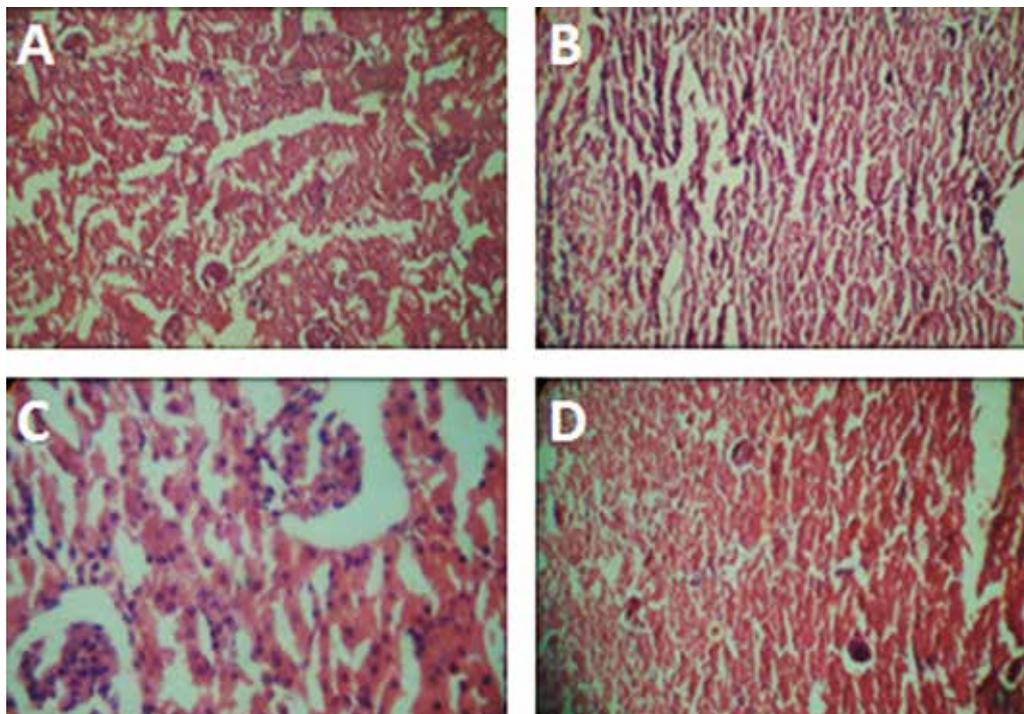


Fig. 6. Photomicrographs of the kidney showing normal renal architecture of control and Lablab purpureus-fed rats. Control rat (A). Rats fed with Rongai brown diet (B). Rats fed with Rongai white diet (C). Rats fed with Highworth black diet (D). Original magn. $\times 240$

when compared with the control. Moreover, the levels of malondialdehyde (MDA) which is an index of lipid peroxidation and H_2O_2 generation were significantly increased in the liver and kidney of animals fed with the three varieties of *Lablab purpureus* when compared with the control.

Hepatic and renal function indices

To investigate the integrity of the liver and kidney in the experimental rats, the levels of biomarkers of hepatic and renal damage were determined. Fig. 4 shows that the serum levels of hepatic dysfunction, namely AST and ALP, as well as renal functional indices, namely urea and creatinine were significantly increased following the consumption of the three varieties of *Lablab purpureus*. AST activity significantly ($P < 0.05$) increased by 24.10 %, 18.90 % and 15.62 % in Rongai Brown, Rongai White and Highworth Black, respectively, compared with the control. ALP activity significantly ($P < 0.05$) increased by 81.70 %, 58.05 % and 61.85 % in Rongai Brown, Rongai White and Highworth Black, respectively, compared with the control. The serum creatinine level was elevated by 1.22 %, 11.59 % and 16.4 %, whereas the urea level increased by 0.71 %, 7.04 %, and 19.92 % in the Rongai Brown, Rongai White and Highworth Black, respectively, compared with the control.

Histology

Histopathological examinations of the kidney sections with the light microscope showed that the control and the *Lablab purpureus*-fed rats had normal renal architecture (Fig. 6). However, while the control liver appeared structurally normal, there were treatment-related lesions identified in the liver of *Lablab purpureus*-fed rats. Histopathological changes observed in the liver of rats fed with Rongai Brown diet include central venous and portal congestion with mild mononuclear cell infiltration. The liver of animals fed with Rongai White diet showed mild periportal cellular infiltration whereas rats fed with Highworth Black diet showed few foci of cellular infiltration with hepatic necrosis when compared with the control (Fig. 5).

DISCUSSION

This study demonstrated that the consumption of unprocessed *Lablab purpureus* caused liver and kidney damage in rats as evidenced by the increases in serum marker

enzymes AST and ALP, along with increases in urea and creatinine levels. Serum levels of liver enzymes including AST and ALP are generally used in the assessment of hepatic damage. Transaminases are used as specific indicators for liver damage because they play a vital role in the amino acid metabolism and biosynthesis of energetic macromolecules for different important cellular functions [17]. The increase in AST activity may be due to liver dysfunction and disruption in the integrity of the liver cell membrane [17], [3]. Alkaline phosphatase (ALP) is a biomarker for evaluating the integrity of the hepatobiliary system and bile flow into the small intestine [3]. The increase in hepatic ALP activity observed in this study indicated obstructive events or cholestatic effects in *Lablab purpureus*-fed rats. Serum urea and creatinine levels were significantly elevated in rats fed with the *Lablab purpureus* seeds. The increase in urea may be due to the insufficiency in renal excretion processes, whereas an increase in serum creatinine level may indicate an impaired kidney function [2].

Lablab purpureus increased the oxidative stress by disrupting the antioxidant defense mechanisms in the liver and kidney of rats. The superoxide radical is converted to H_2O_2 by the SOD, whereas CAT and glutathione peroxidase (GPx) are responsible for the detoxification of H_2O_2 . Moreover, GST is directly involved in the elimination of electrophilic oxidants by conjugating them with GSH [20]. In this study, the consumption of unprocessed *Lablab purpureus* seeds caused a significant decrease in renal SOD but increased hepatic SOD activity along with augmentation in the CAT, GPx and GST activities in both liver and renal tissues, thus suggesting their induction possibly to combat reactive oxygen species (ROS) generation during *Lablab purpureus* metabolism in these tissues. Indeed, increased production of singlet oxygen and peroxy radicals has been demonstrated to directly inhibit SOD activity [8]. Thus, the decrease in the SOD activity is indicative of enzyme inhibition possibly due to excessive ROS production in the treated rats. The observed decrease in the hepatic GSH level in the present study may indicate an increased demand of GSH to detoxify ROS generation in the tissue.

Furthermore, the present investigation revealed that the consumption of *Lablab purpureus* caused oxidative stress in the liver and kidney of rats. The significantly elevated level of H_2O_2 observed in the liver and kidney shows that the increase in antioxidant defense enzymes (CAT, GPx and GST) in rats fed with the three varieties of *Lablab purpureus*

was insufficient to eliminate this noxious substance from the hepatic and renal tissues. Previous studies have shown that CAT is the most adaptive antioxidant enzyme that plays a key role in cell protection during oxidative stress [10], [25], [26], [34]. The observed increases in CAT activity therefore, suggests an adaptive response to high concentrations of H₂O₂ during the treatment with *lablab purpureus* seeds.

Lipid peroxidation (LPO) is known to disrupt the integrity of cellular membranes and consequently results in the tissue injury. The increase in levels of malondialdehyde (MDA), a biomarker of LPO in the liver and kidney of *Lablab purpureus*-fed rats, may be attributed to the increased production of the reactive oxygen species and an altered antioxidant defense system. A previous study indicated that *Lablab purpureus* increased LPO in the testes of rats [31].

Light microscopy showed no treatment-related kidney histopathology in rats fed with the three varieties of *Lablab purpureus*, whereas liver damage was evident within the time course of this investigation. The lack of obvious kidney histopathology may be due to the short duration of the consumption of the feed. The histological changes observed in the liver of rats fed with the Rongai Brown diet include the central venous and portal congestion with mild mononuclear cell infiltration. The liver of rats fed with Rongai White diet showed mild periportal cellular infiltration whereas rats fed with Highworth Black diet showed few foci of cellular infiltration with hepatic necrosis. The alteration in the hepatic architecture in the present study may be attributed to the direct or indirect effects of increased ROS and consequently induced lipid peroxidation which is capable of disrupting its structure and function.

Based on the data presented, it can be concluded that the consumption of raw *Lablab purpureus* seed induced hepatorenal toxicity via a mechanism involving alteration of antioxidant defense systems, elevation in H₂O₂ generation and lipid peroxidation in the liver and kidney of the treated rats.

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VERTEBROBASILAR SYSTEM IN THE EUROPEAN HARE

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ABSTRACT

The aim of this study was to describe the arterial arrangement of the cervical spinal cord in the hare using the corrosion technique. The study was carried out on 10 adult European hares (*Lepus Europeanus*). The arterial system of the cervical spinal cord was injected using Batson's corrosion casting kit No.17. The fusion of the bilateral vertebral arteries was found in 70% of the cases without a connecting branch and in 30% of the cases with one connecting branch just posterior to the fusion. The ventral spinal artery was in connection with the right vertebral artery in 60% of the cases and by means of an anastomosis of two spinal branches arising from the bilateral vertebral arteries in 40% of the cases. Based on the results of this study, it is possible to conclude that there is a high variability of the blood supply to the cervical part of the spinal cord in the hare.

Key words: basilar artery; corrosion cast; rabbit; spinal cord; ventral spinal artery

INTRODUCTION

Injuries of the cervical spinal cord usually result in full or partial tetraplegia. However, depending on the specific location and severity of the trauma, limited function may be retained for example, in the ability to breath, which makes utilization of mechanical ventilators, phrenic nerve pacing etc., unnecessary [3].

Research on the arrangement and variability of the feeding arteries of the spinal cord in several species of laboratory animals [4], [8], [9], [12] and in man [1], [5], [6] is common. The arterial arrangement of the cervical spinal cord in the European hare has not been described so far.

The vertebral artery arises from the subclavian artery and emerges from the thoracic cavity through the cranial thoracic aperture. The artery then passes through the transverse opening of the sixth cervical vertebra into the transverse canal of the cervical vertebrae, in which it continues in the cranial direction. Along its course inside the transverse canal it gives off spinal branches. These spinal branches enter the vertebral canal through the intervertebral foramina. After entering the vertebral canal they send

ventral and dorsal branches to the spinal cord, which participate in the formation of the ventral and dorsal spinal arteries. Bilateral vertebral arteries enter the vertebral canal via lateral vertebral opening of the atlas. They fuse together on the caudal margin of the dorsal surface of the basilar part of occipital bone forming the basilar artery. The basilar artery participates in forming the arterial circle of the brain. In the place of formation of the basilar artery, it is located at the cranial connection of the spinal arteries to the vertebral arteries [4].

The aim of this study was to describe the arterial blood supply of the European hare spinal cord with a focus on the cervical region.

MATERIALS AND METHODS

This study was carried out on 10 adult European hares (*Lepus europaeus*, L. 1758), aged 140 days. Hares (obtained

from ISFA APRC, Nitra, Slovak Republic) of both sexes (female n=5; male n=5) with weights range of 2.5–3.2 kg were used in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15–20°C, relative humidity 45%, 12-hour light period), and fed with a granular feed mixture (O-10 NORM TYP, Spišské krmne zmesi, Spišské Vlachy, Slovak Republic). The drinking water was available to all animals *ad libitum*. The animals were injected intravenously with heparin (50000 IU.kg⁻¹) 30 min before they were sacrificed by the intravenous injection of embutramide (T-61; 0.3 ml.kg⁻¹). Immediately after euthanasia, the vascular network was perfused with a physiological solution. During manual injection through the ascending aorta, the right atrium of the heart was opened in order to lower the pressure in the vessels to ensure an optimal injection distribution. Batson's corrosion casting kit No. 17, using a volume of 50 ml (Dione, České Budějovice, Czech Republic) was used as

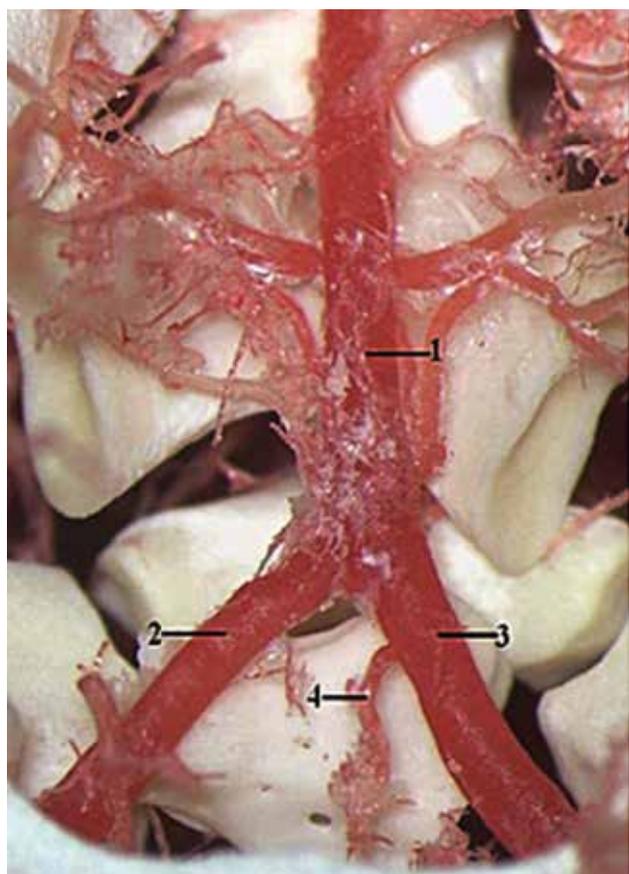


Fig. 1. Formation of the basilar artery without the posterior connection, the connection of the ventral spinal artery to the right vertebral artery. 1 — basilar artery; 2 — left vertebral artery; 3 — right vertebral artery; 4 — ventral spinal artery. Dorsal view. Magn. ×12.5

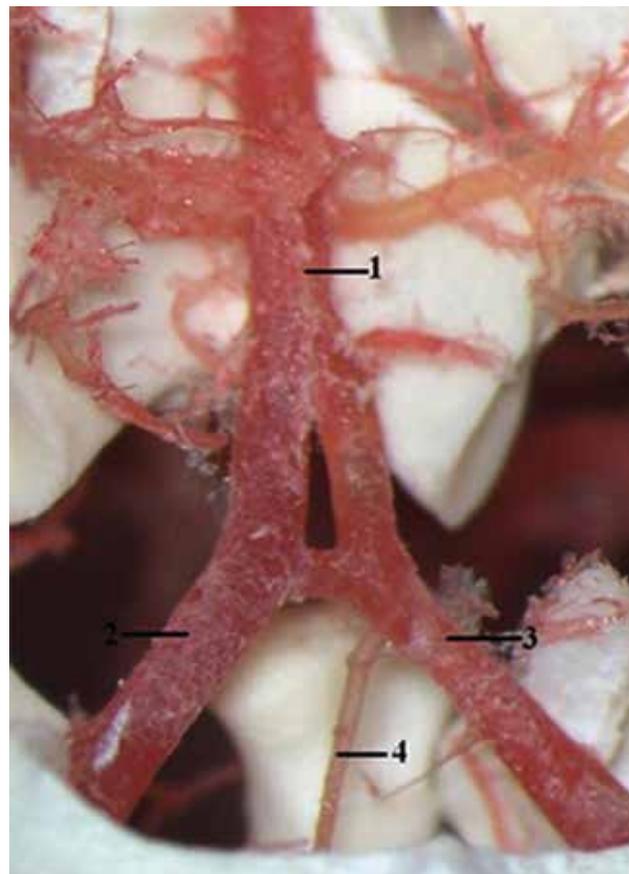


Fig. 2. Formation of the basilar artery with the posterior connection, the connection of ventral spinal artery to the right vertebral artery. 1 — basilar artery; 2 — left vertebral artery; 3 — right vertebral artery; 4 — ventral spinal artery. Dorsal view. Magn. ×12.5

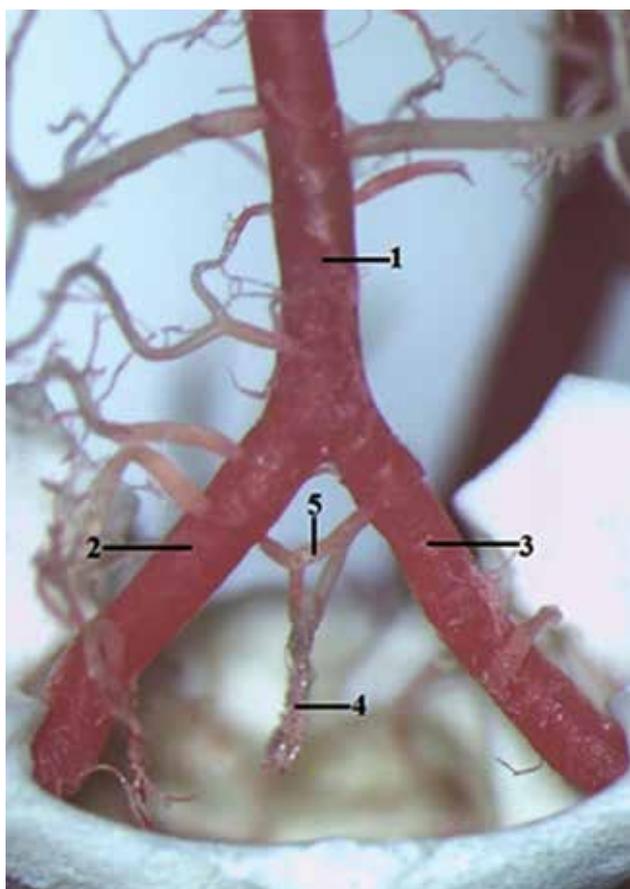


Fig. 3. Connection of the ventral spinal artery to the vertebral arteries by means of anastomosis of two spinal branches, a communicating branch between bilateral spinal branches. 1 — basilar artery; 2 — left vertebral artery; 3 — right vertebral artery; 4 — ventral spinal artery; 5 —communicating branch. Dorsal view. Magn. $\times 12.5$

the casting medium. After polymerization of the medium, maceration was carried out in 2—4% KOH solution for a period of 2 days at 60—70°C. This study was carried out under the authority of decision No.2647/07-221/5.

RESULTS

The blood supply of the cervical spinal cord is more complicated than in the other parts of the body. The vertebral artery, basilar artery and caudal cerebellar artery as branches of the basilar artery give off a lot of small branches participating in the blood supply to the most cranial part of the spinal cord.

The vertebral artery entered the vertebral canal through the foramen vertebrale laterale of the atlas. On the caudal margin of the dorsal surface of the *pars basilaris ossis occipitalis* it was fused with the contralateral vertebral artery.

This fusion formed the basilar artery. The fusion of the bilateral vertebral arteries was found in 70% of the cases without a posterior connection (Figs. 1, 3) and in 30% of the cases with a posterior connection (Fig. 2). At the level of the formation of the basilar artery, different connections of the ventral spinal artery to the vertebral artery were found. It was in connection with the right vertebral artery in 60% of cases (Figs. 1—2) and by means of an anastomosis of two spinal branches with the bilateral vertebral arteries in 40% of the cases. In the case of an anastomosis, a communicating branch was located above the two branches (Fig. 3). Each of these branches were in connection with the medial flank of the vertebral artery on the same side. Using this technique, it was not possible to describe the segmental arteries entering the ventral and dorsal spinal arteries.

DISCUSSION

Based on our study we can conclude that there is a different arterial arrangement in the hare compared with humans. We found the formation of the basilar artery without a posterior connection of the bilateral vertebral arteries in most cases. In humans, the basilar artery is formed by the fusion of the bilateral vertebral arteries without this connection [2]. In hares, the ventral spinal artery (in humans the anterior spinal artery) was in connection with the right vertebral artery, or by means of anastomosis of two spinal arteries originating from both vertebral arteries. These two arteries were originating from the medial flank of the vertebral artery on the corresponding sides. The anterior spinal artery in humans is formed only by the fusion of the anterior spinal branches of the vertebral arteries at the level of foramen magnum [7].

In the rabbit, the formation of the basilar artery was formed by the fusion of the bilateral vertebral arteries without any posterior connection, or two connections [4]. We found the formation of the basilar artery without any posterior connections or with one posterior connection. The origin of the ventral spinal artery was described from the right vertebral artery, from the left vertebral artery and from the anastomosis of two ventral spinal arteries, each originating from the medial flank of the vertebral artery on the same side [4]. In our study, we found the connection of the ventral spinal artery with the right vertebral artery or by means of anastomosis of two spinal branches with

a communicating branch between each other with bilateral vertebral arteries. We did not find the connections with the dorsal spinal arteries in our study.

CONCLUSIONS

The study of the arterial patterns of the spinal cord based primarily on the use of an experimental animal might help to elucidate the principles of how the blood vessels are distributed to the spinal cord. At the same time, such an effort provides additional information concerning the manner of vascularization of the central nervous system in general [10], [11], [13].

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DORSAL BRANCHES OF ABDOMINAL AORTA IN THE RABBIT AND THE EUROPEAN HARE

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ABSTRACT

The aim of this study was to describe the anatomical arrangement of the branches arising from the dorsal surface of the *aorta abdominalis* in the rabbit and the hare. The study was carried out on ten adult rabbits and ten adult European hares using the corrosion technique. After the euthanasia, the vascular network was perfused with saline. After polymerization of the casting medium, the maceration was carried out in a KOH solution. We found different variations in; the number of arteries, level of their origin and arrangement. The *aa. lumbales* of the same level arose by means of a common trunk or their origin was independent. The *aa. lumbales* VI or *aa. lumbales* VI et VII originated also from the *a. sacralis mediana*. By *aa. lumbales* we found an important interspecies difference in; number, diameter, ramification and density of dorsal branches, which are designated for the dorsal muscles of the body stem. All listed parameters of branches were higher in the hare. This anatomical arrangement of dorsal branches is adapted to the higher movement activity of the hare. According to our results,

it can be concluded that the anatomical arrangement of the branches of the *aorta abdominalis* shows a higher number of variations in the domesticated rabbit in comparison with the hare.

Key words: abdominal aorta; *a. sacralis mediana*; *aa. lumbales*, hare; rabbit

INTRODUCTION

One of the most frequently occurring wild animals in Europe is the European hare. Despite its relatively abundant occurrence, there is a significant gap of knowledge about its anatomy in the literature. Up to now, the works dealing with the study of the arterial system in the European hare have only been published sporadically [3], [4].

Variations in the origin, branching and course of several arteries have long received attention of anatomists, surgeons and particularly vascular specialist. The variations in the arterial system of several species are the object of interest in a large number of studies [8]. The arterial arrange-

ment of the branches arising from the *aorta abdominalis* was studied in several experimental animals, such as; dogs [1], cats [2], rabbits [5], rats [17] and guinea pigs [15].

The aim of this study was to compare the variations of the *aorta abdominalis* branches arising from its dorsal surface in the domesticated rabbit and the European hare.

MATERIALS AND METHODS

This study was carried out on 10 adult European hares (*Lepus Europaeus*, L. 1758, age 140 days) and on 10 adult rabbits (*Oryctolagus cuniculus f. domestica*, L. 1758, age 140 days). We used hares (obtained from ISFA APRC, Nitra, Slovak Republic) of both sexes (female n = 5; male n = 5) with a weight range of 2.5–3.2 kg and New Zealand White rabbits (obtained from HYLAPA s.r.o., Prešov, Slovak Republic) of both sexes (female n = 5; male n = 5) with a weight range of 1.8–2.3 kg in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15–20 °C, relative humidity 45 %, 12-hour light period), and fed with a granular feed mixture (O-10 NORM TYP, Spišské krmne zmesi, Spišské Vlchy,

Slovak Republic). The drinking water was available to all animals *ad libitum*. The animals were injected intravenously with heparin (50 000 IU.kg⁻¹) 30 min before they were sacrificed by intravenous injection of embutramide (T-61, 0.3 ml.kg⁻¹). Immediately after euthanasia, the vascular network was perfused with a physiological solution [10]. During manual injection through the ascending aorta, the right atrium of the heart was opened in order to lower the pressure in the vessels in order to ensure an optimal injection distribution. Batson's corrosion casting kit No. 17, using a volume of 50 ml (Dione, České Budějovice, Czech Republic) was used as the casting medium. The maceration was carried out in a 2–4 % KOH solution for a period of 5 days at 60–70 °C. This study was carried out under the authority of decision No. 2647/07-221/5.

RESULTS

The paired *aa. lumbales* arose from the dorsal surface of the *aorta abdominalis* as segmental vessels. They supplied the; lumbar spinal cord, lumbar vertebrae, muscles of the back, inner lumbar muscles and skin of the back. In the rabbit, the independent origin of the *aa. lumbales* was pres-



Fig. 1. *Aa. lumbales* and *a. sacralis mediana* in the rabbit. Origin of *aa. lumbales* VI et VII from *a. sacralis mediana*. 1 — vertebra lumbalis VI; 2 — vertebra lumbalis VII; 3 — aorta abdominalis; 4 — *a. sacralis mediana*; 5 — *aa. lumbales* VI; 6 — *aa. lumbales* VII; 7 — common trunk for *aa. lumbales* V et IV. Macroscopic image, lateral view

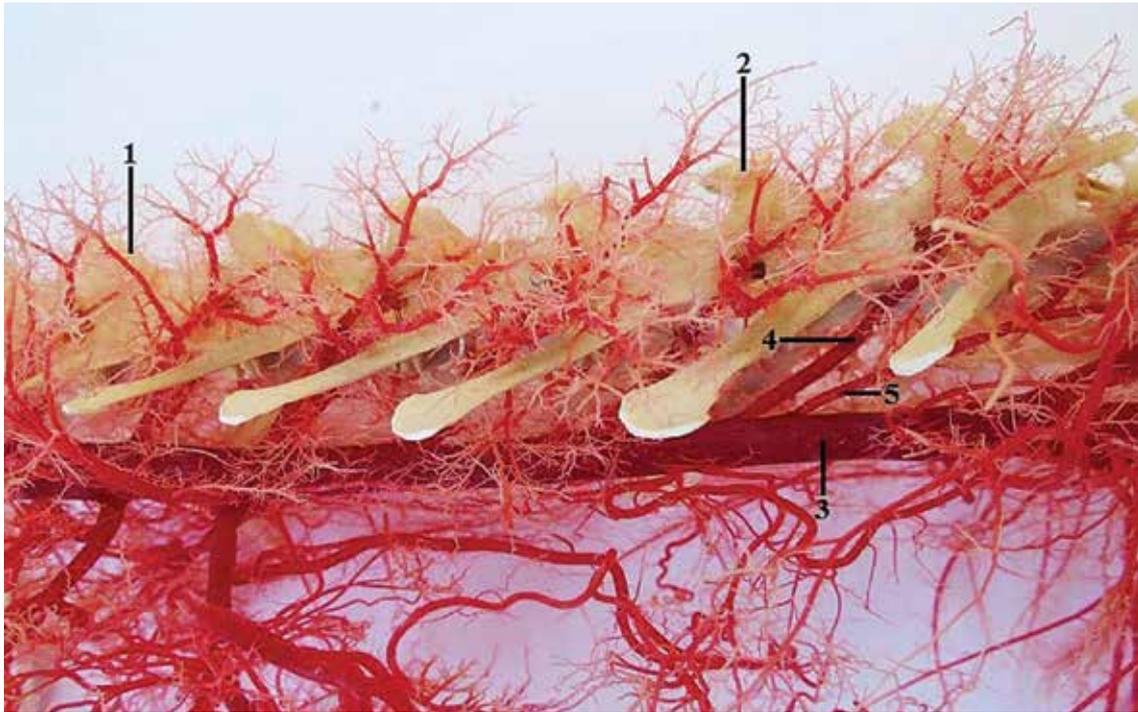


Fig. 2. *Aa. lumbales* and *a. sacralis mediana* in the hare. 1 — *vertebra lumbalis* I; 2 — *vertebra lumbalis* V; 3 — *aorta abdominalis*; 4 — *a. lumbalis* V; 5 — *a. sacralis mediana*. Macroscopic image, lateral view

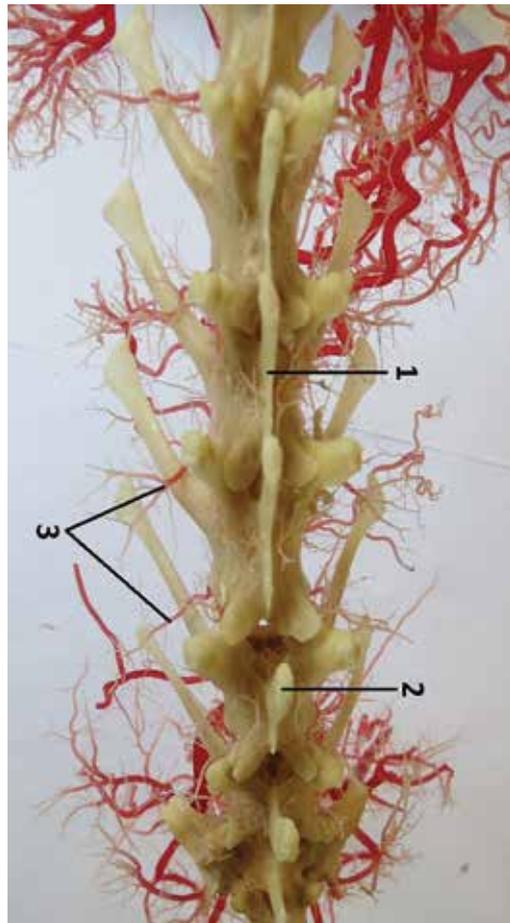


Fig. 3. *Rr. dorsales* arising from *aa. lumbales* in the rabbit. 1 — *vertebra lumbalis* IV; 2 — *vertebra lumbalis* VI; 3 — *rr. dorsales*. Macroscopic image, dorsal view

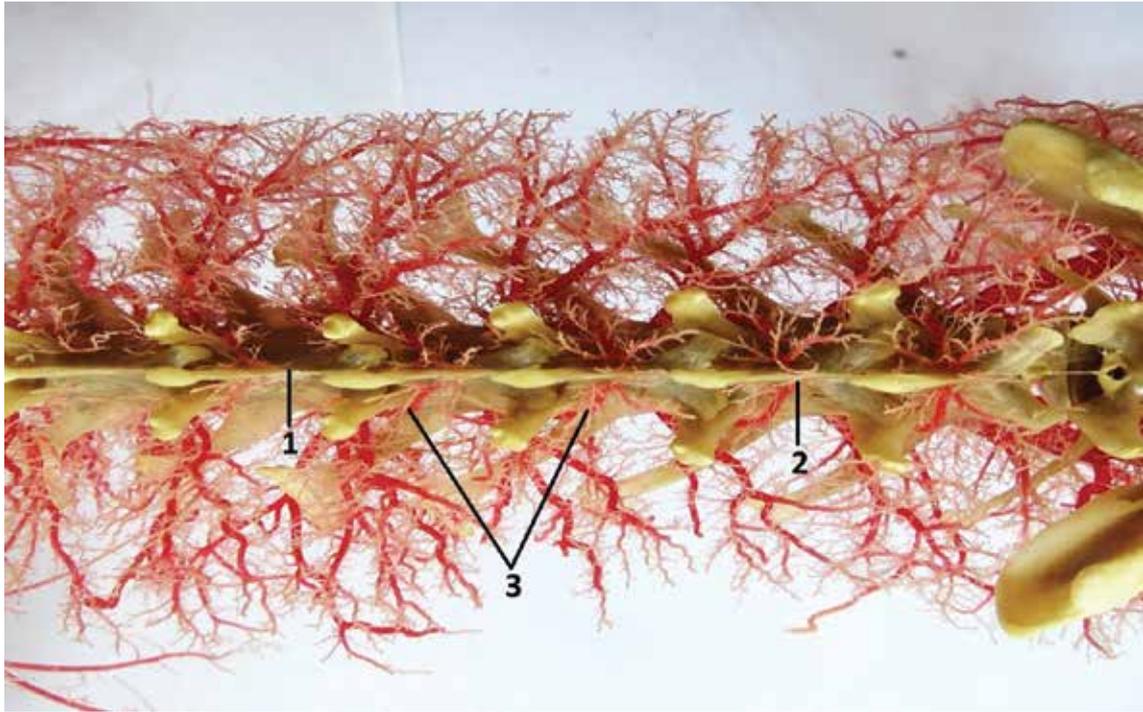


Fig. 4. *Rr. dorsales* arising from *aa. lumbales* in the hare. 1 — *vertebra lumbalis* III; 2 — *vertebra lumbalis* VI; 3 — *rr. dorsales*. Macroscopic image, dorsal view

ent in the first pair in 20% of the cases, in the first two pairs in 20% of the cases and in the first three pairs in 10% of the cases. In the remaining cases, the paired *aa. lumbales* originated by means of a common trunk at each level (Fig. 1). In the hare, the first pair originated independently in 20% of the cases and the first two pairs in 60% of the cases. In the remaining cases, the paired *aa. lumbales* originated by means of a common trunk at each level (Fig. 2). From the *aa. lumbales* arose *rr. dorsales* supplying the muscles of the body stem. In the rabbit, from each *a. lumbalis* arose *rr. dorsales* in number of 3—6 and in the hare in number of 7—9. Also their diameter, ramification and density were higher in the hare in comparison with the rabbit (Fig. 3, 4).

The *a. sacralis mediana* was the direct continuation of the *aorta abdominalis*. It ran ventrally to the sacrum, to reach the coccygeal vertebrae and continued caudally as *a. caudalis mediana*. In the rabbit, *a. sacralis mediana* originated from *aorta abdominalis* at the level of the 6th lumbar vertebra (Fig. 1). From *a. sacralis mediana* in the rabbit arose the last pair of *aa. lumbales* in 30% of the cases and last two pairs in 70% of the cases (Fig. 1). In the hare, the origin of *a. sacralis mediana* was located at the level of the 5th lumbar vertebra in 60% of the cases (Fig. 2) and at the level of the 6th lumbar vertebra in 40% of the cases. From *a. sacralis mediana* in the

hare originated the last pair of *aa. lumbales* in 20% of the cases and last two pairs in 80% of the cases (Fig. 2).

DISCUSSION

The knowledge of anatomical variations is important for radiological and surgical procedures in humans and other animals due to its practical and theoretical significance for experimental research and surgical practice in experimental and domesticated animals [11], [12], [16].

Six pairs of *aa. lumbales* with symmetrical origin from the dorsal surface of the *aorta abdominalis* were found in the rabbit [13]. Krause [9] described, in the rabbit, the *aa. lumbales* as branches arising from the dorsal surface of the *aorta abdominalis* by means of a common trunk. This arrangement was found by the *aa. lumbales* I—VI. The *aa. lumbales* VII were arising by means of a common trunk with an origin from *a. sacralis mediana*. This unpaired artery arose from the dorsal surface of the *aorta abdominalis* at the level of aortic bifurcation. Popesko et al. [13] described *a. sacralis mediana* in the rabbit as an independent branch arising from the dorsal surface of the *aorta abdominalis*. From the *a. sacralis mediana* arose the *aa. lumbales*

VII. In the majority of cases in our study, the *aa. lumbales* arose by means of a common trunk and the *a. sacralis mediana* gave off the *aa. lumbales* VI et VII.

The well-developed *rr. dorsales* arising from the *aa. lumbales* in the hare was the most visible interspecies difference in our study. These differences are associated with the way of life. The wild living hares are better and permanently trained. Training induces dimensional adaptations of the vascular system concerning the vessel diameter, distensibility, density and wall thickness in a proportional manner [7].

There is a very significant gap in the literature concerning the description of the arterial system in the European hare. The anatomical differences between familiar species will help to understand the behavioral differences in the wild and domesticated animals. We hope that this work will be the starting point for possible future studies in this research area. Such knowledge is also critical in comparative studies across species, as well as in the professional's daily practice [6].

CONCLUSIONS

A good understanding of the anatomical variations can facilitate surgical interventions, including the interpretation of a number of modern medical procedures of clinical interest, such as computed tomography and angiography [14]. Therefore, both the normal and variant anatomy of the region should be well known for accurate diagnosis, better treatment and avoidance of iatrogenic injuries during interventional vascular procedures.

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INTEROBSERVER VARIATION IN CANINE HIP DYSPLASIA EVALUATION

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ABSTRACT

Canine hip dysplasia (CHD) is a common disease representing an important problem for many dog breeds worldwide. The screening for CHD and breeding programs have been ongoing for many decades but the incidence of disease have failed to be reduced to the expected level. The early diagnosis of CHD is paramount in order to facilitate the early management strategies and to prevent the breeding of the affected individuals. Generally in this area, the emphasis is placed on the radiographic evaluation process, however this is partly a subjective process suggested to be influenced by the experience of the observers. This study was designed to evaluate the interobserver agreement in CHD evaluation based on the Federation Cynologique International system (FCI system). Ten original radiographs were sent to five different groups of observers, from students to certified veterinarians. They were asked to evaluate the ventro-dorsal radiographs according to the FCI system which is the most common system used in Europe to give the final grades (A, B, C, D, E). The grades were converted to numbers

and the data were analysed using a one-way ANOVA test. The results showed that only in 20% of the cases, the interobserver agreement was statistically higher in the group of the most experienced observers when compared to the less experienced group of observers. This means that the level of experience does not always lead to a higher agreement. This could be a problem of widespread objective evaluations of CHD. In addition, there are several different systems of evaluation used in various countries. It is necessary to understand the intention of dog owners, who when buying a dog may be planning its first breeding. Therefore, the “correct” or “incorrect” assessment of the CHD radiographs may not always result in the elimination of affected individuals. We do not know exactly the situation of the development of the hip in some breeds, because scrutineers are receiving only “negative radiographs” for evaluation. Many owners are very educated about hip and elbow dysplasia. It is a time to tell the scientific truth.

Key words: canine hip dysplasia; dog; FCI; interobserver; screening

INTRODUCTION

Canine hip dysplasia (CHD) is a multifocal disease first described in 1935 when Schnelle published the first radiographic description of this disease as a bilateral congenital subluxation of the coxofemoral joint [13]. Although there are several evaluation systems of CHD in continental Europe, the recommendations of the Federation Cynologique International (FCI) using a five-grade scale from A to E are followed to a large extent [3]. Slow progress in decreasing the incidence of CHD remains a fact [11]. This may be due to several factors such as the low sensitivity in detecting hip joint laxity, high interobserver variations, or degenerative joint disease often is not visible at the age the radiographs are made and therefore breeders continue to use dysplastic dogs for breeding purposes [1], [8], [9].

There have been several studies which evaluated the agreement among radiologists in assigning hip dysplasia grades. One study found that the level of agreement between observers, using a subjective method, was very low [14]. Paster et al. [10] stated that intra- and interobserver variations are significant. Saunders et al. [12] also found a significant difference between radiologists evaluating ventrodorsal or dorsoventral hip-extended views.

In the FCI system for screening CHD, dogs are graded as having hips A, B, C, D, or E. Radiographs should be interpreted and scored by a specialized certified veterinarian, approved by the national kennel club and/or the breed club in which the dog is registered. The scoring system combines the subjective standard hip-extended radiographic evaluation with the Norberg-angle measurement. The five different scores represent the severity of the disease. Dogs with A (no signs of CHD) and B (near-normal hip joints) hips are considered non-dysplastic and these dogs are therefore recommended for use in the breeding process. Dogs with grade C are considered mildly affected and can be used in the breeding program in certain instances, whereas grades D and E are considered clearly dysplastic and such dogs are therefore not considered as breeding material.

The objective of this study was to investigate the interobserver agreements within and between groups of differently experienced observers in determining CHD final grading using the FCI grading system.

MATERIALS AND METHODS

For this study we obtained ten original digital radiographs of dogs from the archives of the Department of Surgery, Orthopaedics, Radiology and Reproduction, University of Veterinary Medicine and Pharmacy (UVMP) in Kosice. All radiographs were taken under a standard intramuscular anaesthesia in a position with extended hind legs, properly marked, and numbered from one to ten. They were sent on CDs to 15 different observers. The Norberg angle (NA) was the only information provided. This was intended to minimize the interobserver disagreement caused by differences in the measurements. The normal NA in hips with no dysplasia must be equal to or greater than 105° according to the FCI classification.

Using the FCI method, dogs were evaluated on the basis of the size of the NA, degree of subluxation, shape and depth of the acetabulum, and signs of secondary joint disease. The minimum age for the radiological screening is 12 months for most breeds and 18 months for large and giant breeds. Dogs should be deeply sedated or anaesthetized to achieve complete muscle relaxation and good diagnostic quality radiographs.

Five different groups of observers with decreasing experience were used:

- Group 1.** Experienced veterinarians certificated for evaluating CHD radiographs.
- Group 2.** Experienced private veterinarians without certification.
- Group 3.** Doctors/Lecturers from UVMP.
- Group 4.** PhD. students from the Department of Surgery, UVMP.
- Group 5.** Students from UVMP.

The observers were asked to evaluate all ten radiographs according to the FCI criteria and to give the final grade (A, B, C, D, E). The grades were converted into numbers to be used in statistical analysis (A-1, B-2, C-3, D-4, E-5). An agreement score was derived based on the FCI score for each radiograph and for each CHD grade. We tested whether the agreement scores differed between the experienced and inexperienced groups of observers and between observers in each group. All data were analysed using one-way ANOVA and $P < 0.05^*$ was considered statistically significant.

RESULTS

There were three observers in each of the 5 groups (Table 1). This table shows the amount of radiographs which each observer assigned to each grade of A, B, C, D, or E. The results showed that the more experienced subgroups graded more radiographs with grade A and the inexperienced subgroups graded more radiographs with grade E.

Comparisons were performed for the average scores (Mean \pm SD) of all ten radiographs for the three members in each group (Table 2). There was no statistical difference in the average grading in the groups 1, 2, 3, 4, and 5 ($P > 0.05$).

Table 2 summarizes the evaluations by the individual observers with the number of radiographs evaluated (N), mean values of CHD grades with the standard deviation (SD) and P value expressing that between the evaluations of individual observers and there was no statistically significant difference ($P > 0.05$).

Although the mean value can be the same in groups 1 and 5, the value of the SD demonstrates low interobserver agreement within the groups. For the more experienced observers, this agreement is slightly higher when compared to

the less experienced groups of observers, which confirmed the hypothesis that agreement is growing with experience.

This can be illustrated also by analysing the pooled standard deviations as a weighted average of each group's standard deviations. Data expressed in Table 3 show that the SD increases with decreased level of experience.

The evaluation of the level of agreement for the 5 groups based on 10 different x-rays expressed the following findings. For radiographs 1, 2, 3, 4, 6, and 7 there was confirmed statistical disagreement by means of the difference with $P < 0.05$ (0.011, 0.027, 0.042, 0.01, 0.015, and 0.00, respectively). For radiograph number 10 the P value was 0.001.

DISCUSSION

The primary goal for the ongoing CHD screening program is to exclude genetically susceptible individuals from the breeding pool. Because CHD is a polygenetic heritable trait [7] and current screening systems rely on interpretation of radiographs, their efficacy in reducing CHD is limited [3], [17]. Despite intensive screening for 4 decades, the

Table 1. Relative agreement between observers

Group of observers	Observer	Grade A [%]	Grade B [%]	Grade C [%]	Grade D [%]	Grade E [%]
1	1.1	–	–	40	40	20
	1.2	–	10	70	10	10
	1.3	10	20	50	–	20
2	2.1	50	20	20	10	–
	2.2	20	20	10	30	20
	2.3	–	60	10	30	–
3	3.1	20	20	10	40	10
	3.2	10	30	20	30	10
	3.3	10	30	–	40	20
4	4.1	10	10	20	20	40
	4.2	10	20	10	30	30
	4.3	–	20	20	10	50
5	5.1	–	20	20	30	30
	5.2	10	20	10	30	30
	5.3	10	10	10	40	30

Table 2. Summary of evaluation by individual observers

Observer	N	Mean	SD	P
1.1	10	3.800	0.789	
1.2	10	3.200	0.789	0.176
1.3	10	3.000	1.227	
2.1	10	2.100	1.287	
2.2	10	3.100	1.524	0.229
2.3	10	2.700	0.949	
3.1	10	3.000	1.414	
3.2	10	3.000	1.247	0.852
3.3	10	3.000	1.418	
4.1	10	3.700	1.418	
4.2	10	3.500	1.434	0.812
4.3	10	3.900	1.287	
5.1	10	3.700	1.160	
5.2	10	3.500	1.434	0.926
5.3	10	3.700	1.337	

Table 3. Values of P in each group and pooled SD

Observer group	P	SD
1	0.176	0.9661
2	0.229	1.275
3	0.852	1.362
4	0.812	1.381
5	0.926	1.315

prevalence of CHD is still as high as 40 % in some breeds [2]. Since dogs judged to have moderate or severe CHD cannot be used for breeding purposes, the screening techniques need to be as uniform as possible with a high intra- and interobserver agreements. However, other studies as well as this article indicate that interobserver agreement is low [15]. We can say, that our results are a consequence of the different levels of experience within the groups of observers. Overall, 70 % of all cases confirmed that the difference in assessing the degree of hip dysplasia is linked to the level of experience.

Because of the impact of age at screening and the use of sedation, not all screening approaches are standardized and interobserver agreements seem to be lower also because there is no gold standard for the diagnosis of CHD. The same hip-extended screening system even differs among countries [16]. When a gold standard is available, sensitivity and specificity can be provided. Moreover, if there are many different clubs for the same breed, the clubs may choose different people to evaluate CHD in the same breed which makes it difficult to have a national standard for determining the CHD statistics within one breed [4].

Disagreement between observers inevitably leads to a considerable number of false-positive (loss of genetic variation) and false-negative dogs (genetically affected). This allows false-negative dogs to breed, maintaining hip dysplasia in the population, whereas false-positive dogs, which could decrease the susceptibility for hip dysplasia, are rejected from the pool. This may explain the slow progress of decreasing hip dysplasia over the past few decades [11].

In our opinion, as well as by other authors, the credibility of the FCI screening method for canine hip dysplasia, using the standard hip-extended radiologic view, as currently applied in most European countries, is questionable. The results of Fortrie et al. [5] also demonstrated that the recognition and presence of the radiologic signs of CHD is highly dependent on the expertise and specialty of the observer. One example to increase the positive impact of screening on dog breeding is in Sweden, where ~50 % of all susceptible breeds are screened annually [6]. This makes it possible to identify the symptoms of a degenerative disease where early stages may be diagnosed by screening at a young age.

CONCLUSIONS

Based on the results achieved, it is clear that there is a need for unification and objectivity of the process of CHD evaluations. Despite years of efforts to eliminate this disease, the incidence remains high, which is currently largely related to the problems in evaluation and grading systems; thus, the screening and examination techniques used by different observers need to be as uniform as possible. There is a clear need for the evaluation of the hip for dysplasia to be changed because radiology is not the optimal method for real confirmation of dysplastic or non-dysplastic dogs.

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INVESTIGATING POTENTIAL SOURCES OF TOXOPLASMOSIS FOR HUMANS FROM SLAUGHTERED FOOD ANIMALS IN IBADAN, NIGERIA

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ABSTRACT

The consumption of undercooked meat by humans is a potential infectious source for Toxoplasmosis. This study was aimed at finding potential infectious sources of *Toxoplasma gondii* for humans by investigating the seroprevalence of *T. gondii* in animals slaughtered in the Ibadan municipal abattoir. Serum samples from 1337 slaughtered animals (477 cattle, 267 sheep, 139 goats, and 454 pigs) were analyzed for the presence of antibodies to *Toxoplasma gondii*. Serological studies using the ELISA method demonstrated the prevalence of *T. gondii* antibodies in cattle, sheep, goats and pigs as 38.9%, 1.9%, 3.6% and 45.2%, respectively. Univariate statistical analysis detected an association between *T. gondii* seropositivity and sheep, goat and sex ($P < 0.05$). In the multivariate logistic regression model, only sheep, goats and pigs had an association with *T. gondii* seropositivity, while sex was a confounding factor. The detection of varying levels of antibodies to *T. gondii* infection in these food animals highlights their potential as a source of *T. gondii* for humans. Efforts should, therefore be di-

rected at preventing the infection during the production and the processing of meat for food.

Key words: cattle; goats; infection; Nigeria; pigs; slaughter; *Toxoplasma gondii*

INTRODUCTION

Toxoplasmosis is one of the most common parasitic zoonosis caused by *Toxoplasma gondii*, an intracellular apicomplexan protozoan that infects almost all warm-blooded animals, including domestic animals and humans [9], [11].

T. gondii infection in humans is influenced by the immune status and genetic composition of the host and the timing of the infection [13]. Acquired *T. gondii* infections are usually asymptomatic, although lymphadenopathy or ocular disease may occur in some patients [5]. *T. gondii* is also an important cause of abortions and stillbirths in infected pregnant women and has been associated with fetal anomalies [25]. Congenital toxoplasmosis may cause retinal and mental retardation, seizures, blindness, and death [17].

Some cases of acute toxoplasmosis have been associated with psychiatric symptoms such as delusions and hallucinations [17] and psychiatric manifestations have been observed in immunocompromised persons with AIDS in whom latent infections with *T. gondii* have been reactivated [16].

T. gondii infects humans through the ingestion of food or water contaminated with oocysts shed by cats (the definitive host) or by eating undercooked or raw meat containing tissue cysts, especially the viscera of the intermediate hosts [13, 8]. Several studies worldwide have identified undercooked meat (lamb, beef or game) as a major risk factor for toxoplasmosis in humans [11], [19]. In addition, the presence of cats, feed source, farm management, methods of rodent and bird control, carcass handling and water quality have also been suggested as risk factors for the infection in livestock [14], [31].

Seroprevalence studies worldwide showed that *T. gondii* infections are widespread in food animals with results varying between and within regions. Furthermore, the outcome of tests depends on sampled species and/or breed, type of test, the cut-off values and most times the sample size of the study [11]. The seroprevalence of *T. gondii* has been found to be higher in pigs, sheep and poultry than in cattle and less in meat product of animals raised indoor than outdoor reared animals exposed to food, water and environment contaminated with oocyst [14].

The seroprevalence of *T. gondii* antibodies in livestock may be an indirect method to predict potential infection sources, since a correlation was determined between antibodies to *T. gondii* and the presence of tissue cysts in sheep and pigs [26]. However, this may not be the case with cattle where tissue cyst are rarely found even when antibodies were detected in up to 92% of the cattle examined [30]. Tissue cysts of *T. gondii* are often detected in the tissues of sheep, goats and pigs compared with other domestic animals [30].

The seroprevalence of *T. gondii* antibodies obtained in human populations has also been found to vary greatly between countries, among different geographical areas within same country and among different ethnic groups living in the same area [30]. In Nigeria, there are several published seroprevalence studies indicating *T. gondii* infection is endemic among both immunocompromised and immunocompetent individuals [27], [28]. Nevertheless, the sources of *T. gondii* infection in humans has not been reported. A study by K a m a n i et al. [18] on the risk factors for *T. gondii* infection among cat and dog owners in Northern Ni-

geria, suggested that contact with pets are not likely to be the main source of infections for humans. While, ingestion of contaminated meat might be a potential source of transmission of *T. gondii* to humans in Nigeria, no report is available to verify it as an infection source. This study was therefore aimed at investigating whether animals slaughtered for food in the Ibadan municipal abattoir could be a potential source of *T. gondii* infections for humans.

MATERIALS AND METHODS

Location and sampling

The sampling site for the study was the Bodija abattoir in Ibadan, Oyo state, Nigeria. Located at 7° 23' 47'' N, 3° 55' 0'' E, Ibadan is one of the largest cities in West Africa with a land area of 3,080 km² and a population of 2,338,659 [5]. The Bodija abattoir is the largest slaughter facility available in Ibadan and accounts for 65.93% of the total animals slaughtered in Oyo state [1]. Blood samples were randomly collected from 1337 animals (267 sheep, 139 goats, 477 cattle, and 454 pigs) at slaughter over a period of one year. The samples were collected into sterile tubes without anticoagulant and left at room temperature (29–33°C) for 1–2 hours. The separated sera were harvested and stored at –20°C until used for serology.

Serology

The serum samples obtained were analyzed using the commercially available ID Screen Toxoplasmosis Indirect Multi-Species ELISA Kits (ID Vet Innovative Diagnostic, Montpellier, France) to determine the presence of *T. gondii* specific IgG antibodies. The procedure for the test and interpretation of the results obtained were performed according to the manufacturer's instructions.

Statistical Analysis

The data were first entered and stored in Microsoft excel spreadsheet and afterwards exported into SPSS 20 statistical software (SPSS Inc., Chicago, IL, USA). The univariate analysis was done to evaluate the strength of the relationship between our outcome variable and the explanatory variables. The independent effects of variables associated with our dependent variable at P < 0.05 on univariate analysis were entered into the multivariate logistic regression model to further examine the predictors' effect on our response vari-

able. Similarly, Negelkerke's R2 was used in our analysis to illuminate how much variance in the dependent variable is explained or affected by predictor variables. The data were therefore analyzed based on the 5 % level of significance.

RESULTS

Table 1 gives a summary of the characteristics of the sampled animals at the Ibadan municipal abattoir. Table 2 highlights the distribution of *T. gondii* infection across species, age and sex of animals slaughtered at the Ibadan

Table 1. Physical characteristics of animals slaughtered at the Ibadan municipal abattoir

Variable	N	%
Age		
Young	25	1.9
Grower	305	22.8
Adult	1007	75.3
Sex		
Male	660	49.3
Female	677	50.6
Pig		
Large white	288	63.3
Durock	96	21.1
Black local	47	10.3
Crossed(LW+D)	23	5.1
Sheep		
Yankasa	30	11.2
Uda	237	88.8
Goat		
Sokoto red	139	100
Cattle		
Kuri	34	7.1
Ndama	10	2.1
Red Bororo	152	31.7
Sokoto gudali	86	18.0
White Fulani	170	35.6
Mixed breed	25	5.0

Table 2. Distribution of *Toxoplasma gondii* infection across species, age and sex of animals slaughtered at the Ibadan municipal abattoir

Variable	N	Positive [%]	Negative [%]
Species			
Swine	454	205 (45.2)	249 (54.8)
Ovine	267	5 (1.9)	262 (98.1)
Caprine	139	5 (3.6)	134 (96.4)
Cattle	477	186 (38.9)	291 (61.0)
Age			
Young	25	10 (40.0)	15 (60.0)
Grower	305	103 (33.8)	202 (66.2)
Adult	1007	287 (28.5)	720 (71.5)
Sex			
Male	660	150 (22.7)	510 (77.3)
Female	677	248 (36.6)	430 (63.4)
Total	1337	401 (29.9)	936 (70%)

municipal abattoir. The analysis of 1337 serum samples obtained from animals slaughtered at the municipal abattoir in Ibadan showed that 401 (29.9%) samples were positive for *T. gondii* antibodies. Based on species, the prevalence of *T. gondii* antibodies were 1.9% (5/267), 3.6% (5/139), 38.9% (186/477) and 45.2% (205/454) for sheep, goats, cattle and pigs respectively. Fig. 1 shows the distribution of *T. gondii* infection across various breeds of animals slaughtered at the abattoir. Of the 267 sheep examined, only five (1.9%) samples were positive. *T. gondii* antibodies were detected in five (3.6%) of the 139 goats sampled. A total of 186 (38.9%) were positive out of the 477 cattle serum analyzed, while 205 (45.2%) were positive for *T. gondii* antibodies of the 454 pigs sampled. There were 287 (28.5%) adult animals positive for *T. gondii* antibodies while 248 (36.6%) were females out of the total of 1337 samples analyzed from the abattoir.

On univariate analysis, *T. gondii* infection was significantly associated with ovine and caprine species, and also the male gender. Multivariate logistic regression model obtained a significant association between *T. gondii* infection and porcine, caprine and ovine species, but sex was identified as a confounder and not a factor in *T. gondii* infection (Table 3).

Table 3. Multivariate logistic regression analysis of independent variables associated with *Toxoplasma* infection in animals slaughtered at Ibadan municipal abattoir

Variables	N	Univariate		Multivariate	
		Crude OR (95 % CI)	P-value	Adjusted OR (95 % CI)	P-value
Age					
Young	25	1.67 (0.74—0.78)	0.211	–	–
Grower	305	1.285 (0.97—1.68)	0.073	–	–
Adult	1007	1	–	–	–
Species					
Swine	454	1.29 (0.99—1.68)	0.051	2.02 (1.41—2.89)	< 0.001
Ovine	267	0.03 (0.01—0.07)	< 0.001	0.03 (0.01—0.08)	< 0.001
Caprine	139	0.05 (0.02—0.47)	< 0.001	0.07 (0.03—0.19)	< 0.001
Cattle	477	1	–	1	–
Sex					
Male	660	0.51 (0.40—0.64)	< 0.001	0.86 (0.64—1.17)	0.353
Female	677	1	–	1	–

Negelkerke's R² = 0.274; OR — Odds Ratio; CI — Confidence Interval

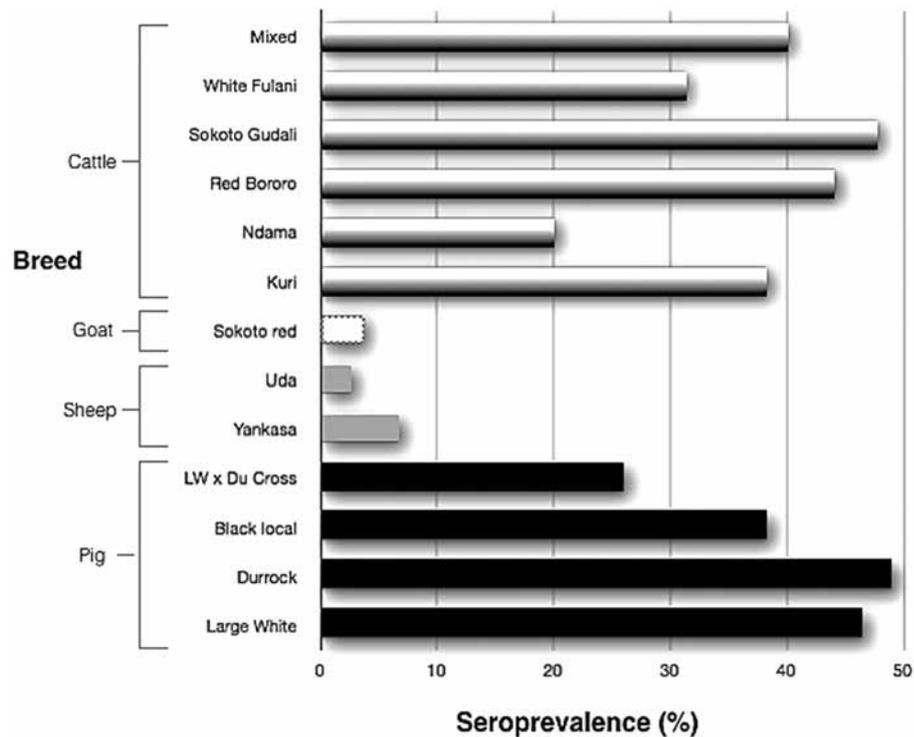


Fig. 1. Chart showing the distribution of *T. gondii* infection across various breeds slaughtered at the Ibadan municipal abattoir

DISCUSSION

This study detected antibodies to *T. gondii* in all of the species of animals (cattle, sheep, pigs and goats) slaughtered at the abattoir. This indicates that *T. gondii* is wide spread across the region and livestock (irrespective of their species) are constantly exposed to *T. gondii* oocyst in their environment.

The comparison of the seroprevalence of *T. gondii* antibodies in our study with other reports elsewhere, show that our findings in pigs is comparable to 30.6% in Chongqing [32], 46% in Ústí nad Labem (Czech Republic) and lower than 76% in Hradec Králové (Czech Republic) [4]. However, our seroprevalence figure is higher than 2.5% in Finland [15], 5% in Sweden [22], 9.8% in North Portugal [20], 11.7% in Nepal [7] and 13% in Mexico [3]. For cattle, the seroprevalence detected in our study was higher than 29.1% in southern Brazil [23], 7.5% in North Portugal [20], 6.0% in Northeast China [21], but lower than 46.4% in Xinjiang, China [24]. For sheep, we obtained a seroprevalence lower than 33.6% in North Portugal [20], 20% in Central Ethiopia [13] and 35% in Iran [29] and 36.4% in Saudi Arabia [2]. For goats, we detected a seroprevalence far less than 15.5%, 18.5%, 30% and 35.3% obtained in Central Ethiopia [13], North Portugal [20], Iran [29] and Saudi Arabia [2].

The seroprevalence of *T. gondii* in food animals varies between and within countries across the world. Several factors including, management systems, climate, population of cat and variation (in sensitivity and specificity) of detection methods usually influence the prevalence of *T. gondii* reported in animals processed for meat [11]. Hence, it is therefore difficult to draw conclusive inferences when comparing *T. gondii* data from several different locations. However, the present findings along with reports from previous investigators suggest that humans consuming animals slaughtered around the world may be potentially exposed to *T. gondii* infection and therefore the disease should be considered in meat inspection procedures worldwide.

We obtained high seroprevalence of *T. gondii* antibodies in pigs brought for slaughter; a situation similar to the reports of previous investigators [32], [4]. Besides, *T. gondii* seropositivity was significantly associated with pigs after a multivariate logistic regression analysis. Although the reason for this observation is not known. However, the management systems under which the pigs were raised

may be an important factor that influenced the outcome of our findings. Pigs in the study area are usually reared in semi-intensive and backyard systems with poor housing facilities that can easily be accessed by rodents and cats. Our previous findings on pig farms in Nigeria (unpublished data) showed that, unlike in the developed countries, domestic and feral cats regularly besiege piggeries and animal feed mills to kill rodents, thereby increasing the probability of pigs contacting viable oocyst of *T. gondii* from cats.

Reports from Europe and the USA showed that organic pigs raised with free access to outdoor environment have higher antibodies titre to *T. gondii*. Furthermore, pork has generally been considered as a major source of *T. gondii* infection in humans [30]. The consumption of contaminated pork was also linked to clinical toxoplasmosis in Korea [6]. With available reports on the evidence of high seroprevalence of *T. gondii* in HIV-infected individuals as well as immunocompetent humans in Nigeria [27], [28], coupled with an increase in the consumption of pork as a cheap source of protein (due to the rising cost of beef, goat meat and chickens); the detection of high levels of antibodies to *T. gondii* in pigs slaughtered for food, raises concern about the possible role of pork in the transmission of *T. gondii* to humans in Nigeria. Moreover, it has been suggested that the probability of isolating viable *T. gondii* parasites increases with high antibody titre in pigs [10]. This implies that high levels of antibodies to *T. gondii* in commercial pigs slaughtered for human consumption could increase the possibility of consumption of viable cyst if the pork is not properly processed. Thus, more studies are needed to investigate the role of pork consumption in the epidemiology of toxoplasmosis.

Our study observed very low antibody levels to *T. gondii* in small ruminants compared to the results from most other investigators [2], [13], [20]. This could be because of the varying types of management systems in our study area. Sheep and goats raised for food in Southwestern Nigeria are mostly reared in confinement with little access to the outdoor environment. This contrast with sheep and goat production from other free-ranging systems where animals are under increased pressure of *T. gondii* infection from oocysts in the contaminated environment [30].

Our study detected a higher prevalence of *T. gondii* antibodies in cattle than in sheep and goat. However, unlike with small ruminants both univariable and multivariable logistic regression analysis showed no significant associa-

tion between *T. gondii* infection and cattle. Previous investigators have suggested that *T. gondii* infection may be of less importance in cattle than in small ruminants because the parasites are rarely isolated from naturally infected cattle [20]. Furthermore, there is evidence to show that tissue cysts are rarely found even in cattle with a seroprevalence as high as 92% [30]. It has also been suggested that cattle might have the ability to eradicate the *T. gondii* from their tissues [12]; hence, unlike in small ruminants, there is no correlation between antibodies to *T. gondii* and the presence of tissue cysts in cattle [26].

Our study found varying level of seroprevalence among age groups, breeds and the sex of food animals, but age and breed were not significantly associated with seropositivity with *T. gondii*. These findings contrasts with some reports that identified age as a risk factor for the infection in some livestock [13], [20]. Our study did not find sex as an independent predictor of seropositivity to *T. gondii*, but a confounding factor (in the multivariable logistic model). It is worth noting that limited inferences can be drawn on risk factors from an abattoir-based study, since we have no information on the management system of animals, presence of cats and rodents on the farm, food and water sources for animals brought for slaughter. Hence, more studies are needed for tracking *T. gondii* infection in livestock meant for human food from farms to slaughter. In addition, there is a need for enforcement of procedures that will ensure that detailed data on livestock brought to the abattoir are obtained before they are slaughtered.

CONCLUSIONS

This study showed that cattle, sheep, goats and pigs slaughtered for human consumption in the studied area had previous varying level of exposure to *T. gondii* infection and may serve as potential infectious sources for *T. gondii* infections in humans, if meat product from them are not properly handled or processed. While, the presence of high antibodies in slaughtered livestock does not directly provide hazards to humans, because the meat may not contain viable *T. gondii* cyst, it is imperative that the risk of zoonosis from infected food animals are not discounted. Preventive measures, especially educating the public (meat handlers and consumers) on zoonotic implication of *T. gondii* is highly recommended.

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A RAT SPINAL CORD INJURY EXPERIMENTAL MODEL

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ABSTRACT

Spinal cord injuries (SCI) with their tragic consequences belong to the most serious pathological conditions. That is why they have stimulated basic research workers, as well as health care practitioners, to search for an effective treatment for decades. Animal experimental models have been essential in these efforts. We have jointly decided to test and standardize one of the spinal cord injury compression models in rats. Twenty-three adult female Wistar rats weighing 250–320 g were utilized. Employing general anaesthesia along with a mixture of sevoflurane with O₂, 2 rats (sham controls) had their vertebral arch of either Th8 or Th9 vertebra removed (laminectomy). The other 21 experimental rats with similar laminectomies were divided into 3 subgroups (n=7) which received compression impact forces of 30, 40 or 50 g (subgroups-1, -2, and -3, respectively) applied on their exposed spinal medulla for 15 minutes. All rats were observed for 28 days after the experimental

procedure and their motor functions were assessed by the Basso, Beattie, Bresnahan (BBB) test 6 hours, 7, 21 and 28 days after the simulated SCI. All 23 rats survived the surgical procedures. The control rats were without any neurological deficits. There were, in every experimental subgroup, 1 or 2 rats with extreme BBB scores. So the rats with the maximum and minimum BBB values were excluded. Then, the results acquired in the residual 5 rats in each group were averaged and statistically analysed by the Tukey multiple comparisons test. Statistically significant intersubgroup differences were found at all survival times equal to or longer than 7 post SCI days. The goal of the SCI experiment was to generate a reproducible and reliable, submaximal spinal cord trauma model. The statistical analyses demonstrated that this objective was best achieved in the subgroup-2 with the 40 g compression.

Key words: compression model; rat; spinal cord trauma

INTRODUCTION

A spinal cord injury (SCI), often produces extensive and persistent motor, and sensory deficits and vegetative states which causes a lifelong disability with major negative impacts on the patients, their families, as well as the society in general [3]. The current global estimates indicate that the incidence of SCI ranges from; 15 per million in Australia to 40 per million in the North America; and 14.7–40 per million in Europe (20 per million in Slovakia), with a mortality rate of 4.4–6.7%, depending on the extent and location of the medullary lesions. The average age at injury is 31.7 years old with the greatest frequency between 15 and 25 years old and with the male to female ratio of 3:1 [4, 7, 18]. The surviving individuals from these SCI experiences, often are confined to a wheelchair and suffer considerable morbidity impacting on their quality of life [2, 4, 18]. The health care costs are significant with a single patient in the USA requiring between 1 and 25 million dollars over a lifetime depending on the severity of his/her injury [5]. So far, despite improvements in the medical and surgical management of SCI, there are no effective therapeutic measures able to improve neurological outcomes following spinal cord trauma. Only progress in neuroscience research, based on animal studies supported with gradable and qualifiable outcomes can hope to change this situation. We collectively decided to test and eventually modify the rat SCI compression model with the intention to standardise this model for further utilisation in planned SCI experiments.

MATERIALS AND METHODS

The experimental protocols were elaborated in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 [1] and approved by the Ethical Commission of the Institute of Neurobiology, Slovak Academy of Sciences in Košice, as well as by the State Veterinary Administration in Bratislava (decision No. 2812/13/221).

The basic experimental plan involved 23 adult female Wistar rats weighing 250–320 g. Two of the rats were kept as sham controls. Twenty-one rats were randomly divided into three subgroups of 7 animals each. A 15 minute compressing force of 30, 40 and 50 g (subgroups-1, -2, and -3, respectively) was applied directly on the intact dural sac containing the spinal medulla which had previously been

exposed by the laminectomy of the 8th or 9th thoracic vertebra. More specific experimental details follow.

After the induction of general anaesthesia by vapours of isoflurane („Forane“, i.e. 1-chloro-2,2,2-trifluoroethylidifluoro-methylether, Abbott Laboratories Ltd., Queensborough, Great Britain) with O₂ in a plexiglass box, all 23 rats were transferred to a heated platform, in order to maintain their rectal temperature between 36.6 and 37.2 °C. The temperature was monitored directly by an inserted rectal thermometer probe. The inhalation anaesthesia (by a mixture of 1.5 % isoflurane with medical oxygen) was delivered by a special mask designated for rodents.

The skin of the dorsal part of the rat's thoracic region was shaved and disinfected by iodine solution („Betadine“, i.e. povidonum iodatum, Egis Pharmaceuticals, Budapest, Hungary), and then the skin, subcutaneous fat and dorsal fascia were incised and an operating microscope Leica M320-F12 (Leica Microsystems CMS, GmbH, Herbrugg, Switzerland) was introduced. With the assistance of an improved illumination and magnification of tiny structures in the operational field provided by the microscope, the paravertebral muscles were separated from the spinous processes as well as the arches of Th8–Th10 vertebrae using microinstruments.

The spinal dural sac containing the spinal medulla was uncovered by laminectomy of either the 8th or 9th thoracic vertebra. In order to enhance the orientation of the operational field, a line connecting both caudal angles of the scapulae, which crossed the spinous process of the Th7 vertebra in a rat was used [10]. With an aim to prevent the constant movements of the spine and spinal cord caused by breathing of the rat, its vertebral column was suspended and fixed to a stereotaxic frame by Kocher clamps attached to the spinous processes of the 7th and 10th vertebrae, or by sutures pulled through the interspinous ligaments.

After a 15 minute exposure of the spinal dural sac to the surrounding environment, the procedure in the control rats were finished by sutures of the surgical wounds. The spinal cord trauma (SCI) to the 21 experimental rats was performed by a steel rod (freely moving up and down in a tube with minimal friction). At the distal end of the rod was a circular impactor with a diameter of 2 mm made of inert material which was placed directly on the exposed spinal dural sac. The metallic rod together with the impactor weighed 10g. On the top of the rod were placed various weights so that the impactor acted on the spinal cord by an exact compression force of 30, 40 or 50 g for 15 minutes (Fig. 1).

After the spinal cord compression, the metallic rod with the impactor was removed and the operational opening was sutured in anatomical layers. Following laminectomy (in 2 sham controls), and the execution of spinal cord compression trauma (in 21 rats), all rats were administered antibiotics ("Amoksiklav", i. e. amoxicillinum natricum + kalii clavulans, Sandoz Pharmaceuticals d. d., Ljubljana, Slovenia) intramuscularly in a total dose of 30 mg. Then, the rats were transferred to disinfected cages, where they were provided with drinking water ad libitum and food granules. The 21 rats belonging to the experimental subgroups were prepared for studies of different aspects of the complex problem of SCI or for testing various therapeutic measures aimed to reduce the extent of the spinal cord damage and neurological deficits.

The rats were observed for 28 days. During this period their general condition, activity, neurological state, healing of surgical wounds and their motor functions by an open field Basso, Beattie, Bresnahan (BBB) test [2] were evaluated by two independent observers 6 hours following the SCI, when they completely recovered from anaesthesia, and then again at the 7th, 14th, 21st and 28th post-SCI day. The maximal and minimal values of the BBB scores obtained from the experimental rats in each subgroup were considered coincidental, so they were left out; then, behavioural data from the five remaining rats belonging to each subgroup were averaged and the intergroup differences analysed statistically by the Tukey multiple comparisons test.

RESULTS

Recovery of rats

The two control rats survived their laminectomies and the 21 experimental rats survived their laminectomies and the compression of the spinal cord trauma. Both the controls and the experimental groups survived the 28-days observation period. Urinary retention developed in only 2 rats from subgroup-2 with medullary lesions caused by the 40 g compression force but in 6 from the subgroup-3 (50 g compression). The retention lasted for 2–5 days, when their urinary bladder had to be emptied by Credé's manoeuvre (manual bladder expression) every 12 hours. Later on, the ability to void spontaneously returned, even in the severely paraparetic or paraplegic rats. The surgical wounds were completely healed in all 23 rats on the 7th day following the surgery.

Behavioural outcomes

In both of the rats from the control group, their BBB score was 21 points (i. e. they did not exhibit any neurological deficits) 6 hours after the surgery.

The SCI rats from subgroup-1 (30 g compression) were paraplegic or severely paraparetic (BBB scores ranged from 0 to 2 points with an average value of 1.6 points) after the surgery. All of the rats from subgroup-2 (40 g compression) were paraplegic (BBB score 0) after the surgery. Five rats from subgroup-3 (50 g compression) were paraplegic (BBB



Fig. 1. Situation following laminectomy of Th9 vertebra in one of the experimental rats. The spinal dural sac containing the medulla spinalis is marked by the arrow No 1. The spinous process of the Th7 is fixed by a Kocher clamp. The metallic rod (arrow No. 2) with an impactor (arrow No. 3) is visible cranially from the laminectomy to enable fixation of the spinous process of the Th10 by another Kocher clamp before the execution of spinal cord compression trauma. A thermometer probe (arrow No. 4) is inserted into the rectum of the rat

score 0) after the surgery. The statistical analyses (by the Tukey multiple comparisons test) revealed no significant differences in the BBB scores among rats from all of the 3 experimental subgroups.

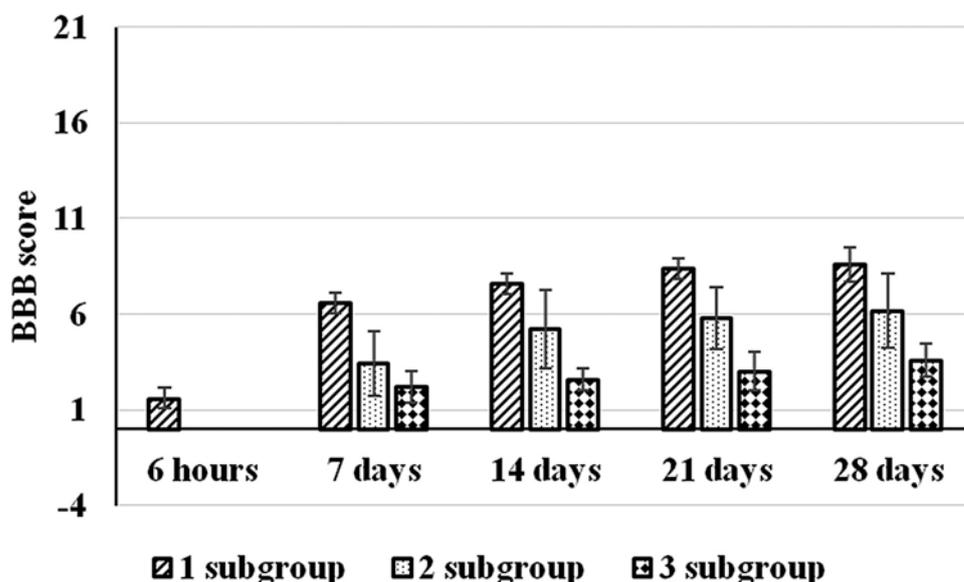
Seven days after the SCI, the BBB score in 5 rats from subgroup-1 was 6–7 points (an average value of 6.6 points), in subgroup-2, it was 1–5 points (average of 3.4 points) and in subgroup-3 it was 1–3 points (average of 2.2 points). The statistical analyses showed a highly significant difference in the improvement of motor functions (expressed by BBB score) in rats belonging to the subgroup-1 *versus* rats belonging to the subgroup-3 ($P=0.0001$) and a significant difference between rats belonging to the subgroup-1 and rats belonging to the subgroup-2 ($P=0.002$). There was no significant difference between rats belonging to the subgroup-2 and rats from the subgroup-3 ($P=0.2502$).

Fourteen days after the SCI, the BBB score in 5 rats from subgroup-1 was 7–8 points (an average value of 7.6 points), in subgroup-2, it was 2–7 points (an average value of 5.2 points) and in subgroup-3, it was 2–3 points (average of 2.6 points). The statistical analyses of the results showed a highly significant difference in the improvement of motor functions (BBB score) between rats belonging to the subgroup-1 *versus* rats belonging to the subgroup-3 ($P=0.0001$), a significant difference between rats belonging to the subgroup-2, *versus* rats belonging to the subgroup-3 ($P=0.017$), and in rats belonging to the subgroup 1, *versus* rats belonging to the subgroup-2 ($P=0.027$).

Twenty one days after the SCI, the BBB score in 5 rats from the 30 g compression (subgroup-1) was 8–9 points (an average value of 8.4 points), in the 40 g subgroup-2, it was 3–7 points (an average value of 5.8 points) and in 50 g subgroup-3, it was 2–4 points (average 3.0 points). The statistical analyses of the results showed a highly significant difference in the improvement of the motor functions between rats belonging to the subgroup-1, *versus* rats belonging to the subgroup-3 ($P=0.0001$), a significant difference between rats belonging to the subgroup-2, *versus* rats belonging to the subgroup-3 ($P=0.0062$) and a significant difference between rats belonging to the subgroup-1 *versus* rats belonging to the subgroup-2 ($P=0.01$).

Twenty eight days after the SCI, the BBB score in 5 rats from the subgroup-1 was 8–10 points (an average value 9.0 points), in subgroup-2, it was 3–8 points (an average value 6.2 points), and in subgroup-3 it was 3–5 points (average 3.8 points). The statistical analyses showed a highly significant difference between rats belonging to the subgroup-1, *versus* rats belonging to the subgroup-3 ($P=0.0002$), a significant difference between rats belonging to the subgroup-2, *versus* rats belonging to the subgroup-3 ($P=0.02$), and a significant difference between rats belonging to the subgroup-1, *versus* rats belonging to the subgroup-2 ($P=0.003$).

The behavioural outcomes expressed as the BBB scores are presented in Graph 1. The significant intergroup differences were found at all survival times longer than 7 days post SCI.



Graph 1. Comparison of the averaged data of locomotor rating scale for open field testing obtained in rats from three experimental subgroups

DISCUSSION

More than a decade ago, spinal cord traumatic lesions meant for patients, the confinement to a wheelchair and different medical complications for the rest of their lives [3], [5], [18]. The treatment armamentarium was limited and the provision of care for individuals with SCI was, in a majority of cases, met with frustration [3], [5], [18]. Progress in neuroscience research has made the idea, that SCI will eventually be repairable, plausible [12], [13]. The use of animal models is critical for devising experimental therapies aimed to restore functions lost due to SCI [14]. Animals such as rabbits, cats, dogs, swine (especially minipigs), non-human primates, as well as rats and mice, are used in different experimental models [8], [9], [14–17], [19], [20]. Due to their cost, accessibility, ease of care and the existence of well-established functional analysis techniques, rat experimental models are the most commonly used at present [2], [6], [15], [19]. At the Institute of Neurobiology, Slovak Academy of Sciences, where we performed our study, they have been breeding Wistar rats for a long time. We decided to use adult female rats of this strain in our experiments.

Different types of SCI protocols address different purposes, therefore each experimental model has its own advantages and disadvantages.

The transection model requires durotomy and partial or complete cutting of the spinal medulla. A lateral hemisection is sometimes preferred because the integrity of one half of the spinal cord is sufficient to maintain urinary bladder and bowel function, and results in less intense postoperative care and an increased animal survival rate. The transection models provide valuable information when axon regeneration is studied or implantation of specific devices are tested. The disadvantage of this SCI model is that transection of the spinal medulla is rarely seen in human patients [3], [7], [18].

The SCI in the contusion model, is induced by hitting the spinal medulla through the exposed dura mater with a blunt contusion force, with a weight dropped from a defined height or with a spinal cord injury computer operated apparatus [20]. These devices allow the measurement of biomechanical parameters such as; impulse, velocity, power and energy. The contusion type of injury better mimics the lesions seen in humans than the transection models [11, 20]. However, it is more difficult to distinguish the spared and regenerated tissues in this model [6].

The SCI in the compression models are induced by compressing the spinal medulla with an aneurysm clip, forceps, balloon or an impactor acting through the intact dura mater. By the help of this model, it is possible to create different degrees of SCI by adjustment of the compression strength and time [19]. The compression model also mimics the neuropathology observed in humans [11]. The disadvantages of compression models are problems with securing reproducibility. The compression force of an aneurysm clip diminishes with each application and every clip closes with a different strength. The compression force of an intraspinally inserted balloon inflated by the exact volume of solution, changes slightly depending upon the diameter of the vertebral canal utilized. We have overcome these problems by utilisation of an impactor compressing the spinal medulla by a defined weight for a defined time interval.

The key element of any SCI study is the ability to acquire reliable, reproducible and worthwhile behavioural data [9], [15], [19]. The most commonly used test to assess motor functions after a SCI is the 21 point BBB locomotor scale [2]. Each point in the BBB score represents a specific set of characteristics of pelvic limb function after thoracic or thoracolumbar spinal cord trauma demonstrated by the rat during spontaneous open field locomotion [2]. We decided to use the assessment of the BBB score in our study.

The goal of all SCI models is to produce submaximal injury, where the outcome can be measurably improved or deteriorated by the therapeutic intervention of interest. The statistical analyses of the data gained in three experimental subgroups demonstrated that these requirements were achieved most reliably in the subgroup-2 of rats following the 40 g compression spinal cord trauma.

CONCLUSIONS

This study confirmed the reproducibility and reliability of the rat spinal cord compression model. The behavioural characteristics expressed by the BBB scores were similar to the consequences of medullary trauma in different experimental animals as well as in humans. The model proved its suitability for preclinical testing of new therapeutic approaches aimed to modulate neuropathological processes participating in secondary injury mechanisms and to improve the outcome in para- or tetraplegic patients.

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SEROLOGICAL AND MOLECULAR SURVEY OF SERIOUS INTRACELLULAR COCCIDIA IN RED FOXES IN EASTERN SLOVAKIA

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ABSTRACT

We tested the sera or meat juices of 215 red foxes (*Vulpes vulpes*), collected during 2009—2014 at different localities of Eastern Slovakia for the presence of anti-*Toxoplasma* and anti-*Neospora* antibodies. We also examined the brains or muscle tissues and uncoagulated blood samples for the presence of both parasite's DNA. The mean seropositivity to *T. gondii* was 72.6% (95% Confidence Interval CI 66.1—78.4) and to *N. caninum* 33.9% (95% CI 27.7—40.7). We observed higher *Toxoplasma*-seropositivity in adults (71.1%) than in juveniles (60.0%). *Neospora*-seropositivity was almost the same in both age groups about of 30%. In general, we observed less frequently the DNA of parasites, *T. gondii* (14.5%) and *N. caninum* (20.3%) in the tissue samples and uncoagulated blood samples. Coccidiosis are considerably common in red foxes and circulate in locations of Eastern Slovakia. The high infection rate in foxes is probably due to their infected prey. On the other hand, the contamination of the environment with oocysts and their subsequent transfer to other farm and wild animals is also possible.

Key words: antibodies, DNA; ELISA; *Neospora caninum*; PCR; red foxes; *Toxoplasma gondii*; Slovakia

INTRODUCTION

The population density of red foxes (*Vulpes vulpes*) after successful oral anti-rabies vaccination has significantly increased throughout Europe. Their expansion to nearby human settlements (the phenomenon of urbanization) has also been going on [6]. Foxes participate in the spread of several parasitic diseases, such as toxoplasmosis and neosporosis.

Toxoplasma gondii and *Neospora caninum* are two closely related protozoan parasites with worldwide occurrence, having an indirect life cycle with cats and other wild feline carnivores as definitive hosts for *T. gondii*; and dogs and other canine carnivores for *N. caninum*. Oocysts are excreted in the faeces of the definitive host to the environment where they mature and can cause infection in some host species, including both domestic and wild animals [4], [7].

Toxoplasmosis is a widespread zoonotic disease, causing serious medical problems especially in pregnant women and immunosuppressed patients [16]. Humans become infected due to the consumption of inadequately cooked meat containing tissue cysts or by ingestion of sporulated oocysts, and also by transplacental transmission [4].

Neosporosis causes repeated abortions in cattle and other farm ruminants and has a negative economic impact on their breeding [3]. Both parasitoses circulate in both sylvatic and domestic cycles. The infections can be transmitted to stables due to poor zoohygiene by rodents or by contact with free living animals.

The aim of this study was to evaluate the occurrence of anti-Toxoplasma and -Neospora antibodies in red foxes from different locations of Eastern Slovakia and to associate the seroprevalence with their DNA evidence.

MATERIALS AND METHODS

Within the period of 2009 to 2014, we collected blood, brain and muscle tissue samples of red foxes from different localities of Eastern Slovakia; in particular from localities of agricultural lands and farms. The blood and tissue samples were obtained from the Department of Pathology of the State Veterinary and Food Institute in Košice from foxes which had been trapped or shot for rabies control and also from individual hunting. Sera were prepared from coagulated blood by centrifugation at 600 g for 10 min. In case when blood samples were absent, meat juices were prepared by freeze-thawing from muscle samples for serological testing. Tissue and uncoagulated blood samples were removed for molecular analysis. All samples were kept at -20°C up to further analyses.

The circulating anti-Toxoplasma antibodies were tested by the multi-species indirect ELISA (ID-Vet, Montpellier, France) using the manufacturer's protocol. We examined a total of 150 serum samples and 65 meat juices. The sera were diluted at 1:10 and meat juices 1:2. Protein G peroxidase labelled conjugate, which recognizes mammalian IgG antibodies and a TMB (3,3',5,5'-Tetramethylbenzidine) substrate, were used. The absorbance (OD) was measured at 450 nm by a spectrophotometer (Thermolabsystem, Opsys MR, U.S.A.). The results were calculated as S/P % index

according to the formula: $S/P\% = (OD_{\text{sample}} - OD_{\text{NC}} / OD_{\text{PC}} - OD_{\text{NC}}) - 100$. The samples with $S/P\% \leq 40\%$ were negative; between 40% and 50% doubtful and with $S/P\% \geq 50\%$, were categorized as positive.

For the detection of the anti-Neospora antibodies, a competitive ELISA (VMRD, Inc., Pullman, USA) test was used according to the manufacturer's instructions. Non-diluted serum samples or meat juices were added to the wells of the antigen-coated plates. The absorbance was measured at 630 nm. A test was considered valid, if the mean of the Positive Controls formed $\geq 30\%$ inhibition of the reaction, $\% I = 100 - (OD_{\text{sample}} / OD_{\text{NC}} \times 100)$. The results were negative when the tested sample produced $< 30\%$ inhibition and positive if they produced $\geq 30\%$ inhibition.

We isolated DNA from the brain and skeletal muscle tissues by NucleoSpin®Tissue kit (Macherey-Nagel, Germany) with a previous overnight digestion with proteinase K (Promega, Madison, WI) at 56°C . Isolation from non-coagulated blood samples was performed by NucleoSpin®Blood kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The isolates were stored at -20°C until the analysis.

The amplification of the TGR1E gene of 191 bp in size repeated itself in the *T. gondii* genome 30–35 times was made using two primers: TGR1E-1 and TIGRIE-25 [13]. DNA-free molecular water was used as the negative and *T. gondii* RH-strain (NRL for Toxoplasmosis, Prague, Czech Republic) as the positive control. The PCR products were analysed in 1.5% agarose gel with GelRed dye and evaluated under UV light.

The detection of the Nc5 gene of *N. caninum* was performed with specific primer pairs: Np6 and Np21 [19]. The amplifications were approved in a gradient thermocycler (Bioer, China). The first Slovak *N. caninum* isolate [14] was used as positive and DNA-free water as negative control. The PCR products were analysed in 1% agarose gel and evaluated under UV lamp using a digital camera (Panasonic AC 40).

The results of the serological investigations were evaluated statistically by the Fisher exact test (GraphPad Software). The prevalence and exact confidence intervals at 95% level were computed. The significance of the differences were evaluated using STATISTICA 6 Base (StatSoft, Inc., 2001).

RESULTS

In 215 foxes from the localities of eight eastern Slovak districts the mean level of anti-Toxoplasma and anti-Neospora antibodies were detected in 72.6% (95% Confidence Interval CI 66.1–78.4) and 33.5% (95% CI 27.2–40.2) of the samples, respectively. The higher Toxoplasma-seropositivity was detected in adults (71.1%; 95% CI 62.4–78.8) compared to the juveniles (60.0%; 95% CI 36.1–80.9). The Neospora-seropositivity was almost the same in the adults and the juvenile categories, or only those of unknown age.

Toxoplasma-infection was present on a similar level in both genders (72.3% in males and 69.7% in females). However, males were more frequently infected with *N. caninum* (39.8%) compared to the females (23.7%). Toxoplasmosis

was detected in all districts at a high level, varying between 50.0% and 78.4%. The level of specific antibodies was high and values of S/P% indexes fluctuated from 96.5% to 260.3%. Neosporosis was present in seven districts with fluctuation from 25.9% to 46.3%. The percentage of inhibition of cELISA varied between 33.0% and 105.5%. The serological follow-up of toxoplasmosis revealed a slight decrease in individual years from 80.6–54.5%. On the other hand, the yearly occurrence of neosporosis was most variable with the significantly lowest seropositivity in 2014 (4.5%; $P=0.0009$) (Table 1). The occurrence and the linear trend of Toxoplasma- and Neospora-seropositivity in red foxes of both examined coccidiosis is shown in Figure 1.

The molecular detection revealed a significantly lower rate of *T. gondii* (14.5%) and *N. caninum* (20.3%) DNA evi-

Table 1. The occurrence of specific antibodies in red foxes according to the year, age, gender and the hunting locality

Years	2009		2010		2012		2014		Total		Total	
	N/n		N/n		N/n		N/n		N/n/%	95% CI	N/n/%	95% CI
	<i>T. g.</i>	<i>N. c.</i>	<i>Toxoplasma gondii</i>		<i>Neospora caninum</i>							
Age category												
Juvenile	2/1	2/0	–	–	17/11	17/6	1/0	1/0	20/12/60.0	36.1–80.9	20/6/30.0	11.9–54.3
Adult	28/23	28/13	–	–	58/44	58/24	42/24	42/2	128/91/71.1	62.4–78.8	128/39/30.5	22.7–39.2
Unknown	6/5	6/0	58/46	58/26	2/2	2/2	1/0	1/0	67/53/79.1	67.4–88.1	67/28/41.8	29.8–54.5
Gender												
Male	17/15	17/6	3/3	3/1	40/32	40/21	23/10	23/1	83/60/72.3	61.4–81.6	83/29/39.8	29.2–51.5
Female	19/14	19/7	1/1	1/0	36/24	36/10	20/14	20/1	76/53/69.7	58.1–79.6	76/18/23.7	14.7–34.8
Unknown	–	–	54/42	54/25	1/1	1/1	1/0	1/0	56/43/76.8	63.6–87.0	56/26/46.4	32.9–60.2
Districts												
Gelnica	–	–	–	–	9/7	9/4	4/2	4/0	13/9/69.2	38.6–90.9	13/4/30.8	9.1–61.4
Košice	31/25	31/11	19/17	19/8	9/8	9/5	15/8	15/1	74/58/78.4	67.3–87.1	74/24/32.4	22.0–44.3
Michalovce	–	–	8/7	8/3	24/18	24/10	9/6	9/1	41/31/75.6	59.7–87.6	41/14/34.1	20.1–50.6
Rožňava	–	–	2/1	2/1	4/2	4/1	–	–	6/3/50.0	11.8–88.2	6/2/33.3	4.3–77.7
S.N. Ves	–	–	13/11	13/5	7/5	7/2	7/4	7/0	27/20/74.1	53.7–88.9	27/7/25.9	11.1–46.3
Sobrance	1/1	1/0	–	–	3/3	3/0	3/1	3/0	7/5/71.4	29.0–96.3	7/0/0	0.0–34.8
Trebišov	–	–	16/10	16/9	21/14	21/10	4/2	4/0	41/26/63.4	46.9–77.9	41/19/46.3	30.7–62.6
Vranov	4/3	4/2	–	–	–	–	2/1	2/0	6/4/66.7	22.3–95.7	6/2/33.3	4.3–77.7
Total N/n	36/29	36/13	58/46	58/26	77/57	77/32	44/24	44/2				
[%]	80.6	36.1	79.3	44.8	74.0	41.6	54.5	4.5	215/156/72.6	66.1–78.4	215/73/33.9	27.7–40.7

T. g. — *Toxoplasma gondii*; *N. c.* — *Neospora caninum*; N — number of animals examined
n — number of positive animals; % — seropositivity

Table 2. Molecular evidence of *Toxoplasma gondii* and *Neospora caninum* in red foxes by the years and examined sample types

Years/ <i>T.g./N.c.</i>	2009			2010			2012		2014		Total N/n/%
	A	B	C	A	B	C	A	B	B	C	
Examined [N]	–	20	20	4	38	–	–	47	40	4	
Positive [n]	–	9	1	0	0	–	–	7	6	2	
Prevalence [%]	–	4.5	0.5	0	0	–	–	14.9	15.0	50.0	
Total <i>T.g.</i> N/n/%	40/10/25.0			42/0/0.0			47/7/14.9		44/8/18.2		173/25/14.5
Examined [N]	1	24	20	9	30	33	13	23	40	4	
Positive [n]	1	11	0	5	12	3	3	4	1	0	
Prevalence [%]	100	45.8	0	55.6	40.0	9.1	23.1	17.4	2.5	0	
Total <i>N.c.</i> N/n/%	45/12/26.7			72/20/27.8			36/7/19.4		44/1/2.3		197/40/20.3

T. g. — *T. gondii*; *N. c.* — *N. caninum*; A — DNA isolated from blood; B — DNA isolated from brain; C — DNA isolated from muscle tissue

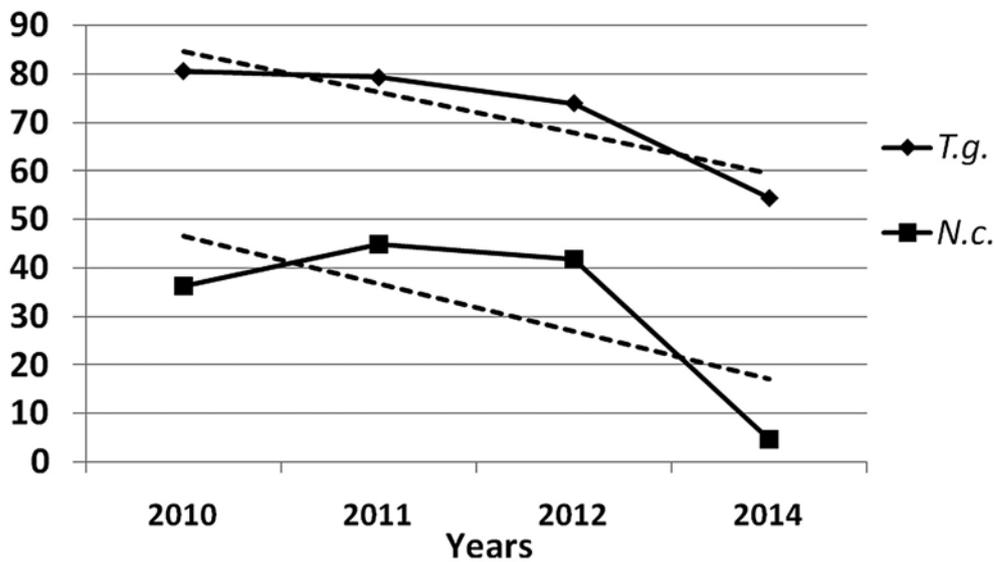


Fig. 1. The occurrence and linear trend of *Toxoplasma*- and *Neospora*-seropositivity in red foxes from different locations of Eastern Slovakia

dence in the tissues and blood samples (Table 2) when compared with the serological evidence. The presence of *T. gondii* was confirmed in 15.2% of the brain tissue samples and 12.5% of the muscle samples. The genomic DNA of *N. caninum* was confirmed in 23.9% of the brain samples, 39.1% in the uncoagulated blood and only 5.3% of the muscle samples (Table 2). Coinfection was detected in three samples, which were simultaneously PCR positive for both parasites.

DISCUSSION

During the past three decades, the rapidly increasing population density of red foxes has had a significant effect on the prevalence of infections, including coccidiosis. This fact has resulted in an increased risk of contact with oocysts due to the greater amount of animals in a small area [10]. The high incidence of toxoplasmosis, and likewise neosporosis, in wildlife is explained by the high abundance of the causative agents in the diets of the foxes. An important prey for foxes represents rodents. On the other hand, the carcasses of the infected foxes can serve as the source of infection for the definitive hosts by contaminating the environment with oocysts. This phenomenon corresponds to our overall detection rate up to 72.6% *Toxoplasma*-seroprevalence in foxes. The occurrences of toxoplasmosis in red foxes from localities of Eastern Slovakia are higher in comparison to other Western European countries where seropositivity vary from 20% to 47% [8, [11], [17], [18]. In accordance with our high seropositivity rate, also in neighbouring Hungary, high (68%) seroprevalence has been detected in red foxes [12]. Similarly, in Central and Eastern Germany, 74.5% and 84.7% of the foxes had antibodies to *T. gondii* [9]. The age and gender of foxes did not influence the seropositivity significantly; however, older animals were infected more frequently than juveniles. We could deduce that the infection is transmitted mainly via infected prey or sporulated oocysts and only sporadically by transplacental transmission.

Despite the high seropositivity rate, the molecular evidence of *T. gondii* was one fifth lower. Similarly, in foxes from Germany 13.4% were PCR-positive [9]. By multiplex real-time PCR, *T. gondii* was detected in 18.8% of the brain samples in Belgian red foxes [2]. In a recent study 6% of *T. gondii* DNA was confirmed in the fox brain tissues from Romania [15].

Concerning the neosporosis in wild carnivores, their natural prey are frequently the source of infections [5]. The presence and distribution of neosporosis in wildlife may represent a high infection risk for livestock, especially during the grazing season. Our results revealed 24.6% mean *Neospora*-seropositivity, which is higher than the prevalence in other countries of Western Europe, where the seropositivity varied between 11% and 17% [1], [18]. In Hungary, only 1.5% *Neospora*-seroprevalence of red foxes was estimated [12]. The absence of significant age-related differences of *Neospora*-seropositivity may suggest that infections could be transmitted mainly via the transplacental way, as in cattle.

CONCLUSIONS

The high occurrence of anti-*Toxoplasma gondii* (72.6%) and anti-*Neospora caninum* (33.9%) antibodies in red foxes from different locations of Eastern Slovakia suggests their important role in the maintenance of sylvatic circulation of both coccidian parasites as a reservoir host. The serological follow-up revealed a reasonable decreasing trend during the examined period. Despite the high serological evidence, DNA of both parasites was significantly less frequently confirmed from tissue and blood samples. A simultaneously positive molecular finding for both coccidia was detected only in three cases. In order to disclose the actual role of red foxes in the epidemiology of toxoplasmosis and neosporosis, more extensive study is required in widespread ranges of natural ecosystems.

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PATHOLOGICAL ALTERATIONS IN HEPATOCYTES OF DAIRY COWS WITH A TENDENCY TO EMACIATION AND FATTENING

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ABSTRACT

The aim of this study was to demonstrate the histochemical and histopathological alterations in the livers of cows with a tendency to become emaciated (body condition score — BCS1 and 2) and a tendency to become fattened (BCS4 and 5) in comparison to the cows of average body condition (BCS3) presented as a control. The histochemical analysis (PAS reaction) showed that the influence of emaciation and fattening in our study was manifested by a decreased occurrence of glycogen and a decreased level of the PAS-positive matter in the hepatocytes of dairy cows with BCS1, 2, 4 and 5. An abundant accumulation of lipids in the form of large lipid droplets, liposomes and lipoproteins observed in the hepatocytes of emaciated and fattened (BCS1 and 5) cows may be related to moderate-severe steatosis. These observations suggest a relationship between liver steatosis and the oc-

currence of lipoproteins in cows with a tendency toward emaciation and fattening.

Key words: body condition score; hepatocyte; liver; steatosis

INTRODUCTION

Several studies indicate that the negative energy balance (NEB) in cows during early lactation may cause excessive mobilization of fatty acids in the liver tissue [7]. In particular, in cows with severe fattening, the cell structure in the liver and their functions are destroyed [22]. These cows are less fertile, have significantly longer time of calving and higher numbers of inseminations per conception compared to cows with moderate fattening [23]. The body condition of dairy cows influences not only ovarian follicle

development [21] but also liver metabolism [23]. Fat in the liver of dairy cows is accumulated in the peri-partum period. According to imposition of lipid droplets in hepatocytes, dairy cows can be classified into two groups: cows with moderate fattening, and cows with heavy fattening [23]. Therefore, the evaluation of the fat content in the liver is relevant to determine the relationship between the body condition and milk performance of dairy cows and their metabolic status.

It is known that the liver plays an important role in the lipid and lipoprotein metabolism and effectively influences metabolic processes throughout the organism. According to Alexander et al. [1], lipoproteins are formed following the fusion of lipid particles of the smooth endoplasmic reticulum (ER) with the apoproteins of rough ER, when B apoprotein and probably other very-low-density lipoprotein (VLDL) apoproteins are bound to the lipid particle to form a nascent lipoprotein. Therefore, it can be assumed that any damage to the hepatocytes (steatosis) is accompanied by changes in the metabolism of lipoproteins [20]. It seems that there are some relationships between glyco-gen, lipid metabolism in the liver and variable nutrition levels in dairy cows. These changes may affect body condition and health status of cows and also may influence their performance. Liver steatosis negatively affects the production of gonadotropic hormones leads to cystic atresia of the ovarian follicles and thus may cause the deterioration of the fertility of cows.

The aim of this study was to elucidate associations between histopathological/ histochemical findings in the liver of dairy cows and their body condition evaluated by a five-point scale of BCS.

MATERIALS AND METHODS

Biological material

As a source of biological material, the livers were acquired at the slaughtering of Holstein dairy cows ($n=23$) at a local abattoir at different times of the post-partum period. The cows were kept under normal feed regimes. The animals were estimated as belonging into certain grades of body condition score (BCS) according to a five-point scale of BCS [8]. Our experimental dairy cows were categorized into four different groups: BCS1 (emaciation; $n=4$), BCS2 (tendency towards emaciation; $n=4$), BCS3 (optimal body

condition status; $n=4$), BCS4 (tendency to fattening; $n=7$) and BCS5 (fattening; $n=4$). The data on these cows were taken from the cow's individual cards on farms and were as follows: average age 6.2 years, 4.1 years, 5.7 years, 5.5 years, 6.36 years; and post-partum period 4.8 ± 0.51 weeks; 4.49 ± 0.59 , 5.82 ± 0.62 ; 10.94 ± 1.37 and 12 ± 2.2 for BCS1, BCS2, BCS3, BCS4 and BCS5, respectively.

Histopathological and histochemical analysis

For the histological analyses of the liver samples ($n=36$) from cows, they were fixed in 10% neutral buffered formalin (Sigma-Aldrich), dehydrated in a rising set of ethanol solutions (70% and 90% for 2 hours and 100% for 1 hour) and embedded into Technovit 7100 resin (Heraeus GmbH, CoKG, Werheim/Ts., Germany) according to the producer's manual. The tissue sections of 1–2 μm in thickness were cut on a Ultracut E (Reichert, Jung, Austria) and two sections obtained from each sample were placed on the standard slides (Bamed, Czech Republic). Afterwards, the first sections were stained with haematoxylin and eosin (HE). The second sections were stained for the detection of glycogen and PAS-positive material according to the PAS-Hotchkiss-McManus methodology (DiaPath Srl., Italy). The samples were described and evaluated from light-microscopy images using a Carl Zeiss AxioScope A1 microscope (Zeiss, Germany). The figures were made using the NIS-Elements AR version 3.0 software (Laboratory Imaging s.r.o., Czech Republic).

Electron microscopy analysis

The liver samples ($n=37$) from cows ($n=17$) were fixed in the aldehyde mixture (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate) at 4°C for one hour and subsequently post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate for one hour. Thereafter, for the lipoprotein visualization, the samples were soaked in 1% *p*-phenyldiamine (Sigma-Aldrich) solution in 70% acetone for 30 min according to Boshier et al. [4]. After the dehydration in acetone, the samples were embedded into Durcupan ACM (Fluka). Ultrathin sections (90 nm) were cut using a Leica EM UC6 ultramicrotome (MIKRO Ltd., Bratislava, Slovak Republic) and then contrasted with uranyl acetate and lead citrate. The contrasted sections were analyzed under a JEM100 CXII transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

The lipids were visualized following osmium post-fixa-

tion. Lipoproteins (VLDL) were visualized by p-phenylene-diamine and localized mostly in the Disse space in the form of lipid particles with a diameter of 80 nm (determined by a scale bar for each electron micrograph) according to [1]. The occurrence of lipid droplets was evaluated from electronograms subjectively as follows; + rare occurrence, ++ moderate occurrence, +++ abundant occurrence, and ++++ very abundant occurrence. This occurrence of lipid droplets is correlated with the definition of steatosis according to Reid et al. [24], who differentiated four degrees of steatosis based on the amount of hepatic fat as follows; healthy dairy cows $\leq 10\%$, moderate steatosis $\leq 20\%$, moderately severe steatosis $\leq 30\%$, and severe steatosis $\geq 30\%$.

RESULTS

Light microscopy and histochemistry

By the evaluation of semi-thin sections of the cow's liver samples, we found sporadic dilatation of hepatic sinuses in cows with BCS2 (Fig. 1A) in contrast to the BCS3 cows, where such dilatation was not revealed (Fig. 1B). These sinuses occurred only occasionally and were filled with erythrocytes. Part of the liver parenchyma, which was composed of radially arranged hepatocytes with regressive changes, was filled with several smaller or larger optically-empty intracytoplasmic vacuoles. In the spaces between adjacent hepatocytes some sinuses contained several cells resembling Kupffer's cells. Normal liver parenchymal tissue with characteristic hepatocytes was observed in BCS3 cows (Fig. 1B).

We have found that the PAS-positive matter is present in the cytoplasm of hepatocytes both in the corpuscular and diffuse form. Histochemically detected PAS-positive material is distributed diffusely (homogenously) in the full range of the hepatic acini. In comparison to the group of BCS3 cows (Fig. 1C), the less PAS-positive matter was found in the hepatocytes of cows with BCS2, 4 and 5 (Table 1, Figure 1D–F).

Electron microscopy analysis of the livers

According to the ultrastructural images of the hepatocytes, the occurrence of glycogen granules in BCS3 cows (Fig. 2A) was higher than in BCS4 cows (Fig. 2B). Ultrastructural images of the hepatocytes demonstrated damages to the organelles in the hepatocytes mainly in cows of

BCS1, 2, 4 and 5. In comparison to the cows of BCS3 (moderate condition), characterized by prominently granulated endoplasmic reticulum surrounding numerous mitochondria and lipid droplets (Fig. 2C), the cows with BCS1 and 5 manifested the increased occurrence of swollen mitochondria or mitochondria with significant electron-optic density, smooth endoplasmic reticulum with cytoplasm vacuolization, absence of polysomes and a rise in the number of lysosomes in portobiliary space of hepatocytes, which indicated on initial stages of liver cell degeneration (Fig. 2C–F). Using subjective evaluation of the electronograms we observed an increased occurrence of lipid droplets and liposomes in the hepatocytes of cows with BCS1 and 5, when compared to the BCS3 cows (Table 1, Fig. 2C–F). In the hepatocytes of cows with BCS1, 2, 4 and 5, the lipofuscin pigments had accumulated (Table 1). Also, in the hepatocytes of the cows of BCS1, 2 and 5 higher accumulations of lipoprotein granules were observed compared to the BCS3 cows (Table 1).

Table 1. The intensity of occurrence of cell inclusions in the hepatocytes of cows with different BCS

Cells inclusions	Body condition score of cows				
	1	2	3	4	5
PAS-positive material ¹	+	+++	+++	++	+
Glycogen ²	+	++	+++	+++	++
Lipid droplets ²	+++	++	+	+++	+++
Liposomes ²	+++	++	+	++	+++
Lipoprotein ²	+++	++	+	+	++
Lipofuscin ²	++	+++	+	++	+++

Occurrence: + — small; ++ — moderate; +++ — abundant

¹ — evaluated by light microscope

² — evaluated by electron microscopy

DISCUSSION

The body condition of dairy cows may affect also liver metabolism [23]. Post-partum depression of feed intake and subsequent energy deficiency causes a rapid loss of body mass and the accumulation of intracellular fat in the liver (lipomobilization, fat infiltration of the liver and other organs, and ketosis). The rapid loss of body reserves

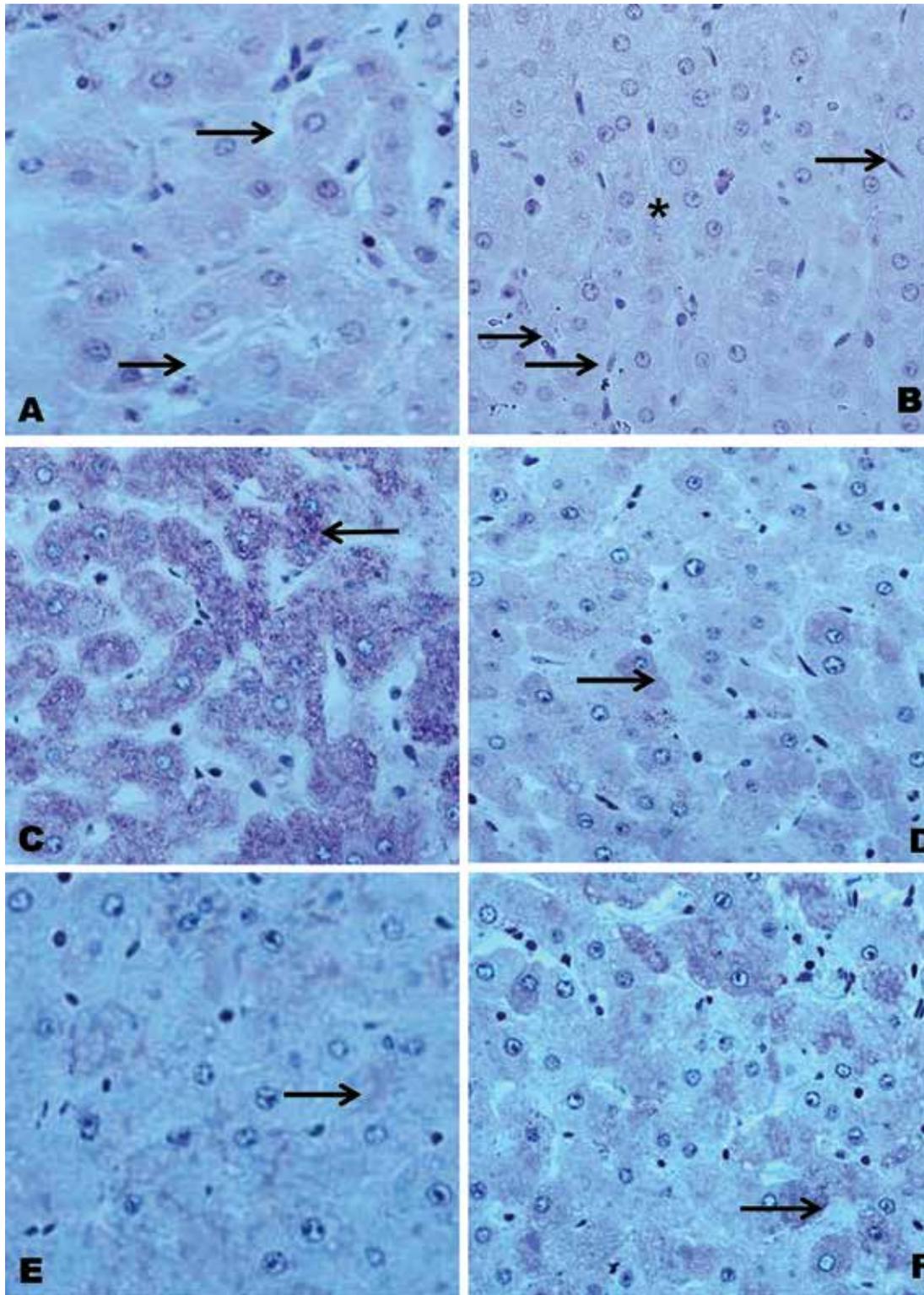


Fig. 1. Microscopical analysis of hepatocytes from dairy cows with different BCS

A (10123P2) — increased occurrence of dilated sinuses (arrows) between radially arranged liver cells of BCS2 cow. **B** (10132P3) — radially arranged hepatocytes of a BCS3 cow with regressive changes in the form of several smaller optically empty intracytoplasmic vacuoles — lipid-like substances (asterisk); there are visible sinuses and Kupffer's cells (arrows) in the spaces between adjacent hepatocytes. **C** (10131P3) — normal parenchyma of the liver of a BCS3 cow with a number of tightly settled PAS-positive hepatocytes in the form of intensive granular reaction (arrow) with the incidence of Kupffer's cells. **D** (10811P) — normal parenchyma of the liver of a BCS4 cow, which consists of numerous similar PAS-positive hepatocytes with a diffuse reaction (arrow). **E** (10142P2) — normal hepatocyte of a BCS2 cow with a moderate diffuse reaction of PAS-positive substances (arrow). **F** (1090P25) — normal parenchyma of the liver of a BCS5 cow, which is composed of a numerous similar in size and shape hepatocytes with more intensive diffuse reaction of PAS-positive substances (arrow). Staining: A, B — haematoxylin-eosin (HE); C—F — PAS-Hotchkiss-McManus methodology. Magn. $\times 400$ (A—F)

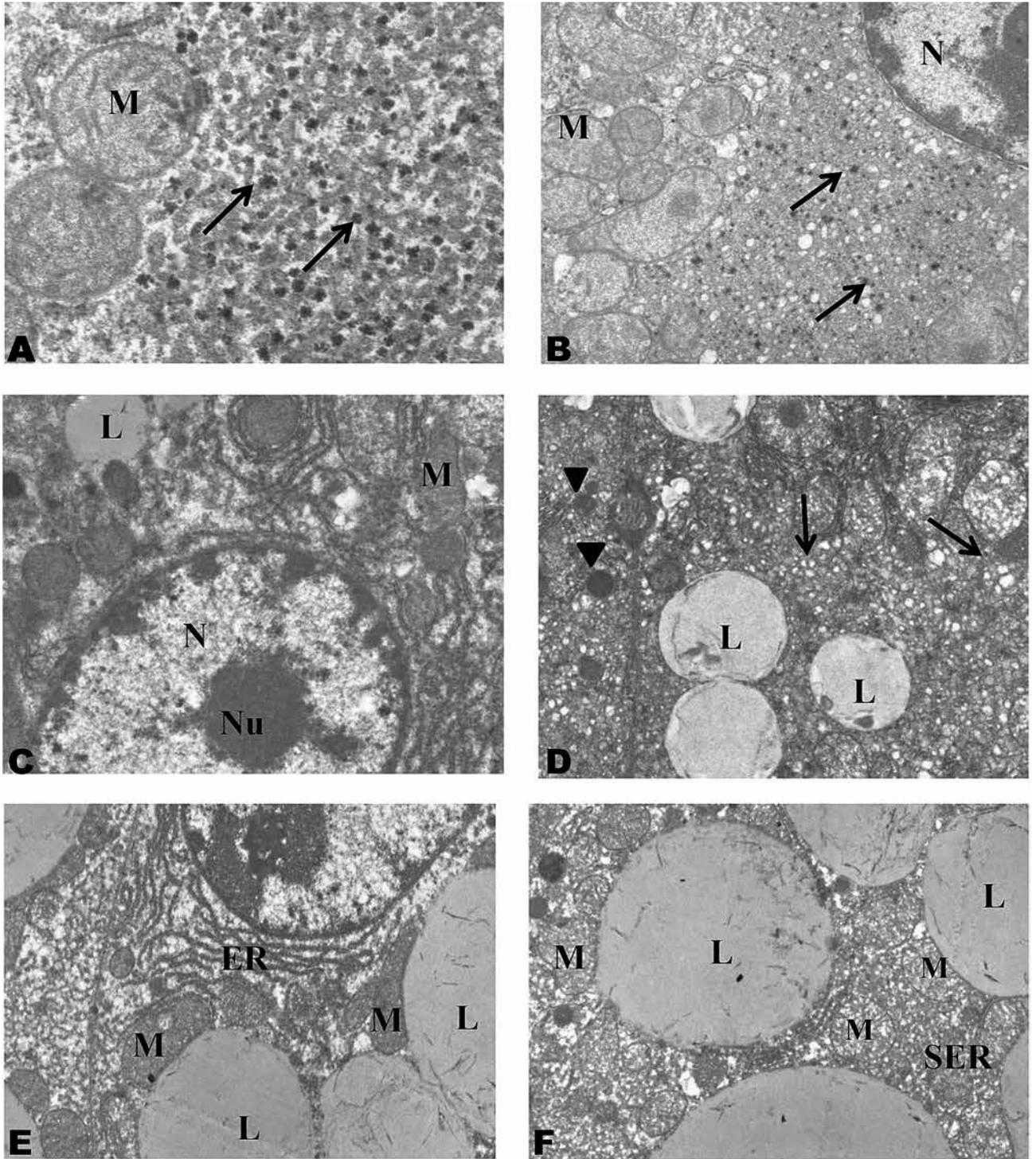


Fig. 2. Ultrastructural image of the liver hepatocytes from dairy cows with different BCS

A (3808) — occurrence of large mitochondria (M); accumulation of large dark granules of glycogen (arrows) in the cytoplasm of the hepatocyte of a BCS3 cow. **B** (3822) — image of the nucleus (N) and mitochondria (M); low incidence of small glycogen granules in the cytoplasm of BCS4 cow's hepatocyte (arrow). **C** (3504) — ultrastructure of the hepatocyte of cows with BCS3; prominently granulated endoplasmic reticulum surrounding numerous mitochondria (M) and lipid droplets (L). The nucleus (N) and nucleolus (Nu) are present in the lower part. **D** (3531) — large lipid droplets in hepatocytes of BCS2 cows; numerous small membrane-enveloped vesicles — liposomes (arrows), large transparent mitochondria (M), several lysosomes in the periportal space (arrowheads). **E** (3532) — large lipid droplets (L) in most of the hepatocyte of BCS1 cow surrounded by the dark elongated mitochondria (M). The nucleus with nucleolus and granulated endoplasmic reticulum (ER) are present in the upper part. **F** (3507) — the hepatocyte of BCS5 cow with large lipid droplets (L) surrounding numerous mitochondria (M) and smooth endoplasmic reticulum (SER). Staining: A–F uranyl acetate and lead citrate. Magn. $\times 19\,000$ (A); $\times 10\,000$ (C); $\times 7\,200$ (B, D, F).

post-partum, besides increased occurrence of metabolic disorders, subsequently disturbs the optimal blood concentration of glucose, insulin and IGF-I [13]. The histopathological images of the livers of emaciated cows (BCS1 and 2) was characterized by; the increase in the diameter of hepatocytes, decline in the volume of rough endoplasmic reticulum, and the number of mitochondria [22]. Starvation in cows results in a restriction of secretory properties of the hepatocytes, as it was found by Cakala and Bieniek [7]. In the liver of starving rats the number of ribosomes decreases [9]. It is generally known, that the content of proteins and phospholipids in the liver of starving animal falls, but the fat level rises, which was also confirmed in our study.

In cows with a tendency to fatten (BCS4) and fattened cows (BCS5), ketones and residual products of lipomobilisation bring the damages to the cell organelles of hepatocytes, especially mitochondria and endoplasmic reticulum and inhibit protein synthesis. The unused fatty acid residues in the hepatocytes generate the triglycerides and very-low-density lipoproteins, which represent a very effective system of endogenous triglyceride transport in the organism. However, when VLDLs are insufficiently produced or released, then triglycerides are accumulated in the hepatocytes resulting in the incidence of steatosis [10], [15]. The pathogenesis of steatosis depends on specific metabolic influences, resulting from the character of existing liver disease, as well as from other individual factors [15], [19], [30].

The livers of cows with BCS1 assessed in our study were, in most cases, similar to those showed in Fig. 2 E, F. In particular, numerous large lipid droplets in several cases occupied a one-third to half of the volume of the hepatocyte cytoplasm. These structures are surrounded by larger or smaller mitochondria with cristae and by granular endoplasmic reticulum with significantly decreased volume. These observations correspond to the findings in [24] about the proteosynthesis in the hepatocytes. Accumulation of lipids is a more common form of morphological alterations in the liver [6]. Fat accumulation is referred to as steatosis when more than 50 % of the hepatocytes contain microvesicular or macrovesicular forms and when the accumulation of the fat is of a diffuse character [15], [17].

The presence of lipids in hepatocytes of cows with a tendency toward emaciation, but also in fattened cows, appeared very often in the form of lipid droplets, liposomes

and lipoproteins. Reid et al. [23], using a stereological method of histological analysis of biopsied liver samples, determined the grades of hepatic steatosis on the basis of percentage of fat as follows: healthy liver — less than 10 %; moderate steatosis — less than 20 %; moderately severe — less than 30 % and severe steatosis — over 30 %. Our subjective estimation on the basis of lipid droplet occurrence in the hepatocytes of cows with BCS1 and 5 as abundant (+++) may be, according to [24], equivalent to a fat content of less than 30 %, which is moderately severe steatosis.

Cholesterol, as a precursor of steroid hormones [16], affects not only the metabolism of lipids but also the reproductive hormones. Steatosis of hepatocytes of emaciated (BCS1 and 2) or fattened dairy cows (BCS4 and 5) negatively affects the formation of gonadotropic hormones and prevents ovulation, which gives rise to cystic atresia of ovarian follicles and finally the formation of ovarian follicular cysts [25]. Therefore, the incidence of steatosis may adversely affect the fertility parameters of dairy cows. The effects of the cow's body condition on the fertility of dairy cows has been demonstrated in several reports [21], [27], [28]. In particular, cultured ovarian granulosa cells from the BCS2 cows manifested increased estradiol secretion [28]. This increased estradiol may inhibit the FSH production, which leads to elevated prolactin level, suppressing ovulation and causing follicular cyst formation. These authors also observed elevated zinc concentration in the blood plasma of BCS2 cows. This trace element is present in every cells of the body. Under the influence of free radicals the intracellular zinc is increased and can be available for various signalling and regulatory cascades, which activates kinases responsible for cellular differentiation, cell survival and apoptosis [12], [29]. Zinc ions enter the cells via the ZIP1 cell transporters, which can be induced by testosterone and prolactin [12]. High levels of zinc ions subsequently suppress terminal oxidation and maintain the cells at a low level of oxidation [11]. We assume that this molecular mechanism relates also to zinc in the hepatocytes, and its increased level may interact on the disorders of lipid or lipoprotein metabolism.

Lipofuscin, as an endogenous lipopigment, is produced in hepatocytes from lipids or lipoproteins probably by their peroxidation [3]. Lipofuscin is deposited in lysosomes [5]. Jean et al. [14] observed a high frequency of simultaneous incidence of steatosis and lipofuscin, especially with fibrotic changes in the liver. Lipofuscin granules visible in

the cytoplasm of hepatocytes are associated with the liver of old age [2], [26]. However, Mak et al. [18] have found that lipofuscin is not specific to the aging liver. It should be considered that the regenerative ability of the liver is well-known and very often even profound alterations, revealed on the level of electron microscopy, have not been confirmed by the functional analyses. The alterations that we found in the livers of cows, besides changes in the BCS status, were not manifested clinically.

CONCLUSIONS

Our observations indicate that in cows with emaciation (BCS1 and 2) or with fattening (BCS4 and 5), the occurrence of glycogen and a PAS-positive material was less apparent. In the hepatocytes of such cows, an intensive accumulation of lipids and lipoproteins was observed. These results suggest that histopathological and histochemical evaluation in combination with ultrastructural studies of the hepatocytes can be useful for the assessment of the body condition status in relation to the metabolism of lipids in cattle. The accumulation of lipids, observed in our study, destroys hepatocyte organelles, and these changes may influence biochemical blood parameters, which could be used for the diagnostics of hepatic steatosis.

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EXCRETION OF CALCIUM, PHOSPHORUS, MAGNESIUM AND SODIUM IN LACTATING SOWS

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ABSTRACT

The aim of this study was to evaluate the excretion of calcium (Ca), phosphorus (P), magnesium (Mg) and sodium (Na) via milk, urine and faeces during the lactation period of sows. Six clinically healthy lactating sows (crossbreed Large White×Landrace) were selected for these experiments and were housed in standard conditions and fed with commercially prepared dry mixture for this category of sows. The blood serum, milk, urine and faecal samples were collected on the 7th, 14th, 21st, and 28th day of lactation. During four weeks of lactation, we recorded the relatively stable and physiological concentrations of Ca, P, Mg and Na in blood serum. The analysis of the sow's milk showed the highest concentration of Ca and P at the end of lactation, while the highest concentration of Mg and Na was observed on the 7th lactation day. The following macro-mineral excretion was recorded in urine: 98.83—194.00 mg.l⁻¹ for Ca; 11.88—53.09 mg.l⁻¹ for P; 171.67—344.05 mg.l⁻¹ for Mg; and 56.50—74.83 mg.l⁻¹ for Na; and in the faeces, 1824.5—3045.5 mg.kg⁻¹ for Ca; 1566.93—2483.2 mg.kg⁻¹ for P;

1916.2—2505.2 mg.kg⁻¹ for Mg; and 516.8—748.2 mg.kg⁻¹ for Na.

Key words: blood serum; faeces; lactating sows; macro-minerals; milk; urine

INTRODUCTION

Minerals are important constituents of any animal's diet. Swine require 15 minerals in their diet and macro-minerals are the minerals that swine need in larger quantities, usually designated as a percent of the diet or in grams. The macro-minerals include calcium, phosphorus, sodium, chlorine, potassium, magnesium, and sulphur. They have many diverse physiological roles within the body, from regulatory to structural functions. The mineral requirements of pigs are influenced by; the type and level of production, the age and breed of the animal, the interrelationship and associations between minerals and other nutrients, as well as the quantity and chemical form of the mineral element themselves [5].

Absorbed minerals are incorporated into body tissue, stored in reserve for later use, or excreted. Pigs through the faeces and urine, normally excrete around 45 to 60% of nitrogen, 50 to 80% of calcium and phosphorous, and 70 to 90% of potassium, sodium, magnesium, copper, zinc, manganese and iron, when fed diets containing commonly used feedstuffs [18]. The level of some minerals, such as nitrogen and phosphorous in pig faeces is very important because of the risk of soil and water contamination [26].

The critical stage in the mineral nutrition of a sow is during late gestation and lactation [23]. The single most important demand on the lactating sow for minerals is for the production and secretion of milk to nourish the rapidly growing neonate [27]. Mammary mineral transfer to milk remain fairly regular even under varying maternal mineral status levels. This sensitive control is necessary in order to prevent toxicities or deficiencies in the offspring. In addition, to meet the neonatal demands, the dam will use up her body mineral reserves and catabolise skeletal tissue before the progeny becomes malnourished [17]. This depletion has been demonstrated in the skeletal demineralization of sows with increasing parity [14], [24]. Thus, lactation can severely deplete the sow's body stores of nutrients.

The aim of this study was to evaluate the concentrations of Ca, P, Mg and Na in the blood serum, milk, urine and faeces of lactating sows during four weeks after parturition.

MATERIALS AND METHODS

Six lactating sows (crossbreed Large White × Landrace) in the age of 2.5–5.0 years (average litter size 12), and average body weight 220 kg, were used in our experiments. During four weeks, the sows were fed with a concentrated feed mixture (OŠ-09) for lactating pigs (Tajba a.s., SR). The diet was based on corn, soybean extracted ground meal, barley and wheat. In addition, OŠ-09 contained per 1 kg of DM: vitamin A, 6000 U; vitamin D3, 600 U; NC, 155 g; DF, 70 g; lysine, 7.5 g; threonine, 4.5 g; methionine and cysteine, 4.0 g; Ca, 7.0 g; P, 5.0 g; Na, 2.0 g; Mg, 0.5 g; vitamin E, 20 mg; Fe, 240 mg; Zn, 110 mg; and Cu, 20 mg. The diet covered the standardized nutritional requirements of lactating pigs [28]. We used a divided type of feeding (three times daily) corresponding to the count of new-born piglets (6.80 kg of concentrated feed mixture per lactating sow with 12 piglets per day). The sows were housed individu-

ally in the standard farrowing pens with free access to water. The blood, urine and faeces were sampled in one-week intervals: 1st, 2nd, 3rd, 4th sampling on 7th, 14th, 21st, and 28th day after farrowing, respectively. The blood was taken from the vena cava cranialis. Samples of milk were collected during the morning sucking of the piglets. The specimens were stored at –24 °C until analysis. All samples were subjected to wet mineralization in a microwave oven. The concentrations of Ca, Mg, and Na in the blood serum, milk, faeces, and urine were determined by the flame AAS method (Perkin Elmer, AAnalyst 100) [4]. The concentration of P in the faeces and milk was determined using a spectrophotometer SPECOL 211 (Carl Zeiss Jena), the concentrations of P in the blood plasma and urine were determined by an automatic biochemical analyser ALIZE (Lisabio) using diagnostic kits (Bio Mérieux, Randox) [12].

The statistical analysis was done using one-way analysis of variance (ANOVA) with the post hoc Tukey multiple comparison tests. Results in tables are presented as means (\bar{x}) and the standard deviation (SD).

RESULTS AND DISCUSSION

Calcium (Ca) and phosphorus (P) are minerals that have important physiological functions in the body such as; muscle contraction, transmission of nerve impulses, enzyme activation, metabolic reactions, protein synthesis, maintenance of osmotic and acid-base balances, components in membranes, and other functions [6], [11]. An important aspect of Ca and P metabolism is plasma homeostasis, which is controlled by absorption, urinary excretion, and bone turnover [3], [13]. A major part of the Ca in swine diets originates from inorganic sources because the concentration of Ca in cereal grains is low. Most of the phosphorus in cereal grains and oilseed meal is in the form of phytic acid (organically bound phosphorus) and is poorly available to pigs. Supplemental phytase, an enzyme that degrades some of the phytic acid in feedstuffs, is commonly added to diets to further reduce phosphorus excretion [8]. However, it's necessary to remember that, high levels of Ca in swine diets reduce the effect of phytase and affect the digestibility of P [19].

Magnesium (Mg) has several important functions, as for example; a cofactor for protein and DNA synthesis, oxidative phosphorylation, cardiovascular tone, and neuro-

Table 1. The concentration of macro-elements in blood serum of lactating sows in mmol.l⁻¹ (x ± SD)

Sampling	Ca	P	Mg	Na
1st	2.45 ± 0.16	1.91 ± 0.57	0.95 ± 0.22	140 ± 5.67
2nd	2.32 ± 0.20	2.23 ± 1.07	1.00 ± 0.22	143 ± 4.78
3rd	2.44 ± 0.27	2.80 ± 0.96	0.91 ± 0.09	145 ± 6.99
4th	2.33 ± 0.18	2.40 ± 0.64	0.91 ± 0.13	145 ± 4.62

Table 3. The concentration of macro-elements in the urine of lactating sows in mg.l⁻¹ (x ± SD)

Sampling	Ca	P	Mg	Na
1st	98.83 ± 92.34	22.05 ± 33.16	171.67 ± 164.51	56.50 ± 51.14
2nd	116.17 ± 85.39	11.88 ± 9.26	344.5 ± 331.69	74.83 ± 57.80
3rd	116.00 ± 75.00	19.36 ± 15.51	282.33 ± 155.10	58.67 ± 32.75
4th	194.00 ± 195.06	53.09 ± 85.22	285.50 ± 233.34	63.33 ± 43.89

Table 2. The concentration of macro-elements in the milk of lactating sows in mg.l⁻¹ (x ± SD)

Sampling	Ca	P	Mg	Na
1st	1287.83 ± 364.95	6.98 ± 4.21	115.83 ± 72.45	695.67 ± 382.92
2nd	1713.33 ± 273.69	9.21 ± 3.00	107.00 ± 41.29	445.83 ± 195.97
3rd	1817.17 ± 244.41	8.96 ± 3.32	106.83 ± 45.56	350.33 ± 53.39
4th	1783.83 ± 320.40	11.4 ± 0.26	103.33 ± 6.88	381.67 ± 101.00

Table 4. The concentration of macro-elements in the faeces of lactating sows in mg.kg⁻¹ (x ± SD)

Sampling	Ca	P	Mg	Na
1st	3045.5 ± 1604.4	1857.9 ± 870.6	2505.2 ± 566.2	517.5 ± 426.8
2nd	2282.1 ± 1585.6	2483.2 ± 1388.3	2170.9 ± 387.3	516.8 ± 447.3
3rd	1824.5 ± 845.3	1566.93 ± 809.3	1919.9 ± 357.4	648.9 ± 510.65
4th	1858.2 ± 1610.2	1656.9 ± 815.3	1916.2 ± 410.5	748.2 ± 610.5

muscular excitability [3]. It is associated with calcium and phosphorus in bone, the largest reservoir of these minerals, from which it can be released to the body as required. Magnesium is readily available in plant sources, and is absorbed from the small intestine. It is thought to be efficiently recycled so that supplementation is not necessary to fulfil most requirements [5]. On the other hand, studies have demonstrated that supplementing Mg to swine diets; alleviated the effects of stress and improved the meat quality [2], [10], and prevented and controlled the occurrence of constipation [29]. Magnesium is stored in bone or excreted in the urine [13], [30].

Sodium (Na) is necessary for; the generation of nerve impulses, for maintenance of electrolyte balance and fluid balance, heart activity and certain metabolic functions [25]. Sodium together with chloride are available from feed grains, but only in low concentration, therefore they are provided as supplements to diets, especially during lactation to maintain the physiological balance of these minerals

[22]. These minerals are provided by common salt, which contains 40 % of sodium and 60 % of chloride. The recommended level of salt is 0.5 % in sow diets [8].

The concentration of Ca, P, Mg and Na revealed no significant differences in the blood serum of lactating sows within the four samplings (Table 1). The concentrations of all these elements was physiological when compared with the physiological range of Ca, P, Mg and Na which is 2.0—3.0 mmol.l⁻¹, 1.30—3.55 mmol.l⁻¹, 0.7—1.6 mmol.l⁻¹, and 135—145 mmol.l⁻¹ [31] for pigs, respectively. But our values were lower than those obtained by Žvorc et al. [32].

The peak daily milk yield is reached at around 21 days of lactation [9], and general milk composition after day 7 is relatively stable for the remainder of lactation. The major components of milk solids (17 to 25 %) are; fat (7 or 8 %), lactose (~4 %), and protein (6 %). The other major components of milk are the ash or mineral contents. Sow's milk contains 0.6 to 1.0 % ash, with the majority of that as calcium and phosphorus [16]. The calcium and phosphorus

composition of milk seems stable despite fluctuations in dietary intake [5]. The body reserves are sufficient to buffer this need, and are utilised especially in sows with high milk production. If the reserves of calcium and phosphorus are not replenished, then the strength and integrity of the sow's bones will decrease [5]. Our results are in accordance with the finding of Mahan and Fetter [21], that sow's milk has a higher concentration of Ca and P in the third and fourth weeks of lactation than the first two weeks. The lowest concentration of Ca was found in the 1st week (1287 mg.l⁻¹; Table 2), and the highest in the 3rd week (1817 mg.l⁻¹). Similarly, the lowest concentration of P was observed in the 1st week (6.98 mg.l⁻¹), and the highest in the 4th week (11.4 mg.l⁻¹). The milk concentrations of Mg and Na ranged between 103.33–115.83 and 350.33–695.67 mg.l⁻¹, respectively.

The kidneys play a central role in the homeostasis of calcium, phosphorus, and magnesium. Gastrointestinal absorption is balanced by renal excretion. When the body stores of these ions decline significantly, gastrointestinal absorption, bone resorption, and renal tubular reabsorption increase to normalize their levels. Renal regulation of these ions occurs through glomerular filtration and tubular reabsorption and/or secretion [3]. Sodium is also excreted primarily in the urine, with only small amounts lost in the faeces and perspiration [20]. The highest nonsignificant urinary excretion was recorded on 28th day of lactation for Ca, P (4th sampling), and on the 14th day of lactation for Mg, Na (Table 3).

The concentration of the macro-minerals in the faeces of lactating sows was without significant differences between the four collections (Table 4). The faecal output of minerals included both unabsorbed dietary minerals and minerals that had been secreted into the intestine and not reabsorbed (usually referred to as endogenous faecal excretion) [1]. The daily faecal production in relation to the body weight (DFP/BW) is around 0.6 % [7] and the dry matter content of faeces (DMF) varies around 30 % [15]. As mentioned above, phosphorus is considered a potential environmental pollutant. Excretion of P in the urine of lactating sows changed from 11.88 to 53.09 mg.l⁻¹ (Table 3). Faecal output of phosphorus varied between 1566.93 and 2483.2 mg.kg⁻¹ (Table 4), but these values were lower than the excretion of P in the category of growing piglets (2600 mg.kg⁻¹) [15]. The highest faecal excretion was recorded on the 7th day of lactation for Ca, Mg; on the 14th day of lactation for P; and on the 28th day of lactation for Na.

Our experimental observations of macro-mineral excretion in the lactating sows can be helpful not only from the view point of milk composition, but also from the environmental aspect, because some minerals (as for example phosphorus) in pig excrement can constitute risks for soil and water contamination.

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