FOLIA VETERINARIA

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

ISSN 0015-5748

3

LVIII • 2014

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

IČO: 397 474

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

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

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

Issued on March 25, 2014

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

IČO: 397 474

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

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

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

Dátum vydania: 25.3.2014

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

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

EV 3485/09

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

Indexed and abstracted in AGRIS, CAB, EBSCO

FOLIA VETERINARIA, 58, 3, 2014

CONTENTS

FOLIA VETERINARIA, 58, 3: 145—152, 2014

ADVERSE EFFECTS OF THREE VARIETIES OF *LABLAB PURPUREUS* SEEDS ON REPRODUCTIVE FUNCTIONS IN MALE RATS

Soetan, K. O.[1,2], Adedara, I. A.[1], Farombi, E. O.[1]

[1]Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry
College of Medicine, University of Ibadan
[2]Department of Veterinary Physiology, Biochemistry and Pharmacology
Faculty of Veterinary Medicine, University of Ibadan
Oyo State

kehinde.soetan@gmail.com

ABSTRACT

This study investigated the reproductive toxicity of three varieties of *Lablab purpureus* seeds (i. e. Rongai brown, Rongai white and Highworth black) in male Wistar rats. Four groups of eight rats per group were studied. Three groups of rats were separately fed with feed containing the Rongai brown, Rongai white and Highworth black for 14 days. A control group was fed only with the standard rats chow for 14 days. There was a significant decrease in the absolute testicular weights of animals exposed to Rongai brown and Rongai white, whereas Highworth black group showed no treatment-related changes. The three *Lablab purpureus* seeds significantly decreased glutathione-S-transferase (GST) activity without affecting superoxide dismutase (SOD) activity. There were significant increases in glutathione (GSH) levels, catalase (CAT) and glutathione peroxidase (GPx) activities with concomitant elevated hydrogen peroxide generation and lipid peroxidation in all *lablab purpureus*-fed rats when compared with the controls. *Lablab purpureus*-fed animals showed a significant decrease in; spermatozoa motility, epididymal spermatozoa number, viability, testicular spermatozoa numbers and daily spermatozoa production with elevated spermatozoa abnormalities when compared with the control animals. Rongai brown fed animals showed disrupted testicular basement membrane of seminiferous tubules and loss of spermatozoa; whereas Rongai white and Highworth black fed animals showed oedema and reduced seminiferous tubular diameters. In summary, induction of oxidative stress may be attributed to the gonadotoxic and spermatotoxic effects of the three varieties of *Lablab purpureus* seeds.

Key words: biochemical; *Lablab purpureus*; oxidative stress; reproductive toxicity

INTRODUCTION

Low productivity of livestock in the tropical and subtropical regions is associated with low digestibility and low nitrogen contents of available feed resources. One of the strategies to alleviate the effects of such under-nutrition is by supplementation with leguminous forages [33]. *Lablab purpureus* is one of the most diverse domesticated legume species and has multiple uses [24]. It is cultivated to provide adequate nutrition in animal production. The wild forms of lablab are believed to have originated in India [11] and were introduced into Africa from southeast Asia during the eighth century [22] and to China about 2000 years ago [8]. Presently, lablab is common in Africa, extending from Cameroon to Swaziland and Zimbabwe, through Sudan, Ethiopia, Uganda, Kenya and Tanzania [37]. The herbage is used as green manure, for erosion control, and as a feed supplement for cattle grazing mature pastures in the dry season [17], while the seed and immature pods are used for human food and herbal medicine [8]. In Nigeria, it is utilized in conjunction with natural pastures and crop residues to reduce the

weight losses common to livestock during the dry season, whereas the seeds are edible and are also used in the preparation of bean cakes "kose" and "moimoi" [28].

Although there are several varieties of *Lablab purpureus*, Rongai white, Rongai brown and Highworth black are well studied and consumed [1], [18], [29]. *Lablab purpureus* has considerable promise as a crop species because its grain yields can be higher than those of cowpea and its spectrum of adaptability to differing ecological conditions is wider than any other leguminous plant [2]. Also, when compared to other tropical forage legumes, lablab was given an excellent production rating based on observations of the dry matter yield, persistency, resistance to drought, pests and diseases [27]. Accessions with good grain and forage yields of high potential for use in the cereal-legume livestock systems in the moist savannah zone of West Africa have been identified [12]. *Lablab purpureus* has a potential to be a source of nutraceuticals and pharmaceuticals [26]. However, the nutritive value of *Lablab purpureus* seeds is masked by concurrence of toxic anti-nutritional factors such as haemagglutinin, trypsin inhibitors, saponins, phytic acids, tannis, alkaloids, cyanogens, amylase inhibitors, lectins and polyphenoilic compounds [1], [19].

In the last few decades or more, large numbers of scientific data have emerged, linking diet and food selection patterns to the maintenance of health and the prevention of some chronic diseases [30]. There is a consensus among physicians that nutrition constitutes an essential aspect of health care. Despite the numerous studies that have been done on this important plant, there is no information in the literature about the positive and/or negative effect of *Lablab purpureus* on male fertility. In the present study, in order to see the effects of *Lablab purpureus* on male fertility, we examined; epididymal spermatozoa characteristics, testicular antioxidant system and lipid peroxidation, along with histology of the testes in rats exposed to the three varieties *Lablab purpureus* for fourteen days.

MATERIALS AND METHODS

Percentage feed composition

Three varieties of *L. purpureus;* Rongai white (NAPRI 4), Rongai brown ($P_1$509114) and Highworth black (Grif 12293) used in this study, were obtained from the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. The pictures of the three varieties of the *L. purpureus seeds* have been presented reported by Nwokocha et al. [29]. The percentage feed composition of the control and test diets are shown in Table 1 below. The diets contain the three varieties of Lablab beans (*Lablab purpureus*), i. e. Rongai brown, Rongai white and Highworth black. All the feeds were calculated to contain 20 % crude protein and 3 kilocalories of energy (iso-proteinous and iso-caloric diet). The proximate analysis of the three varieties of raw *Lablab purpureus* seeds have been reported by Soetan and Fafunso [38].

Vitamin A 10 000 000 IU.2.5 kg^{-1}; Vitamin D_3 2 000 000 IU.2.5 kg^{-1}; Vitamin E 12 000 mg.2.5 kg^{-1}; Vitamin K_3 2 000 mg.2.5 kg^{-1}; Vitamin B_1 1 500 mg.2.5 kg^{-1}; Vitamin B_2 5 000 mg.2.5 kg^{-1}; Vitamin B_6 1 500 mg.2.5 kg^{-1}; Vitamin B_{12} 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 250 mg.2.5 kg^{-1}.

Experimental protocol

Thirty-two adult male Wistar rats of an average weight of 198 ± 5 g were obtained from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan. They were housed in plastic cages placed in a well-ventilated rat house, provided rat pellets and water *ad libitum* and subjected to natural photoperiod of 12-hour light : 12-hour dark**.** All the animals received humane care

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

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 PHS 1996 [32].

The rats were randomly assigned into four groups of eight rats per group. A group of rats were fed with the standard rats chow for 14 days and served as the control. The other groups of rats were fed with feed containing the three varieties of *Lablab purpureus* seeds (i. e. Rongai brown, Rongai white and Highworth black) for 14 days according to Saba et al. [35]. Twenty-four hours after the last treatment, all the animals were killed by cervical dislocation and the testes and epididymis were quickly removed and weighed. Samples from testes were fixed in Bouin's solution, paraffin embedded, sectioned and stained routinely with haematoxylin and eosin for microscopic analysis. All slides were coded before examination with light microscope by investigators who were blinded to which slides were from the control or treatment groups.

Analysis of testicular antioxidant status

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

Eight right testes were homogenised in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15 % potassium chloride, and the homogenate was centrifuged at 10 000 g for 15 minutes at 4 °C. The supernatant was collected for the estimation of catalase (CAT) activity using hydrogen peroxide as substrate according to the method of Clairborne [10]. Superoxide dismutase (SOD) was assayed by the method described by Misra and Fridovich [25]. Glutathione S-transferase (GST) was assayed by the method of Habig et al. [16]. Protein concentration was determined by the method of Lowry et al. [23]. Reduced glutathione (GSH) was determined at 412 nm using the method described by Jollow et al. [20]. Hydrogen peroxide generation was assessed by the method of Wolff [42]. Glutathione peroxidase (GPx) activity was determined by the method of Rotruck et. al. [34] which is based on the reaction between GSH remaining after the action of GPx and 50,50-dithiobis(2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Farombi et al. [13] and expressed as micromoles of MDA per gramme tissue.

Spermatozoa motility and spermatozoa count

Spermatozoa motility was assessed by the method described by Zemjanis [43]. One drop of the spermatozoa samples from the *cauda epididymis* from each group were used for the motility assay. The suspension was placed on a slide and covered with a 24 × 24 mm cover slip.

Histological Examination

The spermatozoa motility was evaluated microscopically under a phase contrast microscope at x 200 magnification within 2—4 minutes of their isolation from the *cauda epididymis* and data were expressed as percentages. Epididymal spermatozoa count was obtained by mincing caudal epididymis in distilled water and filtering through a nylon mesh. The spermatozoa were counted by haemocytometer using the improved Neubauer (Deep 1/10 m; LABART, Darmstadt, Germany) chamber as described by Pant and Srivastava [31].

Determination of daily spermatozoa production and testicular spermatozoa number

Daily spermatozoa production was determined using eight frozen left testes from control and treated rats according to Joyce et al. [21]. Briefly, after the testes had been removed and weighed, they were homogenized for 3 minutes in 25 ml of physiological saline containing 0.05 % (v/v) Triton X-100. Then, 5.5 µl of sample aliquots were placed on the haemocytometer and counted twice at 100× magnification under the microscope to determine the average number of spermatids per sample. These values were used to obtain the total number of spermatids per testis, and this number was then divided by the testes weight to give spermatids per gram of testes. Developing spermatids spend 4.61 days in rats. Thus, the values for the number of spermatids per testis were divided by 4.61 to obtain daily spermatozoa production.

Morphological abnormalities and percentage viability assay

Aliquots of spermatozoa placed on a slide glass were smeared out with another slide and stained with Wells and Awa's stain (0.2 g of eosin and 0.6 g of fast green dissolved in distilled water and ethanol in the ratio 2 : 1)for morphological examination and 1 % eosin and 5 % nigrosine in 3 % sodium citrate dehydrate solution for live/dead ratio according to the method described by Wells and Awa, 1970 [41]. A total of 400 spermatozoa from each rat were examined for the morphological examinations.

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Levels of statistical significance were analysed by ANOVA, followed by the Student's *t*-test to compare the means between *Lablab purpureus*-treated groups and the controls. Significance was set at $P < 0.05$ using Student's *t*-test.

RESULTS

Absolute and relative weights of the testes

The data on the treatment-related effects on the absolute and relative weights of the testes are presented in Figure1. There were significant decreases in the absolute weights of the testes in animals exposed to Rongai brown and Rongai white when compared with the control group. A significant decrease in the relative testes weight was observed only in the Rongai brown. There were no treatment-related changes in the absolute and relative testes weights in Highworth black-exposed animals when compared with the control group.

Testicular antioxidant status

Figures 2—4 reveal the effects of consumption of Rongai brown, Rongai white and Highworth black on antioxidant systems and lipid peroxidation in the testes of rats. There

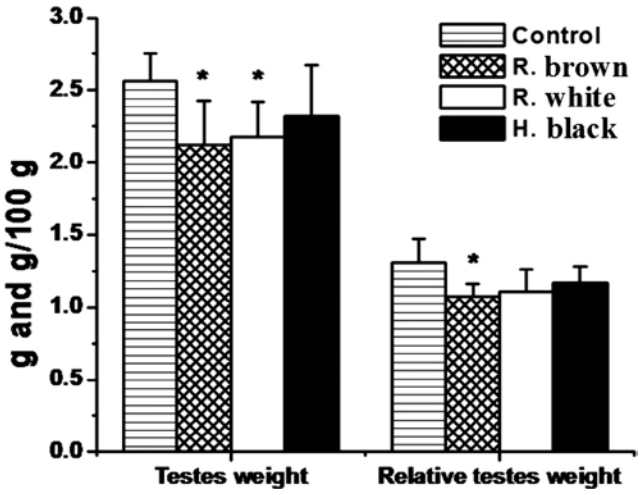

Fig. 1. Effects of Rongai brown, Rongai white and Highworth black on absolute and relative testes weight. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

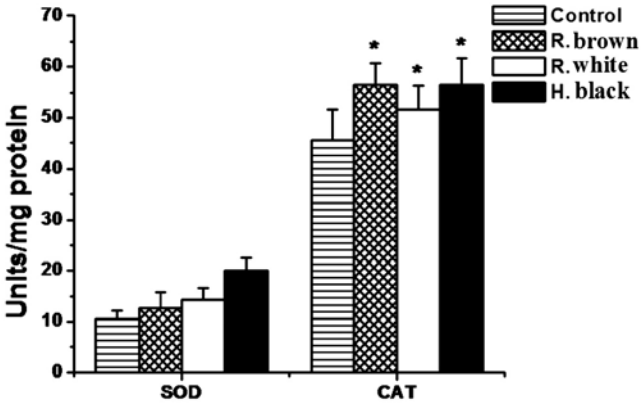

Fig. 2. Effects of Rongai brown, Rongai white and Highworth black on SOD and CAT activities. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

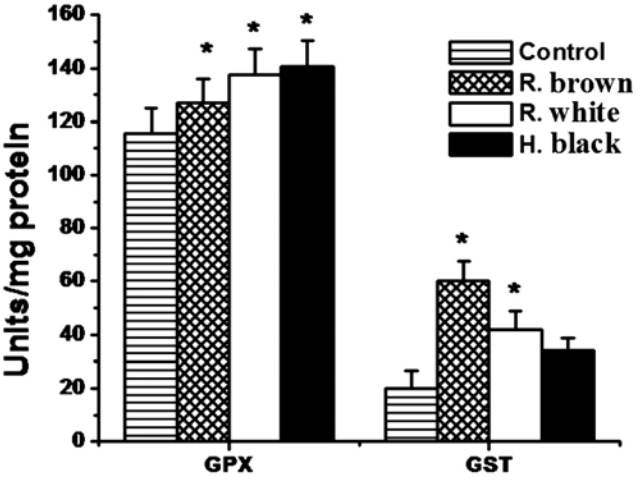

Fig. 3. Effects of Rongai brown, Rongai white and Highworth black on GPx and GST activities. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

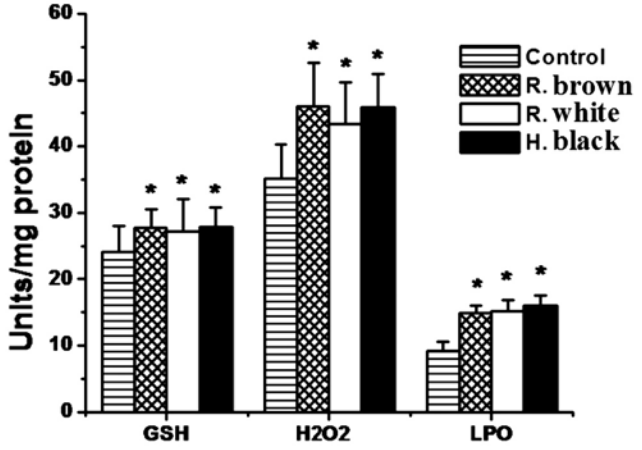

Fig. 4. Effects of Rongai brown, Rongai white and Highworth black on GSH, H_2O_2 and LPO levels. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

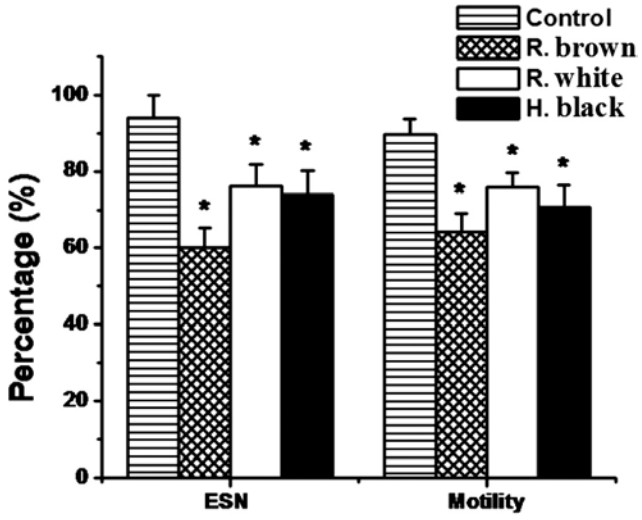

Fig. 5. Effects of Rongai brown, Rongai white and Highworth black on % Epididymal Sperm Number (ESN) and % motility of spermatozoa. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

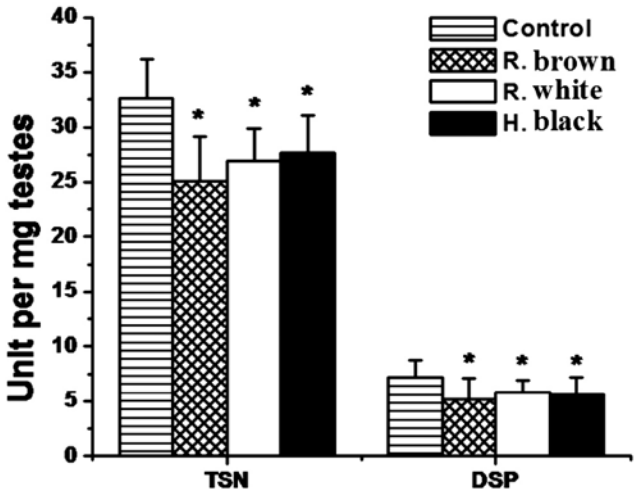

Fig. 6. Effects of Rongai brown, Rongai white and Highworth black on Testicular Spermatozoa Number (TSN) and Daily Spermatozoa Production (DSP). Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

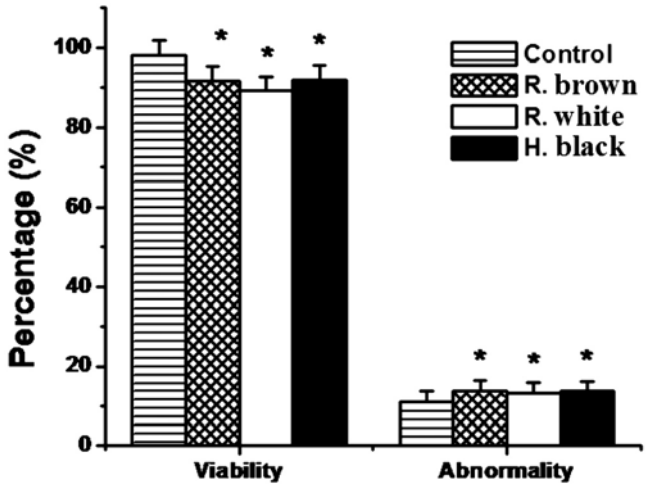

Fig. 7. Effects of Rongai brown, Rongai white and Highworth black on % viability of spermatozoa and % abnormality of spermatozoa. Each bar represents mean ± SD of 8 rats. * — values differ significantly from the control (P < 0.05)

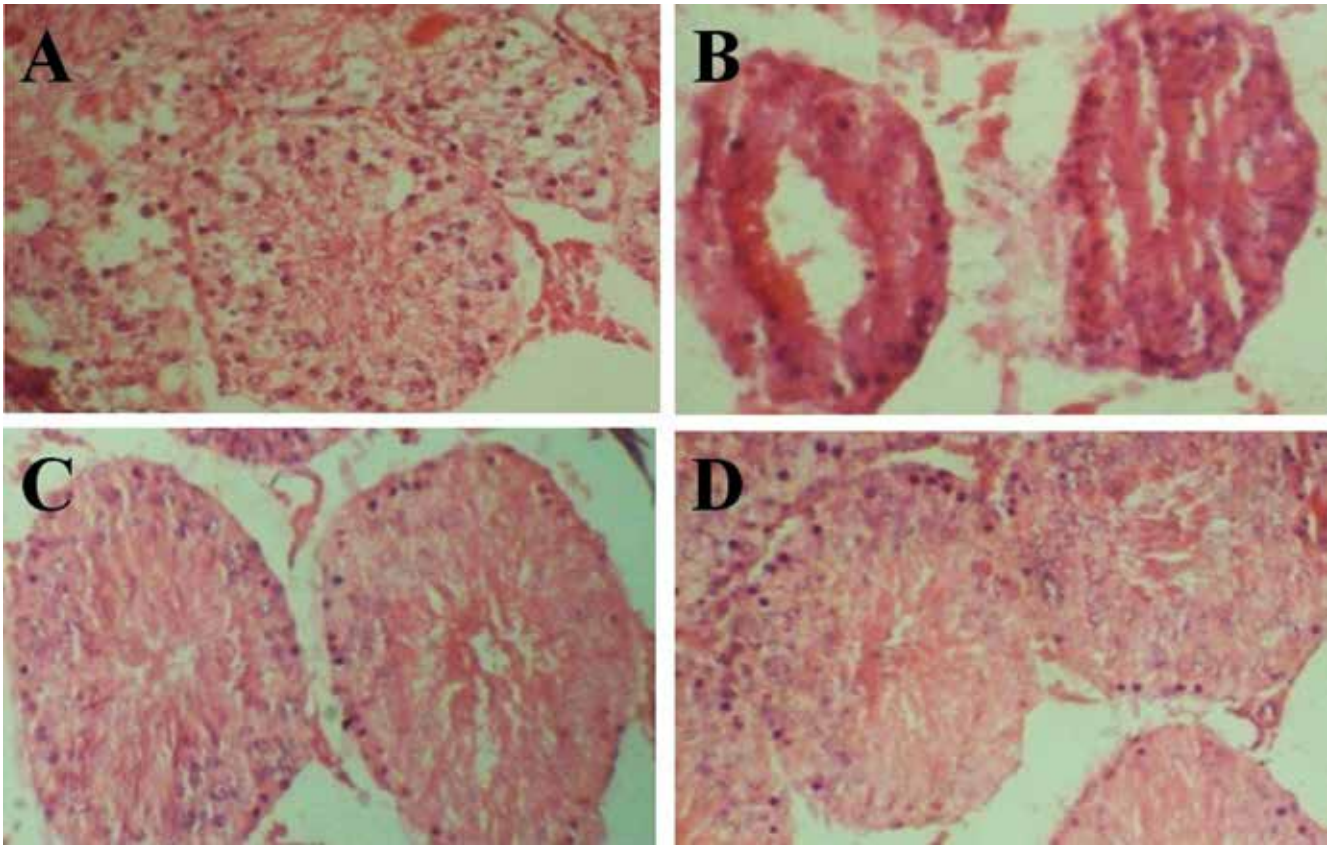

Fig. 8. (A) Control testes with normal architecture; (B) Rongai brown fed testes showed disrupted testicular basement membrane of seminiferous tubules and loss of spermatozoa; (C and D) Rongai white and Highworth black fed animals showed oedema and reduced seminiferous tubular diameters. Magn. ×100 (Hematoxylin and Eosin)

was anon-significant increase in the activity of SOD in all the *Lablab purpureus*-fed rats. The activities of CAT and GPx as well as GSH level significantly increased in all the treated rats, whereas GST activity markedly decreased in all the *Lablab purpureus*-fed rats when compared with the controls. The levels of malondiadehyde (MDA) which is an index of lipid peroxidation and hydrogen peroxide (H_2O_2) generation, were significantly elevated in the testes of animals fed with the three varieties of *Lablab purpureus*.

Sperm analysis

The adverse effects of Rongai brown, Rongai white and Highworth black on spermiogram are shown in Figures 5—7. When compared with the control animals, *Lablab purpureus*-fed animals showed significant decreases in spermatozoa motility, epididymal spermatozoa number (ESN), viability, testicular spermatozoa number (TSN) and daily spermatozoa production (DSP).The percentage of morphologically abnormal spermatozoa was significantly elevated in all the animals fed with the three varieties of *Lablab purpureus*.

Histology

The testicular sections of the control and *Lablab purpureus*-fed rats are shown in Figure 8. Light microscopy revealed that control testes appeared normal whereas treatment-related lesions were identified in the testes of *Lablab purpureus*-fed rats. Histopathological changes observed in the testes of Rongai brown fed animals include disruption of testicular basement membrane of seminiferous tubules and loss of spermatozoa. The testes of animals fed with Rongai white and Highworth black showed reduced seminiferous tubular diameters and spermatozoa when compared with the control.

DISCUSSION

The consequences of toxic anti-nutritional factors inplants used as food/feedstuffs are not their direct toxicityto man and animals alone, but also the inconvenience and the economic loss associated with poisoning of domestic animals and the cost of preventing or reducing such happenings [39]. Antinutritional factors have been reported to increase free radicals levels leading to oxidative stress which is an imbalance between the production of reactive oxygen species (ROS) and the capacity of the antioxidant defense system [15]. In the present study, testicular toxicity of *Lablab purpureus* was characterized biochemically and histologically. Oxidative stress could result in testicular and spermatozoa damage, deformity and eventually male infertility [3], [6]. Superoxide dismutase, the first line of defense against oxygen erived radicals is responsible for the dismutation of superoxide radicals to H_2O_2, whereas catalase metabolically removes $H_2O_2$from the intracellular environment, thereby further reducing the $H_2O_2$and hydroxyl radical generation [4]. The increase in activities of these enzymes observed in this study indicates enzyme induction against accumulation of superoxide anion and peroxide radicals.

Glutathione S-transferase (GST), GPx together with GSH participate in the glutathione redox cycle by converting hydroxyl radical, H_2O_2 and lipid peroxides to non-toxic products [5], [36]. The elevated intracellular GSH concentration and GPx activity in the testes of animals fed with *Lablab purpureus* indicates their induction to decrease tissue damage and promote better survival under the conditions of oxidative stress. However, the decrease in the GST activity may lead to decreased protection against oxidants [7]. Lipid peroxidation is a degenerative pathway of membrane components mediated through free radicals produced in the cell [40]. Elevated levels of oxidative stress indices such as H_2O_2 and MDA observed in this study are indicative of a testicular injury following *Lablab purpureus* consumption which is well supported by the histopathological report.

The adverse effect of *Lablab purpureus* was evident in the significant decrease in the weight of the testes which was more pronounced in the Rongai brown. The weight of the testes is largely dependent on the mass of the differentiated spermatogenic cells [9]. The reduction in the testes of animals fed with *Lablab purpureus* possibly indicates the testicular toxicity on the spermatogenic cells. Interestingly, all the animals fed with the three varieties of *Lablab purpureus* showed significant deteriorations in testicular functions and spermatozoa characteristics. These animals demonstrated a significant decrease in; spermatozoa motility, epididymal spermatozoa numbers, spermatozoa viability, testicular spermatozoa numbers, and daily spermatozoa production with increased abnormal morphology rates. The accumulation of ROS may lead to the destruction of seminiferous epithelium and loss of germinal elements, resulting in a decrease in the daily spermatozoa production which is evident by the significant decrease in epidydimal spermatozoa numbers and testicular spermatozoa numbers seen in *Lablab purpureus* fed animals. Furthermore, increased lipid peroxidation of polyunsaturated fatty acids in the spermatozoa plasma membrane could lead to abnormalities in spermatozoa morphology and impaired spermatozoa motility resulting in infertility due to the failure of the spermatozoa to reach the site of fertilization, as well as their ability to penetrate zonal pellucid [14].

Overall, the gonadotoxic and spermatotoxic effects of *Lablab purpureus* may result in testicular dysfunction and reduced spermatozoa functional competence, culminating in male infertility. Therefore, in order to safeguard the reproductive health of humans and livestock, we recommend adequate and thorough processing of *Lablab purpureus* which is reported to contain high concentrations of anti-nutritional factors.

REFERENCES

1. Abeke, F.O., Ogundipe, S.O., Sekoni, A.A., Adeyinka, I.A., Oni, O.O., Abeke, A., Dafwang, I.I., 2008: Effect of Duration of cooking *Lablab purpureus* beans on its utilization by broiler finishers (4–8 weeks). *Asian J. Animal Vet. Adv.*, 3, 85—91.

2. Adebisi, A.A., Bosch, C.H., 2004: *Lablab purpureus* (L.) Sweet. In **Grubben, G.J.H., Denton, O.A.** (Eds.)**:** *Plant Resources of Tropical Africa (PROTA*), No. 2, vegetables. PROTA Foundation,

Wageningen, The Netherlands/Backhuys, Leiden, The Netherlands/ CTA, Wageningen, The Netherlands, 343—348.

3. Adedara, I. A., Farombi, E. O., 2010: Induction of oxidative damage in the testes and spermatozoa and hematotoxicity in rats exposed to multiple doses of ethylene glycol monoethyl ether. *Hum. Exp. Toxicol*., 29, 801—812.

4. Adedara, I. A., Ebokaiwe, A.., Farombi, E. O., 2013: Tissues distribution of heavy metals and erythrocytes antioxidant status in rats exposed to Nigerian bonny light crude oil. *Toxicol. Ind. Health*. 29, 162—168.

5. Adedara, I. A., Teberen, R., Ebokaiwe, A. P., Ehwerhemuepha T, Farombi, E. O., 2012: Induction of oxidative stress in liver and kidney of rats exposed to Nigerian Bonny light crude oil. *Environ. Toxicol*., 27, 372—379.

6. Agarwal, A., Makker, K., Sharma, R., 2008: Clinical relevance of oxidative stress in male factor infertility: an update. *Am. J. Reprod. Immunol*., 59, 2—11.

7. Amstad, P., Peskin, A., Shah, A. G. et al., 1991: The balance between Cu, Zn superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemist*, 30, 9305—9313.

8. Chang, Y. L., Wang, E. T., Sui, X. H., Zhang, X. X., Chen, W. X., 2011: Molecular diversity and phylogeny of rhizobia associated with *Lablab purpureus* (Linn.) grown in Southern China. *Syst. Appl. Microbiol*., 34, 276—284.

9. Chapin, R. E., Harris, M. W., Davis, B. J. et al., 1997: The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam. Appl. Toxicol*., 40, 138—57.

10. Clairborne, A., 1995: Catalase activity. In **Greewald, A. R.** (Ed.): *Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton, FL, 237—242.

11. Deka, R. K., Sarkar, C. R., 1990: Nutrient composition and anti-nutritional factors of *Dolichos lablab* L seeds. *Food Chemistry*, 38, 239—246.

12. Ewansiha, S. U., Chiezey, U. F., Tarawali, S. A., Iwuafor, E. N. O., 2007: Potential of *Lablab purpureus* accessions for crop-livestock production in the West African savanna. *J. Agric. Sci*., 145, 229—238.

13. Farombi, E. O., Tahnteng, J. G., Agboola, A. O., Nwankwo, J. O., Emerole, G. O., 2000: Chemoprevention of 2-acetylaminofluorene-induced hepatotoxicity and lipid peroxidation in rats by kolaviron- a *Garcinia kola* seed extract. *Food. Chem. Toxicol*., 38, 535—541.

14. Farombi, E. O., Adedara, I. A., Ebokaiwe, A. P., Teberen, R., Ehwerhemuepha, T., 2010: Nigerian bonny light crude oil disrupts antioxidant systems in the testes and sperm of rats. *Arch. Environ. Contam. Toxicol*., 59, 166—174.

15. Gu, C., Qu, H., Han, L., Song, X., Zhao, L., Lu, W., 2011: The effect of raw soybean on oxidative status of digestive organs in mice. *Int. J. Mol. Sci*., 12, 8836—8845.

16. Habig, W. H., Pabst, M. J., Jakoby, W. B., 1974: Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem*., 249, 7130—7139.

17. Hendricksen, R. E., Minson, D. J., 1985: *Lablab purpureus* – A review. *Herbage Abstracts,* 55, 215—227.

18. Ismartoy, O., Dixon, R. M., Slocombe, R. F., Holmes, J. H. G., 1993: *Lablab purpureus* seed as a supplement for Goats fed low quality roughage. *AJAS,* 6, 515—519.

19. Janarthanan, S., Suresh, P., Radke, G., Morgan, T. D., Oppert, B., 2008: Arcelins from an Indian Wild Pulse, *Lablab purpureus*, and insecticidal activity in storage pests. *J. Agric. Food Chem*., 56, 1676—1682.

20. Jollow, D. J., Mitchell, J. R., Zampaglione, N., Gillette, J. R., 1974: Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4 bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology*, 11, 151—169.

21. Joyce, K. L., Porcelli, J., Cooke, P. S., 1993: Neonatal goitrogen treatment increases adult testes size and sperm production in the mouse. *J. Androl*., 14, 448—455.

22. Kay, D. E., 1979: Hyacinth bean – food legumes. Crop and product digest No. 3. *Tropical Products Institute,* 16, 184—196.

23. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J., 1951: Protein measurement with folin phenol reagent. *J. Biol. Chem*., 193, 265—275.

24. Maass, B. L., Knox, M. R., Venkatesha, S. C., Angessa, T. T., Ramme, S., Pengelly, B. C., 2010: *Lablab purpureus* — a crop lost for Africa? *Tropical Plant. Biol*., 3, 123—135.

25. Misra, H. P., Fridovich, I., 1972: The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem*., 247, 3170—3175.

26. Morris, J. B., 2009: Morphological and reproductive characterization in Hyacinth bean, *Lablab purpureus* (L.) sweet germ plasm with clinically proven nutraceutical and pharmaceutical traits for use as a medicinal food. *J. Dietary Suppl*., 6. 263—279.

27. Murphy, A. M., Colucci, P. E., 1999: A tropical forage solution to poor quality ruminant diets: A review of *Lablab purpureus*. *Livestock Res. Rural Dev*., 11, 22.

28. Nasiru, F., 2001: Production of lablab in Nigeria. *Extension Bulletin Number*, 206, 5—24.

29. Nwokocha, L. M., Soetan, K. O., Williams, P. A., 2010: A study of the properties of starch isolated from three varieties of Lablab purpureus seeds. *Carbohydrate Polymers*, 79, 685—693.

30. Oyewole, O. E., Atinmo, T., 2008: Nutrition education in medical training: the need to reconsider the sacrosanctity of medical education in Nigeria. *Afr. J. Med. Sci*., 37, 219—224.

31. Pant, N., Srivastava, S. P., 2003: Testicular and spermatotoxic effects of quinalphos in rats. *J. Appl. Toxicol*., 23, 271—274.

32. PHS 1996: *Public Health Service Policy on Humane Care and Use of Laboratory Animals*. Washington, DC: US Department of Health and Human Services. (PL 99-158. Health Research Extension Act, 1985).

33. Poppi, D. P., McLennan, S. R., 1995: Protein and energy utilisation by ruminants at pasture. *J. Anim. Sci*., 73, 278—290.

34. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hoekstra, W. G., 1973: Selenium: Biochemical role as a component of glutathione peroxidase. *Science*, 179,588—590.

35. Saba A. B., Oridupa, O. A., Oyeyemi, M. O. and Osanyingbe, O. D., 2009: Spermatozoa morphology and characteristics of male Wistar rats administered with ethanolic extract of *Lagenaria breviflora Roberts*. *African Journal of Biotechnology,* 8, 11170—1175.

36. Sanocka, D., Kurpisz, M., 2004: Reactive oxygen species and sperm cells. *Reprod. Biol. Endocrinol*., 2, 1—7.

37. Skerman, P. J., Cameron, D. G., Riveros, F., 1991: Leguminous forages of the tropics. *Food and Agricultural Organization(FAO) Production Year Book, No. 2*. Food and Agricultural Organization of the United Nations, Rome.

38. Soetan, K. O., Fafunso, M. A., 2010: Studies on the Proximate and Mineral Composition of Three Varieties of lablab beans (*Lablab purpureus*). *Int. J. Applied Agric. Res.*, 5, 291—300.

39. Soetan, K. O., Oyewole, E. O., 2009: The need for adequate processing to reduce the antinutritional factors in plants used as human foods and animal feeds: A review. *African Journal of Food Science*, 3, 223—232.

40. Veena, C. K., Josephine, A., Preetha, S. P. et al., 2007: Effect of sulphated polysaccharides on erythrocyte changes due to oxidative and nitrosative stress in experimental hyperoxaluria. *Hum. Exp. Toxicol.*, 26, 923—932.

41. Wells, M. E., Awa, O. A., 1970: New technique for assessing acrosomal characteristics of spermatozoa. *J. Dairy Sci.*, 53, 227.

42. Wolff, S. P., 1994: Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.*, 233, 182—189.

43. Zemjanis, R., 1970: Collection and evaluation of semen. In **Zemjanis, R.** (Ed.)**:** *Diagnostic and Therapeutic Technique in Animal Reproduction*, 2nd Edn., The William- and Wilkins Company, Waverly Press Inc., Baltimore, MD, 139—153.

Received November 8, 2013

FOLIA VETERINARIA, 58, 3: 153—156, 2014

THE INCIDENCE OF YEAST *MALASSEZIA* AND *CANDIDA* IN CATTLE IN THE KOŠICE REGION, EASTERN SLOVAKIA

Link, R.[1], Čonková, E.[2], Reichel, P.[1], Linková, M.[3]

[1]Clinic for swine
[2]Department of Pharmacology and Toxicology
University of Veterinary Medicine and Pharmacy, Komenskeho 73, 041 81 Košice
[3]Department of Social Medicine, Institute of Public Health, Faculty of Medicine
UPJŠ Košice, tr. SNP 1, 040 11 Košice
The Slovak Republic

robert.link@uvlf.sk

ABSTRACT

Samples of hair and smear samples from ears, inguinal region, prepuce, vagina, the rear quarters of the udder, and axilla were obtained to monitor the incidence of *Malassezia* spp. and *Candida* spp. in cattle in the Košice region of Eastern Slovakia. There were 51 calves, 24 cows and 3 heifers included in the study. All animals were clinical healthy and came from agricultural farms near Košice. Samples from different parts of the animals' bodies were taken with sterile swabs and then inoculated on nutrient agar. The yeasts were identified by their macro and microscopic appearances. From the genus *Malassezia,* only *Malassezia pachydermatis* was found in the inguinal region of one healthy calf; in another case, *Malassezia pachydermatis* occurred in one cow's ear. *Candida* spp. was found in one sample from the ear of a cow. There were 8 calves in which *Candida* was found. In most of them, *Candida albicans* was detected (n = 6).

Key words: calves; *Candida;* cows; *Malassezia;* yeast

INTRODUCTION

Malassezia spp. are normal commensals and occasional pathogens of the skin found in many animal species. The genus *Malassezia* comprises lipophilic yeasts that belong to the normal cutaneous microbiota of humans and warm-blooded animals [15], but these yeasts are also associated with a variety of diseases in humans and animals [17].

Malassezia pachydermatis is a very common cause of otitis and pruritic dermatitis in dogs but is of less importance in other veterinary species. Although the dermatophytes and *Malassezia* spp. both exist in the *stratum corneum* of mammalian skin, there are important differences in the epidemiology, pathogenesis, and clinical consequences of infections [1].

The genus *Malassezia* has recently been revised by means of morphology, ultrastructure, physiology, and molecular biology and enlarged to seven species: *M. pachydermatis*, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* [8]. Recently, *M. yamatoensis* was reported as a new species of *Malassezia* [20]. Besides *M. pachydermatis,* the typical zoophilic species are: *M. caprae, M. equina, M. nana* and *M. cuniculi* [3].

M. pachydermatis is the only non-lipid-dependent species in the genus. The species usually colonizes the skin and external ear canal, mainly in wild or domestic carnivores, including dogs, cats, and foxes [9], but it has been found also in cows [7].

Some researchers revealed also the occurrence of lipid-dependent species of the genus *Malassezia* in cows. *M. furfur, M. sympodialis* and *M. restricta* were isolated from the skin surface of healthy cows. Besides them, *M. globosa and M. furfur* were isolated from the external ear canal of healthy cows [4]. *M. caprae* has been revealed in goats [2].

The remaining, lipid-dependent species of *Malassezia* require long-chain fatty acids for *in vitro* growth. The isolation and identification of *Malassezia* spp., mainly lipid-dependent species, continue to be difficult due to low viability, especially with some of the species.

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide [14]. Many species are harmless commensals or endosymbionts of hosts; however, when mucosal barriers are disrupted they can invade and cause disease. *Candida albicans* is the most commonly isolated species. However, there is an increasing incidence of infections caused by *C. glabrata* and *C. rugosa*, which could be because they are frequently less susceptible to the currently used azole antifungals. Other medically important *Candida* species include; *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* [6].

The aim of our study was to monitor the incidence of the genus *Malassezia* and *Candida* yeast in cattle in the Košice region of Eastern Slovakia.

MATERIALS AND METHODS

We obtained samples of hair and smear samples from ears, axilla, prepuce, vagina, inguinal region of calves or the inguinal parts of the rear quarters of cow udders, which came from clinical healthy calves (n = 51), cows (n = 24) and heifers (n = 3) from agricultural farms near Košice. Table 1 shows the sites of sampling. There were 11 Slovak red-spotted, 6 Holstein, 1 Brown and 60 cross-bred animals included in the monitoring. Most animals were females (n = 51), 27 were males.

Samples of ear smears and smears from other parts of the animal bodies were taken with sterile swabs which had been soaked in a suitable medium (Fungi-Quick; Dispolab Žilina, Slovakia) and then inoculated on appropriate nutrient agars.

Laboratory tests were done at the Department of pharmacology and toxicology of the UVMP. The following specific media were used for mycological determination in this study: Sabouraud's dextrose agar with chloramphenicol (HiMedia Laboratories, Mumbai, India) and modified Dixon's agar (30 g malt extract, 15 g agar, 3 g peptone, 20 g dehydrated ox-bile, 10 ml Tween 40,2 ml glycerol, 400 mg cycloheximid, 50 mg chloramphenicol), prepared according to Senczek et al. [19].

Inoculated plates were incubated at 37 °C or 32 °C, for 5 days with daily observations. Yeasts were identified by the macro and microscopic appearances of the colonies (lactophenol blue) and their ability to grow on a lipid-free culture media. Additional phenotypic characterisations were performed: catalase testing; utilization of Tween; and the ability to hydrolyze aesculin.

The identification of yeasts of the genus *Candida* was done by cultivation on HiCrome Candida Agar chloramphenicol (HiMedia Laboratories, Mumbai, India) and by tests API 20C AUX (bioMérieux SA, Lyon, France).

RESULTS

In cows, only one case of *Malassezia pachydermatis* occurred in a sample from an ear and *Candida* spp. was also found in one cow's ear (Table 2).

From the genus *Malassezia,* only *Malassezia pachydermatis* (n = 1) was detected in the inguinal region of one calf (Table 3). There were 8 calves in which *Candida* was found. *Candida albicans* was detected in most of them (n = 6).

In our study we did not find *Malassezia* sp. and *Candida* sp. in samples from heifers.

DISCUSSION

At present, there are many known species of yeasts, which belong to the genus *Malassezia*. Only one of them, *M. pachydermatis,* is a non-lipid-dependent organism. It does not require lipid supplementation in the culture medium.

Table 1. Number of samples from different parts of animals' bodies

Parts of body	Calves (n = 51)	Cows (n = 24)	Heifers (n = 3)	All samples taken from relevant body parts
Hair	48	6		54
Ears	45	22	3	70
Groins	44			44
Prepuce	21			21
Vagina	20			20
Udder		11		11
Axilla		4		4
All samples from one category	**178**	**43**	**3**	**Total = 224**

Table 2. Number and percentage of yeast-like organisms from different parts of the cow's bodies

Isolated species	Ear		Udder		Hair		Axilla		All cows	
	n = 22	%	n =11	%	n = 6	%	n = 4	%	n = 24	%
M. pachydermatis	1	4.5	0	0	0	0	0	0	1	4.2
Candida spp.	1	4.5	0	0	0	0	0	0	1	4.2

Table 3. Number and percentage of yeast-like organisms from different parts of calves

Isolated species of yeasts	Hair		Ear		Inguinal region		Prepuce		Vagina		All calves	
	n = 48	%	n = 45	%	n = 44	%	n = 21	%	n = 20	%	n = 51	%
M. pachydermatis	0	0	0	0	1	2.3	0	0	0	0	1	2.0
C. albicans	0	0	0	0	3	6.8	2	9.5	1	5.0	6	11.8
C. glabrata	0	0	0	0	0	0	1	4.8	0	0	1	2.0
Candida spp.	0	0	0	0	0	0	1	4.8	0	0	1	2.0

Several authors reported *Malassezia* findings in cattle. Duarte et al. [7] isolated *M. sympodialis*, *M. globosa, M. slooffiae* and *M. furfur* from cows with otitis. *M. nana* was revealed both in healthy cows and cows with otitis [10]. *M. furfur, M. sympodialis, M. restricta* and *M. globosa* have been reported in healthy cows [4].

We isolated *Malassezia pachydermatis* from one cow and one calf. Our findings differed from Chengappa et al. [11]. In their study, *Malassezia pachydermatis* was isolated only from dogs. They isolated 229 yeasts from a variety of clinical specimens. Yeasts were identified by using the API 20 C microsystem in conjunction with morphological characteristics. 218 (95.1 %) of them were from bovine, porcine, canine, and equine species. The gastrointestinal and reproductive tracts were the major sources of yeasts, representing 60 (26.2 %) and 28 (12.2 %) isolates, respectively. Their survey revealed that *C. albicans, C. krusei, C. tropicalis* and *C. parapsilosis* were the most frequent species, representing 71.4 % of the total *Candida* isolates.

M. globosa was mainly isolated from pityriasis versicolor [16], *M. restricta* was isolated from seborrheic dermatitis in humans [18], and from a cow's groin [4]. Our animals did not suffer with seborrheic dermatitis, so it may be the reason why we did not find *M. restricta*.

In Slovakia, only the occurrence of *M. pachydermatis* in dogs has been proven [5]; there is no evidence of other *Malassezia* spp. in the Košice region.

Our study, similar to Chengappa et al. [11], revealed more *Candida* spp. than *Malassezia* spp. The two genera can be easily differentiated on CHROM agar *Candida* medium, with vital growth factors for *Malassezia*, on CHROM agar *Malassezia* medium [13].

For further identification, Kaneko et al. [12] presented cost-effective identification method for most clinical laboratories. CHROM with two specific media (Sabouraud's dextrose agar, SDA and Tween 60-aesculin agar) and catalase reactions allowed for the identification of *Malassezia* species easily and quickly. Only *M. pachydermatis* grew on the lipid-free culture medium (SDA). *M. furfur* developed characteristically large pale pink and wrinkled colonies on CHROM and could be differentiated from other *Malassezia* species. *M. restricta* was the only catalase-negative species. With the exception of *M. yamatoensis*, other *Malassezia* species were correctly identified by their system.

Candida spp. and *Malassezia* spp. belong to opportunists. They rarely cause disease in an immunocompetent host, and they are easily treated with triazole derivatives, which exhibit their antifungal activities by inhibiting ergosterol biosynthesis.

In our study, we found *Malassezia pachydermatis* in a cow's ear and *Candida* spp. in a sample from another cow's ear. In calves, *Malassezia pachydermatis* (n = 1) occurred in an inguinal region of one calf. *Candida* sp. was found in 8 calves; in most of them we detected *Candida albicans* (n = 6). None of the animals showed clinical signs of disease, so they did not have to be treated.

ACKNOWLEDGEMENTS

This work was supported by VEGA 1/0537/12 and KEGA 007 UVLF-4/2012.

REFERENCES

1. Bond, R., 2010: Superficial veterinary mycoses. *Clin. Dermatol.* 28, 226—236.

2. Cabañes, F. J., Theelen, B., Castellá, G., Boekhout, T., 2007: Two new lipid-dependent *Malassezia* species from domestic animals. *FEMS Yeast Research*, 7, 1064—1076.

3. Cabañes, F. J., Vega, S., Castella, G., 2010: *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med. Mycol.*, 49, 40—48.

4. Crespo, M. J., Abarca, M. L., Cabanes, F. J., 2002: Occurrence of *Malassezia* spp. in horses and ruminants. *Mycoses*, 45, 333—337.

5. Čonková, E., Sesztáková, E., Smrčo, P., Čellárová, E., Šutiak, V., 2007: *Malasszezia pachydermatis* – occurrence in dogs and its sensitivity to antimycotics (In Slovak). Proceedings of abstracts 4th Czech-Slovak interbranch conference on medical mycology. Pardubice, Czech Republic, 31st May—2nd June, 78.

6. Denfert, C, Hube, B. (Ed.), 2007: *Candida: Comparative and Functional Genomics*. Caister Academic Press. 428 pp.

7. Duarte, E. R., Melo, M. M., Hahn, R. C., Hamdan, J. S., 1999: Prevalence of *Malassezia* spp. in the ears of asymptomatic cattle and cattle with otitis in Brazil. *Med. Mycol.*, 37, 159—162.

8. Guillot, J., Guého, E., Lesourd, M., Midgley, G., Chévrier, G., Dupont, B., 1996: Identification of *Malassezia* species. A practical approach. *J. Mycol. Med.*, 6, 103—10.

9. Guillot, J., Chermette, R., Guého E., 1994: Prévalence du genre *Malassezia* chez les mammiféres. *J. Mycol.Méd.*, 4, 72—79.

10. Hirai, A., Kano, R., Makimura, K., Duarte, E. R., Hamdan, J. S., Lachance, M. A. et al., 2004: *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int. J. Evol. Microbiol.*, 54**,** 623—627.

11. Chengappa, M. M., Maddux, R. L., Greer, S. C., Pincus, D. H., Geist, L. L., 1984: Notes. Isolation and identification of yeasts and yeastlike organisms from clinical veterinary sources. *J. Clin. Microbiol.*, 19, 427—428.

12. Kaneko, T., Koichi, K., Abe, M., Shiota, R., Nakamura, Y., Kano, R. et al., 2007: Revised Culture-Based System for Identification of *Malassezia* Species. *Clin. Microbiol.,* 45, 3737—3742.

13. Kaneko, T., Makimura, K., Onozaki,, M., Ueda, K., Yamada, Y., Nishiyama, Y., Yamaguchi, H., 2005: Vital growth factors of *Malassezia* species: presumptive identification of *Malassezia* and *Candida* species on modified CHROM agar Candida agar. *Med. Mycol.*, 43, 699—704.

14. Manolakaki, D., Velmahos, G., Kourkoumpetis, T., Chang, Y., Alam, H. B., De Moya, M. M., Mylonakis, E., 2010: Candida infection and colonization among trauma patients. *Virulence*, 1, 367—375.

15. Midgley, G., 1989: The diversity of *Pityrosporum* (*Malassezia*) yeasts *in vivo* and *in vitro*. *Mycopathologia*, 106, 143–153.

16. Nakabayashi, A., 2002: Identification of causative species in *Malassezia*-associated dermatoses. *Jpn. J. Med. Mycol.*, 43**,** 65—68.

17. Scott, D. W., Miller, W. H., Griffin, C., 1995: *Muller and G.H. Kirk's Small Animal Dermatology*. 5th edn., Philadelphia, W. B. Saunders Co., 631 pp.

18. Sei, Y., 2006: *Malassezia* related disease. *Jpn.. J. Med. Mycol.*, 47, 75—80.

19. Senczek, D., Siesenop, U., Böhm, K. H., 1999: Characterization of *Malassezia* species by means of phenotypic characteristics and detection of electrophoretic karyotypes by pulse-field gel electrophoresis (PFGE). *Mycoses*, 42, 409—414.

20. Sugita, T., Tajima, M., Takashima, M., Amaya, M., Saito, M., Tsuboi, R., Nishikawa, A., 2004: A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol. Immunol.*, 48**,** 579—583.

Received May 30, 2014

FOLIA VETERINARIA, 58, 3: 157—161, 2014

COMPARISON OF ACID-BASE PARAMETERS IN VENOUS BLOOD OF PIGS BEFORE AND AFTER SHORT-TERM SURGICAL ANAESTHESIA

Novotný, J., Reichel, P., Kovačocyová, K., Raši, T.

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

novotny@uvm.sk

ABSTRACT

The aim of this study was to analyse the influence of short-term injection anaesthesia (azaperon — Stresnil inj., Janssen Pharmaceutica; diazepam — Apaurin inj., Krka; ketamine — Ketamidor 10% inj., Richter Pharma AG) on the pH and concentration of blood gases in the venous blood of pigs. The parameters of; acid-base profile — pH, pCO_2, pO_2, HCO_3, standard bicarbonate SBi, base excess BE, and oxygen saturation, were monitored in two different surgical procedures. Five boars (crossbreed Large White and Landrace, mean body weight 30.4 kg, 3 months old) had surgery outside of the abdominal cavity — castration + inguinal hernia repair. Four gilts (crossbreed Large White and Landrace, mean body weight 78.75 kg, 5 months old) had surgery in the abdominal cavity — ovariectomy. Blood samples were taken before anaesthesia (0 sampling), and 15 minutes (castration) and 120 minutes (ovariectomy) respectively, after the administration of ketamine (1st sampling). The statistical comparison of acid-base parameters before and after castration showed significant increases of partial pressure of O_2 (4.80 to 9.20 kPa; $P < 0.05$) and saturation of O_2 (63.80 to 92.20%; $P < 0.05$). On the other hand, the statistical comparison of samplings before and after ovariectomies showed no significant differences. The comparison between surgical procedures showed significantly lower values of pH (7.32; $P < 0.01$), HCO_3 (23.00 mmol. l^{-1}; $P < 0.05$), SBi (21.25 mmol. l^{-1}; $P < 0.05$) and BE (–3 mmol. l^{-1}; $P < 0.05$) after ovariectomies. The obtained results confirm the differences of blood parameters depending on the duration of the surgery.

Key words: acid-base profile; anaesthesia; pigs; venous blood

INTRODUCTION

The assessment of the acid-base status in an organism, is a functional examination that gives information about intake, formation and excretion of acids and bases. The maintenance of a stable acid-base balance is a vital component of the body's homeostasis. The main mechanisms that maintain acid-base balance are the dilution effects of body fluids, buffer reactions, and functions of lungs, kidneys, skeleton and liver [3].

General anaesthesia is the purposeful inducing of a reversible state, which is characterised by a temporary (total or partial) loss of consciousness, sensitivity and reflexes. However, during this time, important life functions, such as a blood circulation and respiration must be maintained [12].

Pigs are difficult to restrain and anesthetize, due to their size and resistance to sedative drug combinations [15]. Many times, injectable sedative drugs have depression effects on the cardiovascular and respiratory functions. Many variables may affect a swine's response to anaesthetics. Therefore, it is important to analyse the length and seriousness of an adverse effect [1], [14], [17].

The aim of this experimental work was to analyse the influence of short-term surgical anaesthesia on the pH and concentrations of pCO_2, pO_2, HCO_3, standard bicarbonate SBi, base excess BE, and saturated O_2 in the venous blood of pigs.

MATERIALS AND METHODS

Animals

Experimental work was carried out at the Clinic for swine UVMP in Košice. Nine pigs (crossbreed Large white × Landrace) were subjected to short-term surgical anaesthesia. Four gilts (mean body weight 78.75 kg, 5 months old) had surgery in the abdominal cavity — the removal of ovaries (ovariectomy) and five boars (mean body weight 30.4 kg, 3 months old) had surgery outside of the abdominal cavity — castration + inguinal hernia repair. Pigs were housed individually and fed adequate dry feed mixtures (OŠ-06) with 6 hours preoperative fasting before castration and 12 hours fasting before ovariectomy. All animals were in very good health before any surgical interventions and well treated according to all welfare rules.

Experimental design

Before the surgical procedures, the animals were submitted to general anaesthesia. For this purpose, the same combination of tranquilizers and anaesthetics were used in both groups of animals. Azaperone (Stresnil inj., Janssen Pharmaceutica, Belgium — 2 $mg.kg^{-1}$ body weight) was applied simultaneously with diazepam (Apaurin inj., Krka, Slovenia — 0.5 $mg.kg^{-1}$ body weight), after 30 minutes of ketamine (Ketamidor 10 % inj., Richter Pharma AG, Austria — 20 $mg.kg^{-1}$ body weight). All drugs were applied intramuscularly into the dorsal part of the neck region. However, because the anaesthetic effect of this combination of tranquilizers and anaesthetics was maximally 45 minutes, during ovariectomy it was necessary to deepen and prolonged the anaesthesia by twice repeated intravenous application of ketamine (0.3 ml *pro toto*).

All patients were transferred and positioned on the operating tables. Patients undergoing abdominal surgery (ovariectomy) were operated in the right lateral position. Patients undergoing castration were in the supine position. All animals were allowed to breathe spontaneously without artificial ventilation. Also, intravenous, perioperative fluid therapy was done by infusion of crystalloids (0.9 % sodium chloride).

Venous blood was collected into a 2 ml syringe (an anticoagulant — heparin included) just before anaesthesia (0 sampling), and 15 minutes (castration) 120 minutes (ovariectomy) respectively, after administration of ketamine (1st sampling).

Acid-base profile determination

The pH, partial pressure of carbon dioxide — pCO_2, partial pressure of oxygen — pO_2, bicarbonate — HCO_3, standard bicarbonate — SBi, base excess — BE, and oxygen saturation — sat O_2, of venous blood was analysed by a blood gas analyser *Radiometer ABL 5*.

Statistical processing of results

The statistical processing of the results was performed by assessment of the means (x) and standard deviations (SD) in both groups of pigs. The significance (P) of differences in the means of corresponding variables was evaluated by Student's *t*-tests. The measurements before and after a surgical intervention were evaluated by the paired *t*-test. The significance of differences between the groups (ovariectomy and castration) was evaluated by unpaired *t*-test at the same time.

The obtained parameters were compared with a reference range (Table 1) provided by the laboratory that performed the test (Clinic for swine UVMP in Košice).

RESULTS

The mean values of pH before surgeries (0 sampling) were approximately on the same level (7.34; Table 2). Significant differences were found between ovariectomies and castrations at the 1st sampling ($P < 0.01$). The highest mean value (7.41) was recorded at the 1st sampling after castrations; on the contrary, the lowest mean value (7.32) was after the ovariectomies. This value was under the physiological reference values (7.35—7.45).

Short-time injection anaesthesia *non-significantly reduce*d mean values of pCO_2 (1st sampling). Statistical analysis showed no significant changes. The mean values obtained after surgeries were within the reference interval (4.4—6.5 kPa).

Anaesthesia performed before castrations significantly increased pO_2 in venous blood ($P < 0.05$). The highest mean value was recorded at 1st sampling (9.20 kPa). The comparison of ovariectomies and castration did not show any significant differences.

The highest mean value of HCO_3 (27.40 $mmol.l^{-1}$) was detected before castrations (0 sampling). The comparison of values obtained before and after surgical interventions showed non-significant decreases. However, significant differences were found between analysed surgical procedures ($P < 0.05$; 1st sampling).

Table 1. Reference range for acid-base profile of venous blood in pigs

pH	$_pCO_2$ [kPa]	$_pO_2$ [kPa]	HCO_3 [$mmol.l^{-1}$]	SBi [$mmol.l^{-1}$]	BE [$mmol.l^{-1}$]	Sat. O_2 [%]
7.35—7.45	4.4—6.5	–	–	24—8	–5 — +5	–

Table 2. Mean values and standard deviations (x ± SD) of acid-base parameters of venous blood in pigs undergoing castrations and ovariectomies

Blood parameter	Surgical operation	0 sampling	1st sampling
pH	Castrations + hernia repair	7.34 ± 0.09	7.41 ± 0.02**
	Ovariectomies	7.34 ± 0.10	7.32 ± 0.03**
pCO_2 [kPa]	Castrations+hernia repair	7.06 ± 1.17	5.86 ± 0.42
	Ovariectomies	6.53 ± 1.35	6.05 ± 0.34
pO_2 [kPa]	Castrations + hernia repair	4.80 ± 0.83*	9.20 ± 2.16*
	Ovariectomies	4.65 ± 1.96	5.90 ± 2.95
HCO_3 [mmol.l^{-1}]	Castrations + hernia repair	27.40 ± 1.20	27.20 ± 1.83*
	Ovariectomies	25.75 ± 5.80	23.00 ± 2.24*
SBi [mmol.l^{-1}]	Castrations + hernia repair	24.40 ± 2.42	26.60 ± 1.74*
	Ovariectomies	23.50 ± 5.89	21.25 ± 2.49*
BE [mmol.l^{-1}]	Castrations + hernia repair	0.80 ± 2.79	2.40 ± 1.96*
	Ovariectomies	–0.50 ± 6.42	-3.00 ± 2.12*
Sat-O_2 [%]	Castrations + hernia repair	63.80 ± 11.91*	92.20 ± 4.07*
	Ovariectomies	58.75 ± 26.23	64.75 ±25.91

* — $P < 0.05$; ** — $P < 0.01$

The highest mean value of SBi (26.60 mmol.l^{-1}) was recorded at the 1st sampling after castrations. This value was significantly higher ($P < 0.05$) than the mean value after ovariectomies (21.25 mmol.l^{-1}). Both values calculated before and after ovariectomies were below the reference range (24—8 mmol.l^{-1}).

Similar to the *previous analysis*, the highest mean value of BE (2.40 mmol.l^{-1}) was also detected at the 1st sampling after castrations. On the other hand, the lowest value (-3 mmol.l^{-1}) was recorded at the 1st sampling after ovariectomies, and a statistical comparison of this value showed a significant difference ($P < 0.05$). All mean values ranged within the normal limit (–5 — +5 mmol.l^{-1}).

The saturation of venous blood by oxygen, Sat O_2, increased after both surgical procedures. The highest mean value (92.20 %) was recorded at the 1st sampling after castrations. Compared to 0 sampling, this value was significantly higher ($P < 0.05$).

DISCUSSION

Anaesthesia is frequently required during the medical management of animals for both therapeutic procedures and experimental models. However, swine are challenging patients for sedation, chemical restraint and general anaesthesia [2]. Intramuscular administration of injectable anaesthetic combinations involving tranquilizers and dissociative agents is one of the possibilities for anaesthesia in pigs. This combination is less stressful to the animals if the tranquilizer portion of the injection is administered prior to dissociative agents, which tend to be painful [16]. In our experiments, we used a combination of tranquilizers — azaperone, diazepam, and the dissociative agent — ketamine. In general, this combination allows deeper sedation to be achieved and the executions of short surgical procedures.

Azaperone is a butyrophenone neuroleptic drug with sedative and antiemetic effects, which is used mainly as a tranquilizer in veterinary medicine. It is used mainly in pigs and elephants [11]. Azaperone may cause hypotension (peripheral vasodilation and heat loss) and while it has minimal effects on the respiration in pigs, high doses in humans can cause respiratory depression, which may be why it is rarely used in humans.

Diazepam belongs to a benzodiazepines group and it possesses anxiolytic, anticonvulsant, hypnotic, sedative, skeletal muscle relaxant, and amnestic properties [10].

Ketamine is a drug used in human and veterinary medicine, mainly for starting and maintaining general anaesthe-

sia. Ketamine provides good analgesia and causes only a mild cardiovascular depression in healthy animals. If used alone, the swallowing reflex remains intact but the drug can cause muscular hypertonus and so should be used with a centrally acting muscle relaxant such as a benzodiazepine. The sole use of ketamine has been associated with excessive salivation and excitement during recovery.

In our experiments, acid-base changes have been studied on anaesthetized patients undergoing surgeries of ovaries and testicles. An average time for castration and inguinal hernia repair procedure of boars was 15 minutes. On the other hand, the time necessary for carrying-out ovariectomies in gilts was longer (120 minutes – a demonstration of surgery for university students). The position of the animals during surgery was different, which could also influence the results. Patients undergoing abdominal surgery (ovariectomy) were operated in the lateral position, while castration and hernia repair was done in the supine position. Of course, repeated administration of ketamine during ovariectomy could also influenced the obtained results (bradypnoea). However, during the experiments, none of the clinical disturbances of respiration and circulation which sometimes accompany anaesthesia were found.

The statistical comparison of values measured before (0 sampling) and after completion of ovariectomies (1st sampling) did not show any significant differences. But, a significant increase of oxygen partial pressure and oxygen saturation ($P < 0.05$) was recorded after castrations. These results probably have a connection with a faster respiratory frequency (different age and gender of pigs), shorter duration of surgical intervention and the supine position during surgery. Respiratory obstruction is a major concern when sedating pigs. Brachycephalic breeds, such as Vietnamese pot-bellied pigs, are more prone to this. Placing the pig in dorsal recumbency (supine position) may also alleviate the obstruction [6]. On the other hand, the lateral position can leads to a generally well tolerated increase in ventilation-perfusion inequality but can cause hypoxemia in compromised patients [7].

In comparison with a reference limit of Clinic for swine UVMP (pH 7.35—7.45; pCO_2 4.40—6.50 kPa; BE –5 to +5 mmol.l^{-1}; SBi 24—28 mmol.l^{-1}), lower mean value of pH (7.32 — *acidaemia*) was determined after completion of ovariectomies. Also, a lower concentration of SBi in venous blood of gilts before and after ovariectomy (23.5 mmol.l^{-1} respectively 21.25 mmol. l^{-1} — *metabolic acidosis*) was recorded. This state could be associated with longer preoperative *fasting* (12 hours) before abdominal surgery. As discussed by Lewis [9], the causes of metabolic acidosis include accumulation of ketones and lactic acid, renal failure, and drug or toxin ingestion (high anion gap) and gastrointestinal or renal HCO_3^- loss (normal anion gap). On the other hand, values ascertained after completion of castration ranged within a physiological range. According to Dahan and Teppema [5] or Knill and Clement [8], the acidosis which occurs during anaesthesia is mainly respiratory in origin because of the depressant effect of the anaesthetic agents on the respiratory centre and the paralysing effect of relaxants on the muscles of respiration. Elevation of pCO_2 (*hypercapnia*) is a direct consequence of hypoventilation and it is a relatively common state during anaesthesia [4], [13]. However, our examinations showed an insignificant decrease in pCO_2 immediately after finishing both surgical procedures.

CONCLUSIONS

The comparison between ovariectomies and castration showed significantly lower values of pH ($P < 0.01$), HCO_3, SB and BE ($P < 0.05$) after ovariectomies. The results obtained confirm the differences of blood parameters depending up the duration of the surgery.

Because, the porcine animal model has gained massively in importance in biomedical research during the past few years, more extensive research needs to be conducted to determine the exact levels of acid base parameters and possible influences and complications for particular types of anaesthesia in pigs.

ACKNOWLEDGEMENT

This investigation was supported by Grant agency KEGA 007 UVLF-4/2012 and VEGA 1/0537/12.

REFERENCES

1. Andersen, J. B., Wang, T., 2002: Effects of anaesthesia on blood gases, acid – base status and ions in the toad Bufo marinus. *Comp. Biochem. Physiol. A,* 131, 639—646.

2. Bîrłoiu, I. A., Badea, R., Sîrbu-Boełix, P. M., Efrimescu, C., 2008: Different anesthesia protocols used for experimental swine surgery. *Lucrări Stiinłifice Medicină Veterinară*, Timisoara, 41, 526—529.

3. Bouda, J., Jagoš P., 1990: Disorders in the acid-base balance. **Vrzgula, L., Kováč, G., Bartko, P. et al.** *Metabolic Disorders and their Prevention in Farm Animals* (In Slovak). 2nd. edn., Bratislava, 241—262.

4. Carlston, G. P., 1997: Fluid, electrolyte and acid-base balance. In **Kaneko, J. J., (Ed):** *Clinical Biochemistry of Domestic Animals.* Academic Press, San Diego, 492—493.

5. Dahan, A., Teppema, L., 1999: Influence of low-dose anaesthetic agents on ventilatory control: where do we stand? *Br. J. Anaesth.*, 83, 199—201.

6. Hodgkinson, O., 2007: Practical sedation and anaesthesia in pigs. *Practice*, 29, 34—39.

7. Knight, D. J. W., Mahajan, R. P., 2004: Patient positioning in anaesthesia. *Continuing Education in Anaesthesia, Critical Care & Pain,* 4, 160—163.

8. Knill, R. L., Clement, J. L., 1985: Ventilatory responses to acute metabolic acidemia in humans awake, sedated, and anesthetized with halothane. *Anaesthesiology,* 62, 745—753.

9. Lewis, J. L., 2013: Metabolic acidosis. In *Merck Manual.* http://www.merckmanuals.com/professional/endocrine_and_metabolic_disorders/acid-base_regulation_and_disorders/metabolic_acidosis.html

10. Mandrioli, R., Mercolini, L., Raggi, M. A., 2008: Benzodiazepine metabolism: an analytical perspective. In *Curr. Drug Metab.,* 9, 827—844.

11. Mikota, S. K., 2006: Azaperone, www.elephantcare.org

12. Němeček, L., 1996: Anaesthesiology. In **Janda, J. et al.:** *General Veterinary Surgery.* Košice, 80—93.

13. Speirs, V. C., 1980: Arteriovenous and arteriocentral venous relationship for pH, $_pCO_2$ and actual bicarbonate in equine blood samples. *Am. Journal Vet. Res.,* 41, 199—203.

14. Svendsen, P., Carter A. M., 1989: Blood gas tensions, acid-base status and cardiovascular function in miniature swine anaesthetized with halothane and methoxyflurane or intravenous metomidate hydrochloride. *Pharmacol. Toxicol.,* 64, 88—93.

15. Swindle, M. M., 2007: *Swine in the Laboratory: Surgery, Anesthesia, Imaging, and Experimental Techniques*. 2nd edn., CRC Press, Boca Raton, Florida, 59—61.

16. Swindle, M. M., 2008: Anesthesia and analgesia in swine. In *Sinclair Research Technical Bulletin*, 1—7.

17. Weiskopf, R. B., Bogetz, M. S., Roizen, M. F., Reid, I. A., 1984: Cardiovascular and metabolic sequelae of inducing anesthesia with ketamine or thiopental in hypovolemic swine. *Anesthesiology*, 60, 214—219.

Received May 30, 2014

FOLIA VETERINARIA, 58, 3: 163—167, 2014

CANCER IMMUNITY — ROLE AND CHARACTERISTICS A REVIEW

Kissová, V., Ševčíková, Z., Revajová, V., Herich, R., Levkutová, K.

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

Viktoria.Kissova@student.uvlf.sk

ABSTRACT

A tumour is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and it persists in the same excessive manner even after the cessation of the stimuli which evoked the alteration. Tumours can and do arise in the presence of a functional immune system. We now appreciate that the immune system, through its interaction with tumours, can, in part, sculpt the cancer phenotype, developing less immunogenic variants that ultimately facilitate the outgrowth of the tumour. The immune system can prevent expansion of proliferating tumour cells. One of the essential parts of the immune system is the tumour-associated macrophage (TAM). They are a major component of the leukocyte infiltrate into a tumour's stroma and have served as a paradigm for cancer-related inflammation. Tumour-infiltrating lymphocytes (TIL), are considered to be a component of an inflammatory host response to the tumour. TILs participate in the host defence mechanisms against tumour cells and are frequently present in human solid tumours. Chronic inflammation has long been associated with an increased risk of tumour onset and progression. It is known to enhance angiogenesis and tissue remodelling, as well as, promoting protein and DNA damages through oxidative stress; processes which are integral to tumour progression. The immune system may also protect against nascent cancers by destroying malignant cells before they developed into detectable tumours, a concept that has become the immune surveillance hypothesis.

Key words: immune system; inflammation; tumours

INTRODUCTION

The basic function of the immune system is to collect information from the internal and external environment. The immune system's logical processing and response, results in a general defensive-adaptation response, but sometimes it may also damage some of its own tissues, cells and structures by an autoimmune or other immunopathological responses. The most important characteristic of the immune system is its ability to recognize their own molecular structures from extraneous structures [15]. Cancer immune surveillance is considered to be an important host protective process to inhibit carcinogenesis and to maintain cellular homeostasis. First of all, it is necessary to discuss what tumours are [33].

A tumour is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change [40].

The history behind the study of the immune reaction to cancer is a long and often controversial one, most extensively reviewed

by Dunn et al. [11]. Briefly, in 1909, Paul Ehrlich first hypothesized that a key function of the immune system was to protect the host from cancer. However, the lack of immunological tools and knowledge about the immune system meant that this hypothesis was not tested experimentally. Since then, an extensive amount of experimental data from various mouse models of cancer, together with convincing, correlative clinical data from human patients, has provided unequivocal evidence that cells of the immune system, innate and adaptive, have three primary roles in the prevention of cancer [38].

First, the immune system can protect the host from virus-induced tumours by eliminating or suppressing viral infections. Second, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumorigenesis. Third, the immune system can specifically identify and eliminate tumour cells on the basis of their expression of tumour-specific antigens or molecules induced by cellular stress. The third process is referred to as tumour immune surveillance, whereby the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm [14].

The immune system is an important determinant of the tumour microenvironment. Indeed, the complex interplay between cancer cells and the host immune response has been extensively investigated during the past few decades. Several immunological deficiencies have been linked with enhanced tumour development in mouse models as well as in humans [8].

PHASES OF TUMOUR IMMUNOEDITING

Cancer immunoediting is a multistep process comprising different phases; elimination, equilibrium, and escape [26]. The elimination phase corresponds to the original concept of cancer immune surveillance, whereby cancer cells are successfully recognized and eliminated by the immune system, thus returning the tissues to their normal state of function [14], [38]. When the elimination process is unsuccessful, tumour cells are capable of colonizing sites in the tissue microenvironment and enter the equilibrium phase, in which they may either be maintained chronically or be immunologically induced to change and produce new populations of tumour variants that are less immunogenic or possess mechanisms to control immune activation. In this phase, tumour cells can grow, although the immune system is still capable of controlling tumour progression. The escape phase refers to the final outgrowth of tumours that eventually evolve into a state in which they can effectively evade, suppress, and overcome control by the immune system. Importantly, the escape from immune control is now acknowledged to be one of the hallmarks of cancer [5]. The cancer immunoediting hypothesis predicts that tumours arising in immune-deficient individuals will be less immunogenic than tumours that develop in immune-competent individuals [29].

EFFECTIVE IMMUNE CELLS

Multiple lineages of immune cells are involved in the antitumor responses. Tumour-infiltrating immune cells include mainly tumour associated macrophages (TAM) and tumour infiltrating lymphocytes (TIL) (7). Dendritic cells, M1 macrophages, Th1 CD4 T- cells, cytotoxic CD8 T-cells, and natural killer cells present in the tumour bed tend to reduce cancer growth. In contrast, M2 macrophages, myeloid-derived suppressor cells, neutrophils, Th2 and Th17 CD4 T-cells, and regulatory T (Treg) cells are suspected to stimulate cancer growth, although this functional distinction has to be placed into context for each tumour type [35], [41].

The first assumption of immune and tumour cells interaction is dependent on the recognition of surface tumour antigens. These antigens are tumour-specific (molecules that are unique to the cancer cells) or tumour-associated antigens (molecules that are expressed differently by cancer cells and normal cells) [17].

Tumour-specific antigens represent fragments of novel proteins that are presented at the cell surface, bound to the major histocompatibility complex (MHC) class I molecules. In this form they are recognized by T lymphocytes (T-cells) and eliminated [9].

Tumour-associated antigens on tumour cells are not qualitatively different in structure from antigens found on normal cells, but they are present in significantly greater amounts. Because of their abundance, they are often shed into the bloodstream. Elevated levels of these antigens can be used as tumour markers — that is, as indicators of a tumour [18].

THE ROLE OF TUMOUR-ASSOCIATED MACROPHAGES (TAM) AND POLYMORPHONUCLEAR NEUTROPHILS (PMNS)

Macrophages represent up to 50 % of the tumour mass, and they certainly operate as fundamental actors. Macrophages constitute an extremely heterogeneous population; they originate from blood monocytes, which differentiate into distinct macrophage types, schematically identified as M1 (or classically activated) and M2 (or alternatively activated) [31]. Low macrophage infiltration into the tumour mass correlates with the inhibition of tumour growth and metastatic development in different animal models [24]. In the tumour milieu, TAM carry on their pro-neoplastic role by influencing the fundamental aspects of the tumour biology. For instance, they: produce molecules that affect neoplastic cell growth directly (e. g., epidermal growth factor EGF); enhance neoangiogenesis; tune inflammatory responses; affect adaptive immunity; catalyze structural components; and cause substantial changes of the extracellular matrix (ECM) compartment [31]. Another hallmark of TAM is their tendency to accumulate into necrotic regions of the tumours, characterized by low oxygen tension [22]. A high TAM content is generally correlated with a poor prognosis [32], however, depending on their stage of differentiation and activation, tissue macrophages have the ability to promote or inhibit neoplasia [28]. T-cells can also exert both tumour-suppressive and tumour-promoting effects [16].

Polymorphonuclear leukocytes (PMN), also called neutrophils exhibit an increase in the production of reactive oxygen species (ROS), NADPH oxidase and myeloperoxidase (MPO) [20]. Polymorphonuclear neutrophils are the most abundant circulating blood leukocyte. They provide the first-line of defence against infection and are potent effectors of inflammation. In addition, their release of soluble chemotactic factors guides the recruitment of both nonspecific and specific immune effector cells [23]. They are the most abundant leukocyte population in the blood, comprising 50—60 % of the circulating leukocytes (25×10^9 cells) [34]. PMNs are critical components of the innate immune response that are essential in protecting the host from microbial pathogens, while also minimizing deleterious effects mediated by dying or injured cells. (PMN are elegantly adapted to perform a variety of antimicrobial functions such as degranulation and phagocytosis [2].

THE ROLE OF TUMOUR-INFILTRATING LYMPHOCYTES (TIL)

The TIL cells infiltrating the tumour are effective in the destruction of the tumour. They are cytotoxic, especially for the cells of the tumour from which they were isolated [15]. It has been suggested that TIL elaborate cytokines and growth factors necessary for tumour growth and that tumours produce chemotactic factors that actively recruit mononuclear cells, mainly lymphocytes and macrophages in humans, to the tumour sites [3]. CD4 glycoprotein, is a typical surface characteristic of auxiliary Th-lymphocytes. It participates in the presentation of exogenous antigens presented by antigen-presenting cells in the form of a complex with HLA antigens II. class and serves as a receptor for HIV, which causes AIDS. CD 8 transmembrane glycoprotein, is a typical surface characteristic of Tc cytotoxic and suppressor Ts lymphocytes. They participate in the recognition of antigens on the surface of target cells, where they are located in the form of complexes, immunogenic peptides to HLA class I antigens [13].

Type 1 CD4+ T-cells (Th1) facilitate tissue destruction and tumour rejection by providing help to cytotoxic CD8+ T-cells. Type 2 CD4+ T-cells (Th2) facilitate antibody production by B cells and polarize immunity away from a beneficial cell mediated antitumor response CD4+ T-cells. T regulatory cells (T regs), which are naturally occurring or antigen-induced tumour immunity, blocks the activation of CD8+ cytotoxic T-cells. [4], [36]. In many cases, CD4+ T-cells will not be able to recognize cancer cells directly, due to the lack of MHC class II on most (solid) cancers [27]. Clearly the CD4+T-cells represent a unique branch of the adaptive immune system, that is crucial in achieving a regulated effective immune response to pathogens, and their proper functioning is critical for survival. Through their distinct phenotypes with their respective cytokine profile, they modulate the functions of the innate immune cells, as well as, the members of the adaptive immune system [6]. CD4+ T-cells are more plastic and play dual roles; thus CD4+ T-cells can convert from antitumor to pro-tumour activity (36). The role of Treg cells in immune evasion remains unclear. The number of Treg cells increase in the peripheral blood of patients and they are present within the primary tumours [30]. Their effect on tumour progression vary according to the tumour type in humans: Treg cells have a negative effect on survival in pancreatic, liver, or ovarian carcinoma patients, whereas they may exert a beneficial role in follicular lymphoma or head and neck carcinoma or have no effect on survival (anal squamous cell carcinoma) [21].

INFLAMMATION *VS* CANCER

Inflammation also affects immune surveillance and responses to therapy. Yet, it is only during the last decade that clear evidence has been obtained that inflammation plays a critical role in tumour genesis and some of the underlying molecular mechanisms have been elucidated [19]. A role for inflammation in tumour genesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumours, including some in which a direct causal relationship with inflammation has not yet been proven [25]. Although it is now well established that the induction of inflammation by bacterial and viral infections increases cancer risk [10], recent work has shown that in addition to being a tumour initiator by virtue of its high carcinogen content, tobacco smoke is also a tumour promoter because of its ability to trigger chronic inflammation [39]. Along with its protumourgenic effects, inflammation also influences the host immune response to tumours and can be used in cancer immunotherapy [12] and to augment the response to chemotherapy [41]. Yet, in some cases, inflammation can diminish the beneficial effects of therapy [1]. The tumour microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) and adaptive immune cells (T- and B-lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells) [11]. These diverse cells communicate with each other by means of direct contact or cytokine and chemokine production and act in autocrine and paracrine manners to control and shape the tumour growth. Other immune cells also affect tumorigenesis. Not surprisingly, chronic inflammation, which has long been associated with increased tumour risk, is involved in polarizing immunity toward those effectors that facilitate tumour growth [37].

CONCLUSION

In conclusion, the tumour immunity is the result of interactions between the different cells of the immune system and tumour cells. Tumour cells can become structurally and/or functionally modified; however, the immune system generally fails to successfully destroy most tumours. This suggests that for many cancers, the immune system becomes tolerant to tumour antigens and allows the progression of the neoplastic process. This tolerance may be mediated by specific populations of regulatory cells ("tumour suppressor cells"), which may eventually serve as sensible targets for the development of novel cancer immunotherapy. A tumour is a very complex structure, and as it is evident from this review, not only the type of infiltrating cells are important by the location involved, but moreover, the specific phenotype and function of those cells in the particular environment may play a dominate role. This is a major challenge for our

understanding of lymphocyte infiltration and the impact on clinical outcomes. Whether the immune system limits or promotes tumour growth depends on a delicate balance between opposing forces.

REFERENCES

1. Ammirante, M., Luo, J. L., Grivennikov, S., Nedospasov, S., Karin, M., 2010: B cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature*. 464, 302—305.

2. Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., Zychlinsky, A., 2012: Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.,* 30, 459—489.

3. Balkwill, F., 2004: Cancer and the chemokine network. *Nat. Rev. Cancer*, 4,540—50.

4. Baratelli, F., Lin, Y., Zhu, L., Yang, S. C., Heuze-vourc'h, N., Zeng, G., et al., 2007: NK cells negatively regulate antigen presentation and tumour-specific CTLs in a syngeneic lymphoma model. *J. Immunol.,* 178, 6140—6147.

5. Chew, V., Toh, H. C., Abastado, J. P., 2012: Immune microenviroment in tumour progression: characteristics and challenges for therapy. *Journal of Oncology*, Epub 2012, Aug. 8, doi:10.1155/2012/608406.

6. Chow, M. T., Möller, A., Smyth, M. J., 2012: Inflammation and immune surveillance in cancer. *Seminars in Cancer Biology*, 22, 23—32.

7. Crawford, N. P. S., Colliver, D. W., Galandiuk, S., 2003: Tumour markers and colorectal cancer: utility in management. *Journal of Surgical Oncology,* 84, 239—248.

8. de Martel, C., Franceschi, S., 2009: Infections and cancer: established associations and new hypotheses. *Crit. Rev. Oncol. Hematol.,* 70, 183—194.

9. de Visser, K. E., Eichten, A., Coussens, L. M., 2006: Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer,* 6, 24—37.

10. Dougan, M., Dranoff, G., 2012: *Immune Therapy for Cancer. Innate Immune Regulation and Cancer Immunotherapy.* Springer, New York,, 391—414.

11. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., Schreiber, R. D., 2002: Cancer immunoediting: from immunosurveillance to tumour escape. *Nat. Immunol.,* 3, 991—998.

12. Dunn, G. P., Old, L. J., Schreiber, R. D., 2004: The immunobiology of cancer immunosurveillance and immunoediting. *Immunity,* 21, 137—148.

13. Ferenčík, M., Rovenský, J., Nyulassy, Š., 1999: *Basic Terms and Definitions* (In Slovak). Slovak Academic Press s.r.o., Bratislava, 190 pp.

14. Fridman, W. H., Pages, F., Sautes-Fridman, C., et al., 2012: The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev. Cancer,* 12, 298—306.

15. Graziano, D. F., Finn, O. J., 2005: Tumour antigens and tumour antigen discovery. *Cancer Treat. Res.*, 123, 89—111.

16. Hanahan, D., Weinberg, R. A., 2011: Hallmarks of cancer: the next generation. *Cell,* 144, 646—674.

17. Hořejší, V., Bartůňková, J., 2005: *Basis of Immunology* (In Czech). 3rd edn., Triton s.r.o., Prague, 280 pp.

18. Karin, M., 2006: Nuclear factor-kappaB in cancer development and progression. *Nature*, 441, 431—436.

19. Klink, M., Jastrzembska, K., Nowak, M., Bednarska, K., Szpakowski, M., Szyllo, K., Sulowska., Z. 2008: Ovarian cancer cells modulate human blood neutrophils response to activation in vitro. *Scand. J. Immunol.,* 68, 328—36.

20. Kobayashi, N., Hiraoka, N., Yamagami, W., et al., 2007: FOXP3+ regulatory T-cells affect the development and progression of hepatocarcinogenesis. *Clin. Cancer Res.,* 13, 902—11.

21. Lewis, C., Murdoch, C., 2005: Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. *Am. J. Pathol.,* 167, 627—635.

22. Lillard, J. W. J. R., Boyaka, P. N., Chertov, O., Oppenheim, J. J., Mcghee, J. R., 1999: Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc. Natl. Acad. Sci., USA,* 96, 651—656.

23. Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., et al., 2006: Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.,* 66, 11238—11246.

24. Luckheeram, R. V., Zhou, R., Verma, A D., Xial, B., 2012: CD4+T-cells: differentiation and functions. *Clinical and Developmental Immunology*, Article ID 925135, 12 pp.

25. Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008: Cancer-related inflammation. *Nature,* 454, 436—444.

26. Matsushita, H., Vesely, M. D., Koboldt, D. C., et al., 2012: Cancer exome analysis reveals a T-cell dependent mechanism of cancer immunoediting. *Nature,* 482, 400—404.

27. Mendez, R., Aptsiauri, N., Del, C. A., Maleno, I., Cabrera, T., Ruiz-Cabello, F., et al., 2009: HLA and melanoma: multiple alterations in HLA class I and II expression in human melanoma cell lines from ESTDAB cell bank. *Cancer Immunol. Immunother.,* 58,1507—1515.

28. Montuenga, L. M., Pio, R., 2007: Tumour-associated macrophages in non-small cell lung cancer: the role of interleukin-10. *Eur. Respir. J.,* 30, 608—610.

29. O'Sullivan, T., Saddawi-Konefka, R., Vermi, W., Koebel, C. M., Arthur, C., White, J. M., et al., 2012: Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J. Exp. Med.,* 209, 1869—1882.

30. Perez, S. A., Karamouzis, M. V., Skarlos, D. V., et al., 2007: CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. *Clin. Cancer Res.,* 13, 2714—2721.

31. Pollard, J. W., 2009: Trophic macrophages in development and disease. *Nat. Rev. Immunol.,* 9, 259—270.

32. Quatromoni, J. G., Eruslanov, E., 2012: Tumour-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am. J. Transl. Res.,* 4, 376—389.

33. Ryungsa, K., Manabu, E., Kazuaki, T., 2007: Cancer immunoediting from immune surveillance to immune escape. *Immunology.* 121, 1—14.

34. Sadik, C. D., Kim, N. D., Luster, A. D., 2011: Neutrophils cascading their way to inflammation. *Trends Immunol.,* 32, 452—460.

35. Sharma, M. D., Hou, D. Y., Liu, Y., Koni, P. A., Metz, R., Chandler, P., et al., 2009: Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumour-draining lymph nodes. *Blood*, 113, 6102—6111.

36. Schreiber, R. D., Old, L. J., Smyth, M. J., 2011: Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science,* 331, 1565—70.

37. Smyth, M. J., Dunn, G. P., Schreiber, R. D., 2006: Cancer immune surveillance and immunoediting: the roles of immunity in suppressing tumour development and shaping tumour immunogenicity. *Adv. Immunol.,* 90, 1—50.

38. Swann, J. B., Smyth, M. J., 2007: Immune surveillance of tumours. *J. Clin. Invest.,* 117, 1137—1146.

39. Takahashi, H., Ogata, H., Nishigaki, R., Broide, D. H., Karin, M., 2010: Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cel.,* 17, 89—97.

40. Willis, R. A., 1948: *Pathology of Tumours*. xxiii + 992 pp. Remove from marked Records Pathology of tumours. CAB Direct.

41. Zitvogel, L., Kepp, O., Aymeric, L., Ma, Y., Locher, C., Delahaye, N. F., et al., 2010: Integration of host-related signatures with cancer cell-derived predictors for the optimal management of anticancer chemotherapy. *Cancer Res.,* 70, 9538—9543.

Received June 26, 2014

FOLIA VETERINARIA, 58, 3: 169—174, 2014

EFFECTS OF PIRIMICARB ON CELL PROLIFERATION IN IMMATURE 3D CELL CULTURES

Petrovová, E.[1], Luptáková, L.[2], Krešáková, L.[1], Maloveská, M.[1] Vdoviaková, K.[1], Elias, M. J.[3]

[1]Department of Anatomy, Histology and Physiology
[2]Department of Biology and Genetics
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic
[3]Faculty of Veterinary Medicine, Eduardo Mondlane University, Maputo
Mozambique

eva.petrovova@uvlf.sk

ABSTRACT

In the past decades, carbamate pesticides have been extensively used in agriculture on a worldwide basis. They are considered xenobiotics, acting on the central nervous system without a tendency to accumulate in the body. The central nervous system is a frequent target of the toxic action of various pharmaceutical preparations and dangerous substances which may be released from industry and/or the environment. The aim of this study was to investigate the neurotoxicity of pirimicarb at three different doses, focusing on the effects of this pesticide on the proliferation of immature cells of the rat brain *in vitro*. We observed changes in mitotic activity of nerve cells by means of labelling with radioactive thymidine and by gene expression of the specific proliferation marker Ki67. The experiments included also measurements of the intracellular activity of lactate dehydrogenase (LDH) as a reliable indicator of cellular integrity and viability. The results obtained *in vitro* indicated the moderate developmental neurotoxicity of pirimicarb. The incorporated radioactive thymidine as well as gene expression of the proliferation marker Ki67, indicated significantly decreased mitotic activity of the cells at the highest concentration of pirimicarb (10^{-3} mol.l^{-1}). However, according to the measured lactate dehydrogenase (LDH) activity level, the total number of cells did not change significantly.

Key words: embryonic rat brain, gene expression, neurotoxicity, pirimicarb, proliferation

INTRODUCTION

Original or synthesized toxic substances may cause irreversible damages to the nervous tissue or even death of nerve cells [7]. Investigations of a wide range of substances with neurotoxic potential requires the use of *in vitro* methods which combine organ-specificities with robustness and high reproducibility. Aggregating brain cell cultures (AGGR) obtained by mechanical dissociation of the rat embryonic brain fulfil all these conditions. These AGGR have been extensively characterized over three decades with respect to the normal developmental events. It was the first neural cell culture to be grown in a chemically defined medium [10]. The dissociated cells were kept in suspension for several weeks by rotational mixing. In this way, spherical aggregates were formed spontaneously and individual cells in these aggregates regrouped and maturated. Important morphogenetic changes, such as migration, proliferation, differentiation, synaptogenesis and myelinisation occurred in parallel [12]. Individual cell types found in original tissues are also present in these aggregates. This applies to nerve stem cells, nerve progenitor cells, immature neurons and glial and microglial cells. The AGGR acquire organ-specific functions, for example, activity-dependent consumption of glucose, spontaneous electric activity and brain-specific inflammatory responses. Such 3D cell cultures can therefore serve as a unique model for observation of neurotoxicity during the development as well as during advanced cell differentiation [9]. Therefore, serum-free AGGR were included in the ACuteTox program as a representative model system for the detection of organ- and CNS-specific toxicants. These three-dimensional

cell cultures have been shown to reach a highly differentiated phenotype that is maintained for at least two months [23]. AGGR have been used successfully for neurotoxicological investigations, e.g., the study of the neurotoxicity of heavy metals [22], neuron-specific toxicants [10], [11], organophosphorous compounds [21], mycotoxins, and ammonia [2], as well as, to investigate developmental neurotoxicity [6].

Pirimicarb (2-dimethylamino-5,6-dimethyl-4-pirimidinyldimethylcarbamate) as an anti-cholinesterase pesticide [17] is an important carbamate used in agriculture mostly to control aphids. Pirimicarb is classified by the U. S. EPA as "likely to be carcinogenic to humans". It has been commonly detected in food surveys [14]. Pirimicarb has been generally recognized as a nongenotoxic in bacteria, yeast, fungi, and mammalian cells [3]. Whereas, the pathways underlying the adverse effects at its cytotoxic concentrations on the development of the nervous system, remains unknown, we have used aggregating brain cell cultures in pirimicarb developmental neurotoxicity testing.

For the time being, the most attractive approach is the use of human stem cells. However, despite the extraordinary recent progress in this domain, numerous problems persist to mimic the complex spatial and temporal niche signalling for the maintenance of stem cells, to direct stem cells to acquire the differentiated phenotypes of various progenitor cells, and to direct them to form functional tissues [5].

Lifelong neurogenesis in vertebrates relies on stem cells producing proliferation zones that contain neuronal precursors with distinct fates [8]. In the mammalian brain, proliferation is a typical feature especially of immature nerve cells. This ability of cells decreases dramatically with their maturation (down to 5 % of their original proliferation activity) when the cell proliferation is limited only to mature nerve stem cells and their progeny [20].

The aim of this study was to investigate the neurotoxicity of pirimicarb at three different doses (10^{-3} mol.l^{-1}, 10^{-4} mol.l^{-1}, 10^{-5} mol.l^{-1}), focusing on the effects of this pesticide on the proliferation of immature cells of the rat brain *in vitro*.

MATERIALS AND METHODS

The methods used in this study allow one to detect parameters important for the monitoring of the influence of toxic substances during critical development periods.

Preparation of aggregate cell cultures for the testing of toxicity of xenobiotics

The dissociated cells prepared from the brains of 16-day embryonic rats re-aggregate spontaneously into even-sized spheroids, which are kept in serum-free Dulbecco's modified Eagle's medium (DMEM) and incubated (gyratory agitation; 68 rpm, 10 % CO_2, 90 % humidified air, 37 °C; Fig. 1).

For the three independent experiments, randomized immature (days 6 and 7 of cultivation *in vitro*, DIV 6—7) replicate cultures (200—300 aggregates in each replicate culture; Fig. 2) were taken to test acute toxicity of pirimicarb. For the 24 and 48 hours of exposures, triplicates were taken for each pirimicarb concentration (10^{-3} mol.l^{-1},10^{-4} mol.l^{-1}, 10^{-5} mol.l^{-1}) and solvent controls.

For the preparation of the replicate cultures, the free-floating aggregates contained in six original flasks were pooled and redistributed in 1.5 ml aliquots to 25 ml culture flasks containing 2.5 ml of fresh pre-equilibrated culture medium. Thus, each replicate culture contained an average of 150—200 aggregates (about 20 µg of total RNA) in 4 ml of culture medium. After their preparation, replicate cultures were equilibrated for 2 hours under normal culture conditions prior to the addition of the pirimicarb [23]. The experiment was carried out on 24 replicate cell cultures exposed to three different subcytotoxic concentrations of pirimicarb (10^{-3} mol.l^{-1}, 10^{-4} mol.l^{-1}, 10^{-5} mol.l^{-1}). We used three replicate cultures for one concentration of pirimicarb in one experiment. The experiments were repeated 3 times. Six control replicate cell cultures (CTR) per experiment were exposed to 0.1 % ethanol, which was used as a solvent in preparation of the basic solution of pirimicarb. Both pirimicarb and 0.1 % ethanol were applied to cells on day 5 of cultivation and the subsequent analyses were performed on days 6 (DIV 6) and 7 (DIV 7). After the application, the cell cultures were maintained under standard culturing conditions.

Measurement of mitotic activity

DNA synthesis was observed by means of metabolic labelling with radioactive thymidine [^{14}C-methyl]thymidine (New England Nuclear-DuPont, USA). Subsequent radioactivity was measured using a modified filtration technique of Mans and Novelly [13] and trichloroacetic acid (Sigma-Aldrich, USA). Radiation intensity was measured by a liquid scintillation counter (Tri-Carb 3110TR, PerkinElmer, USA) and the results were expressed in disintegrations per minute (DPM) per µg of protein per hour.

Measurement of LDH activity

LDH catalyses transformation of pyruvate to lactate and oxidation of NADH to NAD^+ takes place in parallel. The absorbance measured by a spectrophotometer is directly proportional to the activity of LDH. Homogenised samples of cell cultures (DIV 6 and DIV 7) were diluted with a solution containing 50 nM Tris-HCl (pH 7.4), 75 mM pyruvate and 12 mM NADH, and analysed by a spectrophotometer Spectro Beckmann DU 640 at 350 nm. The absorbances of LDH activity were recalculated per total amount of proteins (mg) in each cultivation bottle.

Gene expression of specific markers

The RNA was extracted from each cell culture replicate by means of QIAshredder and RNeasy kits (Qiagen, Switzerland). For quantification of RNA we used a spectrophotometer NanoDrop ND-1000 and subsequent reverse transcription by means of a High Capacity cDNA kit (Life Technologies, USA). The PCR analyses were performed on 3.2 ng cDNA per one well, in a total volume of 10 µl, using the following PCR mixture of contained primers (150—300 nM; see Table 1), SYBR Green PCR master mix (Life Technologies, USA), and a Taqman probe (Life Technologies, USA) Ki67: Rn01451446.m1^{-1}.

Statistical analysis

Data, from minimally two independent experiments, were processed statistically by the one-way ANOVA test and Dunnett's comparison test using software GraphPad Prism 6.0.

Table 1. Primer sequences of selected markers

Gene	Forward (5´-3´)	Reverse (5´-3´)
NF-H	CAGGACCTGCTCAACGTCAA	CTTCGCCTTCCAGGAGTTTTCT
GFAP	CCTTGACCTGCGACCTTGAG	GCGCATTTGCCTCTCCAA
MBP	GCACGCTTTCCAAAATCTTTAAG	AGGGAGGCTCTCAGCGTCTT
HSP32	AGGTGTCCAGGGAAGGCTTT	TCCAGGGCCGTATAGATATGGT

NF-H — (Neurofilament Heavy Chain); GFAP — (Glial Fibrillary Acid Protein)
MBP — (Myelin Basic Protein); HSP 32 — (Heat Shock Protein)

RESULTS AND DISCUSSION

After application of pirimicarb, 3D cultures of immature cells obtained from 16-day old rat embryos were labelled with radioactive thymidine. Its incorporation offers a sensitive and reliable method for quantification of mitotic activity (proliferation) of cells. We observed a significant decrease in proliferation activity of the cells exposed to E-3 M pirimicarb. The two lower concentrations tested (10^{-4} mol.l^{-1} and 10^{-5} mol.l^{-1}) elicited no changes in the DNA synthesis (Fig. 3). The results of the mitotic activity were obtained by the employed method based on the incorporation of radioactive thymidine (Fig. 3).

Decreased proliferation activity of nerve cells was indicated also by decreased gene expression of the specific proliferation marker Ki67 (Fig. 4). The proliferation activity decreased with increasing concentration of pesticide and the decrease was significant at concentration E-3 M.

The measurement of intracellular LDH activity allowed us to observe the total number of cells at individual concentrations of pirimicarb. The lowest LDH activity was measured at a concentration of 10^{-3} mol.l^{-1}. The other two tested concentrations of pirimicarb produced no changes in total number of cells and thus their levels of LDH activity did not differ from those of the control cells (Fig. 5).

In our study, we also used specific biomarkers in order to observe the developmental neurotoxicity of pirimicarb; to individual types of nerve cells (neurons, astrocytes, oligodendrocytes; Table 2), to cell proliferation, and on cell stress factor. The measurement of gene expression at the level of mRNA by means of quantitative RT-PCR offers reliable markers for the detection of adverse effects of subcytotoxic

Table 2. Pirimicarb-induced changes in neural cell-relevant mRNA expression in immature 3D brain cell cultures

	Relative mRNA expression of neural cells specific markers							
	24 h				48 h			
Gene	CTR	E-5 M	E-4 M	E-3 M	CTR	E-5 M	E-4 M	E-3 M
NF-H	1.00 ± 0.07	0.96 ± 0.06	0.89 ± 0.10	0.96 ± 0.06	1.00 ± 0.04	1.09 ± 0.08	0.95 ± 0.07	1.27 ± 0.08
GFAP	1.00 ± 0.07	1.35 ± 0.20	1.40 ± 0.26	1.88 ± 0.31	1.00 ± 0.06	1.22 ± 0.15	1.61 ± 0.22	3.28 ± 0.45
MBP	1.00 ± 0.10	0.92 ± 0.02	0.92 ± 0.08	1.00 ± 0.13	1.00 ± 0.04	0.86 ± 0.16	0.96 ± 0.09	1.44 ± 0.18
HSP32	1.00 ± 0.08	1.02 ± 0.08	1.03 ± 0.09	1.41 ± 0.07	1.00 ± 0.01	1.60 ± 0.27	1.45 ± 0.26	2.10 ± 0.16
Ki67	1.00 ± 0.09	0.97 ± 0.08	0.81 ± 0.09	0.36 ± 0.03	1.00 ± 0.08	1.16 ± 0.06	1.09 ± 0.05	0.34 ± 0.04

CTR — Control; E-5 M — (10^{-5} mol.l^{-1}); E-4 M — (10^{-4} mol.l^{-1}); E-3 M — (10^{-3} mol.l^{-1})

Fig. 1. Cultivation of aggregating brain cell cultures

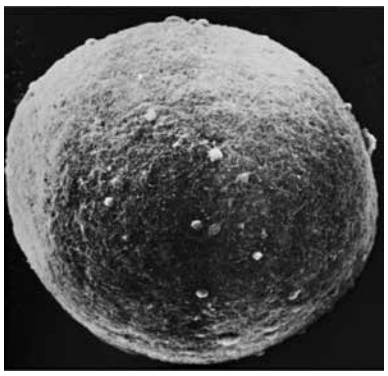

Fig. 2. Brain cell aggregate (200 μm)

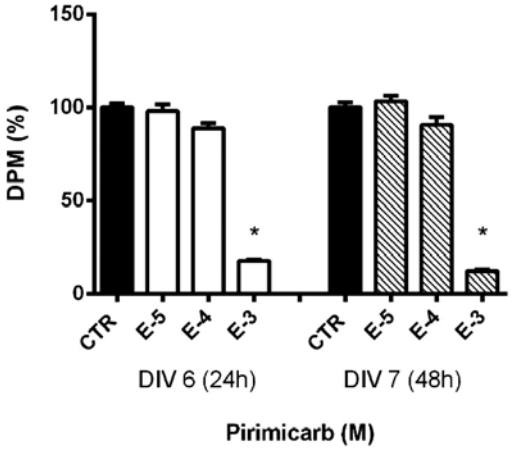

Fig. 3. Proliferation detected by the radiolabelled thymidine. Significant decrease of proliferation is marked with an asterisk. The data were expressed as disintegration per minute (DPM; μg protein^{-1} h^{-1})

CTR (control); E-5 M (10^{-5} mol.l^{-1}); E-4 M (10^{-4} mol.l^{-1})
E-3 M (10^{-3} mol.l^{-1}); DIV (cultivation days *in vitro*)

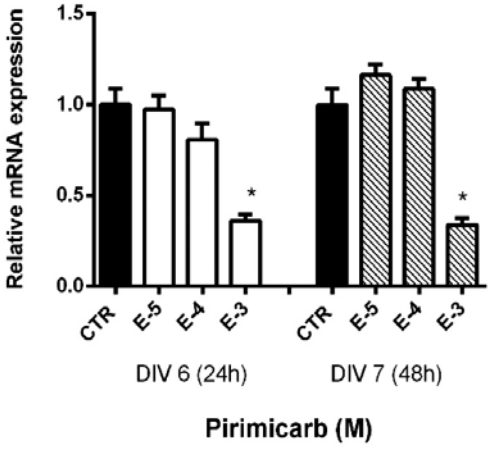

Fig. 4. Relative mRNA expression of proliferation marker Ki67. Significant decrease of proliferation is marked with an asterisk

CTR (control); E-5 M (10^{-5} mol.l^{-1}); E-4 M (10^{-4} mol.l^{-1})
E-3 M (10^{-3} mol.l^{-1}); DIV (cultivation days *in vitro*)

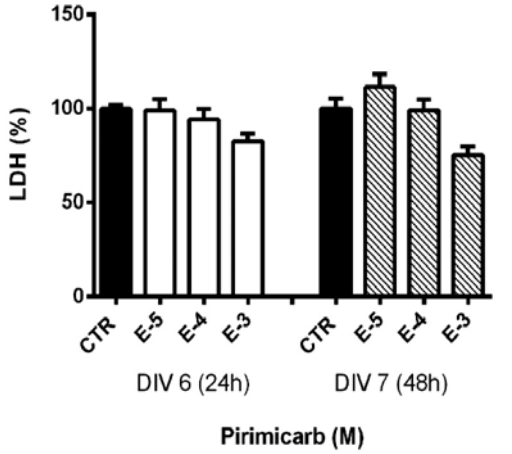

Fig. 5. Detection of the cell death by the LDH activity measurement

CTR (control); LDH (lactate dehydrogenase); E-5 M (10^{-5} mol.l^{-1})
E-4 M (10^{-4} mol.l^{-1}); E-3 M (10^{-3} mol.l^{-1}); DIV (cultivation days *in vitro*)

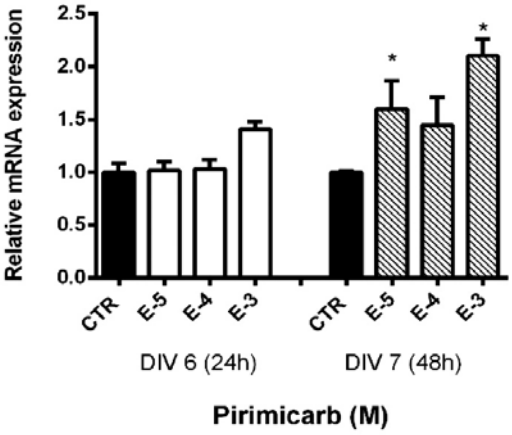

Fig. 6. Relative mRNA expression of cell stress marker HSP 32. Significant decrease of proliferation is marked with an asterisk

CTR (control); E-5 M (10^{-5} mol.l^{-1}); E-4 M (10^{-4} mol.l^{-1}); E-3 M (10^{-3} mol.l^{-1}); DIV (cultivation days *in vitro*)

concentrations of xenobiotics. Changes in gene expression of specific marker for neurons (NF-H) were not observed. Astrogliosis (increased gene expression of specific protein in astrocytes; GFAP) was observed in astrocytes at the highest concentration of pirimicarb as a proof of toxic action of pesticides on the central nervous system, however, without direct action on astrocytes. Expression of a specific indicator for oligodendrocytes (MBP) was increased only at 10^{-3} M concentration and only 48 hours after application of pirimicarb. The expression of cell stress factor (HSP32) after 24-hour exposure was significantly increased only at the highest concentration of pirimicarb, but after 48 hours there was an increase also at the lower concentrations. Thus the immature brain cells were more stressed by the longer action of pesticide (Fig. 6).

Soleneski and Laramendy [16] observed cytotoxic and genotoxic action of pirimicarb on Chinese hamster ovary cells (CHO-K1). Pirimicarb did not induce chromosomal alterations in rat bone marrow cells after oral administration. But an increased frequency of chromosomal aberrations in the peripheral lymphocytes from occupational exposed workers was observed [3]. However, the mechanism of action of pirimicarb at subcytotoxic concentrations during development of the nervous system remains unknown [19].

In vitro methods were developed for the initial identification of potentially neurotoxic substances. Aggregated cell cultures from the brain of rat embryos are suitable for the detection and study of the influence of neurotoxic substances with regard to their developmental, acute and chronic neurotoxicity [4]. An advantage of 3D cell cultures include their high reproduction ability and the possibility of simultaneous evaluation of several parameters of developmental neurotoxicity.

Observation of cell proliferation is an important experimental tool. The methods most frequently used for this purpose include incorporation of radioactive thymidine or labelling with bromodeoxyuridine (BrdU) in the S-phase of the cell cycle [18]. The cytosolic enzyme lactate dehydrogenase is a reliable indicator of cell integrity and viability. Photometric determination of intracellular LDH activity was selected to observe the number of nerve cells at three concentrations of pirimicarb and the results measured were directly proportional to intracellular LDH activity. Generally, when measuring the LDH activity, attention is not paid to differences between individual cells of the nervous system, although the specificity of enzymes within brain cells has been recognised [15].

The measurement of gene expression at the level of mRNA by quantitative RT-PCR offers reliable markers for the detection of adverse influence of subcytotoxic concentrations of antibiotics. In order to detect the influence of pirimicarb on cell proliferation of 3D cell cultures, we observed the expression of Ki67 antigen. This specific marker of cell proliferation is a non-histonic nuclear protein expressed during the active phases of the cell cycle, but absent during the quiescent (G0) phase [1].

CONCLUSIONS

The results of our *in vitro* study indicate a moderate dose-dependent developmental neurotoxicity of pirimicarb, particularly after 48-hours of exposure. Pirimicarb exposure reduces cell proliferation, as detected both by the radiolabelled thymidine and endogenous marker of the cell cycle Ki67 at the highest concentration of E-3 M. Correspondingly, the induction of the stress protein HSP32 was observed after 48-hour exposure. The LDH activity measurements showed the lowest number of cells at this concentration, but the difference was insignificant compared to the control. The increase in expression of glial fibrillary acidic protein (GFAP) may be due to an indirect effect of pirimicarb on astrocytes. But we used higher concentrations ($> 2.38\ mg.kg^{-1}$), therefore the acceptable daily intake of pirimicarb (ADI, $0.02\ mg.kg^{-1}$) seems to be safe for the developing nervous system.

ACKNOWLEDGEMENT

This study was conducted in collaboration with the Department of physiology of the University in Lausanne (Switzerland), and was supported by the organization Centre for Alternatives to Animal Testing, Baltimore (USA) and the project MŠ SR VEGA 1/0111/13.

REFERENCES

1. Bartoš, V., Kullová, M., 2012: Contribution to proliferation activity as a histologic and prognostic parameter of malignant skin melanoma (In Slovak). *Dermatological Practice* (Dermatologická prax), 6, 145—147.

2. Cagnon, L. Braissant, O., 2009: CNTF protects oligodendrocytes from ammonia toxicity: intracellular signalling pathways involved. *Neurobiol. Dis.*, 33, 133—42.

3. Candioti, J. V., Natale, G. S., Soloneski, S., Ronco, A. E., Larramendy, M. L., 2010: Sublethal and lethal effects on RhinellaArenarum (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida. *Chemosphere*, 78, 249—255.

4. Defaux, A., Zurich, M. G., Honegger, P., Monnet-Tschudi, F., 2010: Inflammatory responses in aggregating rat brain cell cultures subjected to different demyelinating conditions. *Brain Res.*, 1353, 213—224.

5. Daley, G. Q., Scadden, D. T., 2008: Prospects for stem cell-based therapy. *Cell*, 132, 544—8.

6. Eskes, C., Honegger, P., Jones-Lepp, T., Varner, K., Matthieu, J. M., Monnet-Tschudi. F., 1999: Neurotoxicity of dibutyltin in aggregating brain cell cultures. *Toxicology in Vitro*, 13, 555—560.

7. Grandjean, P., Landrigan. P., 2006: Developmental neurotoxicity of industrial chemicals. *Lancet*, 368, 2167—2178.

8. Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., Brand, M., 2006: Neural stem cells and neurogenesis in the adult zebra fish brain: Origin, proliferation dynamics, migration and cell fate. *Developmental Biology*, 295, 263—277.

9. Honegger, P., Defaux, A., Monnet-Tschudi, F., Zurich, M. G., 2011: Preparation, maintenance, and use of serum-free aggregating brain cell cultures. In **Costa, L. G., Giordano, G., Guizzetti, M.,** (Eds.)**:** *In vitro Neurotoxicology*, Humana Press, London, UK, 81—97.

10. Honegger, P., Lenoir, D., Favrod, P., 1979: Growth and differentiation of aggregating foetal brain cells in a serum-free defined medium. *Nature*, 282, 305—308.

11. Honegger, P., Pardo, B., Monnet-Tschudi, F., 1998: Muscimol-induced death of GABAergic neurons in rat brain aggregating cell cultures. *Developmental Brain Research*, 105, 219—225.

12. Honegger, P., Zurich, M. G., 2011: Preparation and use of serum-free aggregating brain cell cultures for routine neurotoxicity screening. In **Aschner, M., Sunol, C., Bal-Price, A.,** (Eds.): *Cell Culture Techniques*, Humana Press, London, UK, 105—128.

13. Mans, R. J., Novelly, G. D., 1961: Measurement of the incorporation of radioactive amino acids into proteins by a filter paper disk method. *Arch. Biochem*., 94, 48—53.

14. Oliveira, T. M., Barroso, M. F., Morais, S., de Lima-Neto, P., Correia, A. N., Oliveira, M. B., Delerue-Matos, C., 2013: Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification. *Talanta,* 106, 137—143.

15. Renner, M., Zurich, M. G., Kopp-Schneider, A., 2013: Stochastic time-concentration activity models for cytotoxicity in 3D brain cell cultures. *Theoretical Biology and Medical Modelling*, 10, 19.

16. Soleneski, S., Larramendy, M. L., 2010: Sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO-K1) cells treated with the insecticide pirimicarb. *J. Hazard Mater.*, 174, 410—415.

17. Wang, P., Wang, H. P., Xu, M. Y., Liang, Y. J., Sun, Y. J., Yang, L., et al., 2014: Combined subchronic toxicity of dichlorvos with malation or pirimicarb in mice liver and serum: A metabonomic study. *Food and Chemical Toxicology*, 70, 222—230.

18. Warren, M., Puskarczyk, K., Chapman, S. C., 2009: Chick embryo proliferation studies using EdU labelling. *Developmental Dynamics*, 238, 944—949.

19. Zhang, G., Hu, X., Pan, J., 2011: Spectroscopic studies of the interaction between pirimicarb and calf thymus DNA. *Spectrochimica Acta,* Part A, 78, 687—694.

20. Zurich, M. G., Honegger, P., 2011: Ochratoxin A at nanomolar concentration perturbs the homeostasis of neural stem cells in highly differentiated but not in immature three-dimensional brain cell cultures. *Toxicol. Letters*, 205, 203—208.

21. Zurich, M. G., Honegger, P., Schilter, B., Costa, L. G., Monnet-Tschudi, F., 2004: Involvement of glial cells in the neurotoxicity of parathion and chlorpyrifos. *Toxicol. Appl. Pharmacol.*, 201, 97—104.

22. Zurich, M. G., Monnet-Tschudi, F., Honegger, P., 1994: Long-term treatment of aggregating brain cell cultures with low concentrations of lead acetate. *Neurotoxicology*, 15, 715—720.

23. Zurich, M. G., Stanzel, S., Kopp-Schneider, A., Prieto, P., Honegger, P., 2013: Evaluation of aggregating brain cell cultures for the detection of acute organ-specific toxicity. *Toxicol. In Vitro*, 27, 1416—1424.

Received June 27, 20014

FOLIA VETERINARIA, 58, 3: 175—178, 2014

DETECTION OF *BLASTOCYSTIS HOMINIS* SUBTYPE 3 IN THE BROWN BEAR IN THE SLOVAK REPUBLIC

Valenčáková, A., Kandráčová, P., Kalinová, J. Danišová, O., Hasajová, A.

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

alexandra.valencakova@uvlf.sk

ABSTRACT

Wild carnivores can be involved in the epidemiology of many important diseases and often act as reservoirs of pathogens for domestic animals and humans. We investigated the role of the brown bear (*Ursus arctos*) in the circulation of *Blastocystis* spp. *Blastocystis* spp. is an important enteric pathogen which can cause clinical symptoms ranging from mild to severe diarrhoea and dehydration in immunocompromised individuals. Sixteen faecal samples collected in Poloniny National Park were examined qualitatively for the presence of *Blastocystis* spp. antigens in the faeces by nested PCR. Overall, 50 % (8/16) of the faecal samples from brown bears were positive. We identified *Blastocystis hominis* subtype 3 (ST3). Current data suggest that subtype 3 is the only subtype of human origin and, therefore, is the true *B. hominis*. Our preliminary results revealed the prevalence of *B. hominis* among brown bears in Slovakia and the potential risk of transmission to humans who use the countryside for professional or recreational purposes.

Key words: *Blastocystis hominis;* brown bear (*Ursus arctos*), nested PCR; Slovakia

INTRODUCTION

Poloniny National Park is the least populated national park in Slovakia. It is situated in the East Carpathian Biosphere Reserve, which is positioned near the boundaries of Poland, Ukraine and Slovakia. Poloniny National Park is the territory with the highest occurrence of large forest animals, such as the brown bear. The number of brown bears (*Ursus arctos*) living in Slovakia is unknown, though it will probably not exceed 750. The East Carpathian population of brown bears lives in the northeast area of Slovakia (Low Beskyds and Bukovske hills), where it inhabits coherent coniferous and mixed forests at an altitude of 700—1500 m. Brown bear resides in an area of about 1,000 hectares, which represents 23 % of the territory of Slovakia [5].

Nowadays bears are threatened by the environmental changes caused by humans. The territories of the bears are constantly divided and shrinking which accelerates their loss of habitat. These changes result in economic damage, risk for the people and possible transfer of zoonotic diseases from sylvatic and synanthropic foci. Bears are, despite their zoological classification, omnivores and parts of their diet are rodents, small vertebrates, fish and carrion. Some bears can prey upon sheep from sheep-folds, and goats or heifers from peripheral barnyards. The possibility of the infectious transfer between predator and prey exists as well as the potential spread of these infections in the environment.

This study was aimed to detect human origin of *Blastocystis* spp., particularly *Blastocystis hominis* in the faeces of brown bears. Recent epidemiological studies and *in vivo* and *in vitro* data have shown that *Blastocystis* spp. is a pathogenic micro-organism. Subtypes ST1 and ST3 of *Blastocystis hominis* are pathogenic for humans, but clinical symptoms were also described in infections caused by subtypes ST2 and ST4. *Blastocystis* spp. causes infections

with a wide variety of clinical symptoms, from intestinal symptoms (abdominal pain, watery diarrhoea, bloating and weight loss) to skin lesions such as urticaria. These pathogens are often detected in patients with irritable bowel syndrome (IBS), in which subtypes ST5, 6, 7 and 8 were also identified. *Blastocystis* spp. can play an important role in the pathogenesis of inflammatory bowel disease (IBD) and its most common types — Crohn's disease and ulcerative colitis [4], [15].

The aim of this study was to investigate the role of the brown bear (*Ursus arctos*) in the circulation of *Blastocystis* spp. in Poloniny National Park in Slovakia by examining brown bear faeces for the presence of *Blastocystis* spp. antigens by nested PCR.

MATERIALS AND METHODS

Sixteen samples of bear faeces that were collected in Poloniny National Park, in Eastern Slovakia, were examined with Nested PCR for the presence of the protozoan parasite *Blastocystis hominis*. Extraction of the pathogen was done by the Sheather's flotation method [16].

DNA isolation

For DNA isolation, we used the commercial DNA-sorb-B Nucleic acid Extraction kit (AmpliSens). Prior to the isolation, samples with higher densities were diluted with distilled water. Into each sample (0.1 ml) we added 300 µl of Lysis Solution and 0.5 mm of glass beads. Subsequently, homogenization was performed at 6 500 rpm for 90 seconds with the use of a homogenizer Bertin-Precellys 24. After homogenization we proceeded according to the manufacturer's manual. Isolated DNA was stored at –16 °C until the PCR analysis.

Nested PCR

DNA isolates were processed by the nested PCR method, for which we used two pairs of primers NDIAG2R/F and CPB-DIAGR/F [6]. The outer primer pair amplifies fragments 658—662 bp in length. By the second PCR reaction, where the template was the primary PCR product, we amplified fragments of length 435—450 bp.

The total reaction volume was 50 µl for both, primary and secondary PCR reactions. Each reaction contained: 1× Buffer B, PCR H_2O, 25 mM $MgCl_2$, 10 m MdNTPs, 10 pmol primers, and FIREPol DNA Polymerase 5 $U.\mu l^{-1}$. To the first PCR reaction mix, we added a DNA isolate and to the second PCR mix we added the primary PCR product.

The first PCR reaction was implemented at an annealing temperature of 60 °C and the second reaction at 55 °C in a thermocycler XP Thermal Cycler Blocks.

Electrophoresis and sequencing

The final PCR product was loaded on a 1.5 % agarose gel stained with Red gel. Then the positive PCR products were sent for sequencing and the results were compared with the sequences in Gen Bank database in NCBI using BLAST programme.

RESULTS

The Nested PCR analysis of sixteen examined samples showed 50 % positivity. The eight positive PCR products were sent for sequencing and the results were compared with the sequences in the Gen Bank database in NCBI using the BLAST programme. We identified *Blastocystis hominis* subtype 3 (ST3), deposited under accession number HQ909889.1 in the Gen Bank. Current data suggest that subtype 3 is the only subtype of human origin and, therefore, it is the true *Blastocystis hominis*.

DISCUSSION

Blastocystis spp. has a worldwide distribution with higher numbers being found in developing countries probably due to poor sanitation [8].

Up to 17 subtypes have been described with subtype (ST) 1—9 being found in humans [10]. ST3 is the predominant ST found in most human epidemiological studies [11], [15, [16]. Recent surveys incorporated genotype information by PCR of *Blastocystis* DNA from the faeces or from stool cultures. Such studies are now shedding light on the distributions of genotypes among human populations and animal hosts and also provide information on transmission routes or sources.

A study by Yoshikawa et al. [19] employed the PCR-based genotype classification to study the distribution of *Blastocystis* genotypes among isolates from Bangladesh, Germany, Japan, Pakistan, and Thailand. The most dominant subtype among four populations (except Thailand) was subtype 3 (41.7 to 92.3 %), followed by either subtype 1 (7.7 to 25 %) or subtype 6 (10 to 22.9 %). Similar genotype distributions were reported in Singapore (78 % subtype 3 and 22 % subtype 1) [17], China (60.4 % subtype 3 and 24.5 % subtype 1) [7], Greece (60 % subtype 3 and 20 % subtype 1) [9], Germany (54 % subtype 3 and 21 % subtype 1) [3], and Turkey (75.9 % subtype 3) [12]. In most studies, other genotypes were identified at lower frequencies. Collectively, those studies suggest that subtype 3 is the subtype of human origin and that there is no correlation between *Blastocystis* geographic origin and genotype. Therefore, the proposed standardization of *Blastocystis* terminology to improve communication and correlate research results has been proposed. All mammalian and avian isolates are designated *Blastocystis* spp. and assigned to one of nine subtypes. The current data suggest that subtype 3 is the only subtype of human origin and, therefore, is the true *Blastocystis hominis*. In our study we identified only human origin 3 subtype *B. hominis* in the samples of faeces from the brown bear.

There are very few studies concerning *Blastocystis* spp. in wild animals. The role of the bears as a potential source of infection for people must be taken seriously because species found in bears are also pathogenic for humans. Collectively, there is an increasing body of evidence suggesting that *Blastocystis* is pathogenic or is an opportunistic pathogen, with immunocompromised populations being more susceptible to infection and the associated symptoms.

The literature describes cases of zoonotic transmission of parasites, including *Blastocystis* spp., mainly to wild primates in Great Britain or Nigeria, where authors reported a high prevalence of *Blastocystis* spp. (70 to 80 %), especially subtypes ST1, ST2 and ST3, in Mona monkeys, chimpanzees and gorillas [2], [8]. A higher prevalence (80—85 %) was found by the same authors in animals living in the Zoo. The prevalence of *Blastocystis* spp. in the brown bear has not been described, therefore we cannot compare our results with the results of other authors. Anyway, our results show a relatively high prevalence (50 %) in brown bears in Slovakia, thus making *B. hominis,* the pathogen that is important to focus on.

Due to the ubiquitous presence and wide host range of this parasite, it is important to assess the zoonotic potential of *Blastocystis* spp. Which is dependent on: the correct and precise detection of pathogen; identification and distinguishing between species and subspecies; understanding and analysis of various factors involved in the transmission, such as: the source of infection, transmission routes, infectivity of cysts, contact with faeces, contamination of soil, water and food, coprophagy and possible infection of animals and humans.

Species from the genus *Blastocystis* poses considerable challenges for the diagnostic laboratory. Firstly, the uncertain pathogenesis of the parasite discourages many clinicians from considering *Blastocystis* spp. to be the etiological agent of disease. Secondly, the polymorphic nature of the organism in wet mounts can result in confusion with the *Cyclospora* spp., yeast particules, or fat globules. The classical vacuolar forms may not predominate in fresh faecal specimens [14], while the smaller faecal cyst, when present, may be difficult to identify. Direct microscopy is not sufficient for diagnosis. Molecular PCR-based diagnostic approaches for *Blastocystis* spp. identification and genotyping have been described. Such an approach has been shown to be useful for epidemiological studies, providing information on the distribution of various genotypes among human and animal populations [1], [7], [18], [19] and on the zoonotic nature of certain genotypes. A method for the detection of *Blastocystis* spp. directly from unpreserved stool samples was described and provides a rapid diagnostic tool for *Blastocystis* spp. identification [13].

CONCLUSIONS

Our current knowledge of *Blastocystis hominis* and the putative disease it causes is insufficient to determine the significance of the parasite in humans. In the past 12 years our knowledge of this interesting parasite has increased tremendously. The term *B. hominis* is no longer applicable to all human isolates, since we now know that humans can be infected by numerous genotypes, many of which are zoonotic. Hence, laboratories should report the presence of the parasite in the animal samples as being *Blastocystis* spp. instead of *B. hominis.* A standardization of *Blastocystis* terminology to improve communication and correlate research results has been proposed. All mammalian and avian isolates are designated *Blastocystis* spp. and assigned to one of nine subtypes. The current data suggest that subtype 3 is the only subtype of human origin and, therefore, is the true *B. hominis*. There are limited studies of the molecular and cell biology of *Blastocystis* spp. Despite accumulating evidence indicating that the parasite is pathogenic and that proteases are involved in pathogenesis, not a single virulence factor gene has been identified, cloned, and characterized. As more studies are conducted, the roles of each *Blastocystis* subtype in disease will become clearer. A key research priority is to elucidate the pathogenic potential of the parasite and to understand its pathogenesis. Once these are clarified, there will be a surge in interest in other relevant aspects of *Blastocystis* spp. biology, including diagnosis and treatment.

The connection of *Blastocystis* spp. to severe bowel infections (IBS, IBD) in humans, though still unclear, confirms the pathogenicity of this parasite and can increase its clinical significance.

ACKNOWLEDGEMENTS

The submitted work was supported by the project VEGA No. 1/0063/13, funded by the Ministry of Education, Science Research and Sport of the Slovak Republic.

REFERENCES

1. Abe, N., 2004: Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. *Vet. Parasitol.*, 120, 235—242.

2. Alfellani, M. A., Jacob, A. S., Perea, N. O., Krecek, R. C., Taner-Mulla, D., Verweij, J. J. et al., 2013: Diversity and distribution of *Blastocystis* spp. subtypes in non-human primates. *Parasitology*, 140, 966—71.

3. Böhm-Gloning, B., Knobloch, J., Walderich, B., 1997: Five subgroups of *Blastocystis hominis* from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA. *Trop. Med. Int. Health,* 2, 771—778.

4. Engsbro, A. L., Stensvold, C. R., Nielsen, H. V., Bytzer, P., 2012: Treatment of *Dientamoeba fragilis* in patients with irritable bowel syndrome. *Am. J. Trop. Med. Hyg.*, 87, 1046—52.

5. Goldová, M., Ciberej, J., Rigg, R., 2003: Brown bear (*Ursus arctos*) and parasitic zoonoses (In Slovak). *Folia Venatoria*, 33, 123—126.

6. Johnson, D. W., Peniazek, N. J., Griffin, D. W., Misener, L., Rose, J. B., 1995: Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Appl. Environ. Microbiol.,* 61, 3849—3855.

7. Li, L. H., Zhang, X. P. L. S., Zhang, L., Yoshikawa, H., Wu, Z. et al., 2007: Cross-sectional surveys and subtype classification of human *Blastocystis* isolates from four epidemiological settings in China. *Parasitol. Res.,* 102, 83—90.

8. Mbaya, A. W., Udendeye, U. J., 2011: Gastrointestinal parasites of captive and free-roaming primates at the Afi Mountain Primate Conservation Area in Calabr, Nigeria and their zoonotic implications. *Pakistan Journal of Biological Sciences,* 14, 709—14.

9. Menounos, P. G., Spanakos, G., Tegos, N., Vassalos, C. M., Papadopoulou, C., Vakalis, N. C., 2008: Direct detection of *Blastocystis s*pp. in human faecal samples and subtype assignment using single strand conformational polymorphism and sequencing. *Mol. Cell. Probes*, 22, 24—29.

10. Navarro, C., Domínguez-Marquez, M. V., Garijo-Toledo, M. M., Vega-García, S., Fernandez-Barredo, S., Perez-Gracia, M. T. et al., 2008: High prevalence of *Blastocystis* spp. in pigs reared under intensive growing systems: frequency of ribotypes and associated risk factors. *Vet. Parasitol.*, 153, 347—358.

11. Noel, C., Dufernez, F., Gerbod, D., Edgcomb, V. P., Delgado-Viscogliosi, P., Ho, L. C. et al., 2005: Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *J. Clin. Microbiol.,* 43, 348—355.

12. Özyurt, M., Kurt, O., Mølbak, K., Nielsen, H. V., Haznedaroglu, T., Stensvold, C. R., 2008: Molecular epidemiology of *Blastocystis* infections in Turkey. *Parasitol. Int.,* 57, 300—306.

13. Stensvold, R., Brillowska-Dabrowska, A., Nielsen, H. V., Arendrup, M. C., 2006: Detection of *Blastocystis hominis* in unpreserved stool specimens by using polymerase chain reaction. *J. Parasitol.,* 92, 1081—1087.

14. Stenzel, D. J., Boreham, P. F., McDougall, R., 1991: Ultrastructure of *Blastocystis hominis* in human stool samples. *Int. J. Parasitol.,* 21, 807—812.

15. Tan, K. S., Mirza, H., Teo, J. D. W., Wu, B., MacAry, P. A., 2010: Current views on the clinical relevance of *Blastocystis* spp. *Curr. Infect. Dis. Rep.,* 12, 28—35.

16. Zajac, A. M., Conboy, G. A., 2012: Veterinary clinical parasitology. Wiley-Blackwell publishing, Iowa, 354 pp.

17. Wong, K. H., Ng, G. C., Lin, R. T., Yoshikawa, H., Taylor, M. B., Tan, K. S., 2008: Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. *Parasitol. Res.*, 102, 663—670.

18. Yoshikawa, H., Abe, N., Wu, Z., 2004: PCR-based identification of zoonotic isolates of *Blastocystis* from mammals and birds. *Microbiology*, 150, 1147—1151.

19. Yoshikawa, H., Wu, Z., Kimata, I., Iseki, M., Ali, I. K., Hossain, M. B., Zaman, V., Haque, R., Takahashi Y., 2004: Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitol. Res.*, 92, 22—29.

Received June 30, 2014

FOLIA VETERINARIA, 58, 3: 179—184, 2014

ANATOMICAL STUDY OF ARTERIAL BLOOD SUPPLY OF THE THORACOLUMBAR PART OF THE SPINAL CORD IN THE GUINEA PIG

Maženský, D.[1], Petrovová, E.[1], Supuka, P.[2]
Krešáková, L.[1],Supuková, A.[3]

[1]Department of Anatomy, Histology and Physiology
[2]Institute of Nutrition, Dietetics and Feed Production
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
[3]Institute of Experimental Medicine, Faculty of Medicine, UPJŠ Košice, Trieda SNP 1, 041 01 Košice
The Slovak Republic

mazenskyd@gmail.com

ABSTRACT

The aim of this study was to describe the arterial blood supply of the thoracolumbar spinal cord in guinea pigs. The study was carried out on twenty adult English self guinea pigs. The arterial system of the thoracolumbar spinal cord was injected by using Batson's corrosion casting kit no. 17. The branches entering the ventral spinal artery along the entire thoracic and lumbar spinal regions, we observed as left-sided in 63.8 % and as right-sided in 36.2 % of the cases. In 60 % of the cases, the artery of Adamkiewicz arose from the left fifth lumbar artery. In 30 % of the cases, the artery of Adamkiewicz originated from the fifth right-sided and fourth left-sided lumbar artery. In 10 % of the cases, the artery of Adamkiewicz originated from the right and left fifth lumbar arteries. The dorsal branches along the entire thoracic and lumbar spinal regions, were left-sided in 56.8 % of the cases and right-sided in 43.2 % of the cases. On the dorsal surface, we found two irregular longitudinal dorsal spinal arteries lying in the *sulcus lateralis dorsalis* bilaterally in 60 % of the cases and in 40 % of the cases there were present three irregular longitudinal dorsal spinal arteries receiving the dorsal branches of the *rami spinales*. Based on our results, we can conclude that there is a high variability of the blood supply in the thoracolumbar part of the spinal cord in guinea pigs.

Key words: artery of Adamkiewicz; dorsal branch; guinea pig; ventral spinal artery

INTRODUCTION

Laboratory animals have been used in several experimental studies of spinal cord damages. The more detailed knowledge of anatomy of the spinal cord blood supply with a focus on all possible variations can contribute to the protection of the spinal cord.

Guinea pigs, as laboratory animals, are frequently used in studies of the spinal cord ischemia injuries [5], [10], [11]. The arterial supply of the thoracolumbar part of the spinal cord in guinea pigs has been described in only a few studies [7], [16]. The description of the arterial pattern of the spinal cord has been described in several species of laboratory animals [15], [17], [18], [19] and in man [1], [13].

The aim of this study was to point to some variations in the segmental arterial supply of the spinal cord in the respective regions. We also focused on the spinal arteries supplying blood to the dorsal and ventral part of the spinal cord in the corresponding regions.

MATERIALS AND METHODS

This study was carried out on 20 adult guinea pigs (age 220 days).We used English self guinea pigs of both sexes (female n = 10; male n = 10) with an average weight of 0.8—1 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 a granular feed mixture (FANTASIA). Drinking water was available to all animals *ad libitum*. The

animals were injected intravenously with heparin (50 000 IU.kg^{-1}) 30 minutes before they were sacrificed by an intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}).

Immediately after killing, 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 in order to ensure an optimal injection distribution. Batson's corrosion casting kit No. 17 using a volume of 50 ml (Dione, Česke Budějovice, Czech Republic) was used as the casting medium. After polymerisation of the medium, 10 % formaldehyde was injected into the vertebral canal between the last lumbar vertebra and the sacrum, and between the last cervical and first thoracic vertebra to fix the spinal cord. After 1-week fixation, the vertebral canal was opened by removing the vertebral arches in the thoracic, lumbar and sacral spinal regions. The prepared spinal cord was fixed in 10 % formalin. This study was carried under authority decision No. 2647/07-221/5.

RESULTS

The rami spinales as branches arising from *arteriae intercostales dorsales* and *arteriae lumbales* entered the vertebral canal through the intervertebral foramen. Their entering was associated with the respective spinal nerve roots. The *rami spinales* were divided after entering the vertebral canal into

Table 1. Occurrence of the ventral branches of the arterial spinal branches in the thoracolumbar region of the spinal cord (dissection technique; ten guinea pigs)

Occurrence of arterial spinal branches (number of blood vessels)		
Level	**Right**	**Left**
Th 1	3	6
Th 2	3	3
Th 3	3	10
Th 4	0	3
Th 5	0	5
Th 6	5	5
Th 7	0	3
Th 8	3	6
Th 9	0	5
Th 10	5	5
Th 11	3	3
Th 12	0	3
L 1	0	0
L 2	0	9
L 3	3	0
L 4	6	3
L 5	5	5
L 6	5	0
L 7	3	9

L — lumbar segment of the spinal cord
Th — thoracic segment of the spinal cord

Table 2. Occurrence of the dorsal branches of the arterial spinal branches in the thoracolumbar region of the spinal cord (dissection technique; ten guinea pigs)

Occurrence of arterial spinal branches (number of blood vessels)		
Level	**Right**	**Left**
Th 1	5	5
Th 2	5	6
Th 3	3	3
Th 4	0	3
Th 5	3	5
Th 6	3	3
Th 7	3	5
Th 8	3	0
Th 9	1	3
Th 10	3	5
Th 11	3	9
Th 12	6	3
L 1	5	3
L 2	3	3
L 3	0	3
L 4	0	3
L 5	0	6
L 6	9	10
L7	3	10

L — lumbar segment of the spinal cord
Th — thoracic segment of the spinal cord

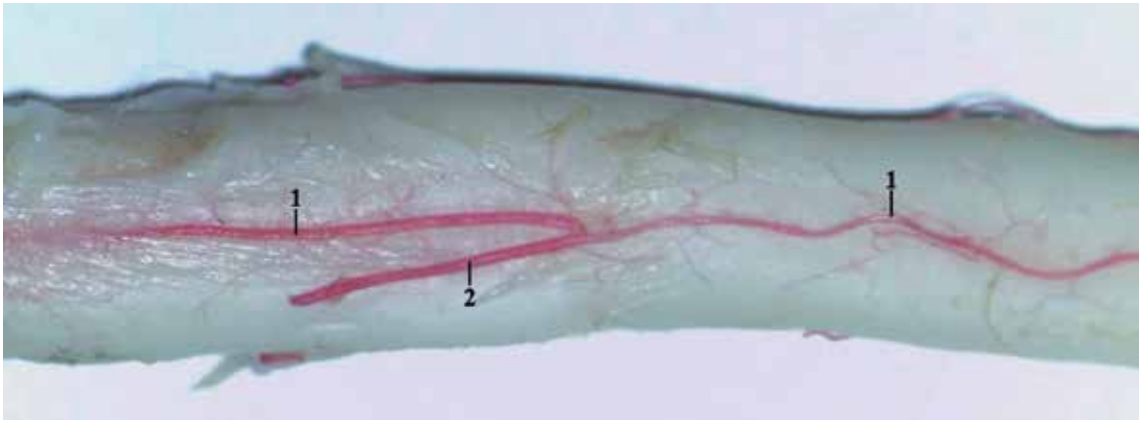

Fig. 1. Left-sided localization of the artery of Adamkiewicz
(1) *arteria spinalis ventralis;* (2) artery of Adamkiewicz. Ventral view
Magn. ×8

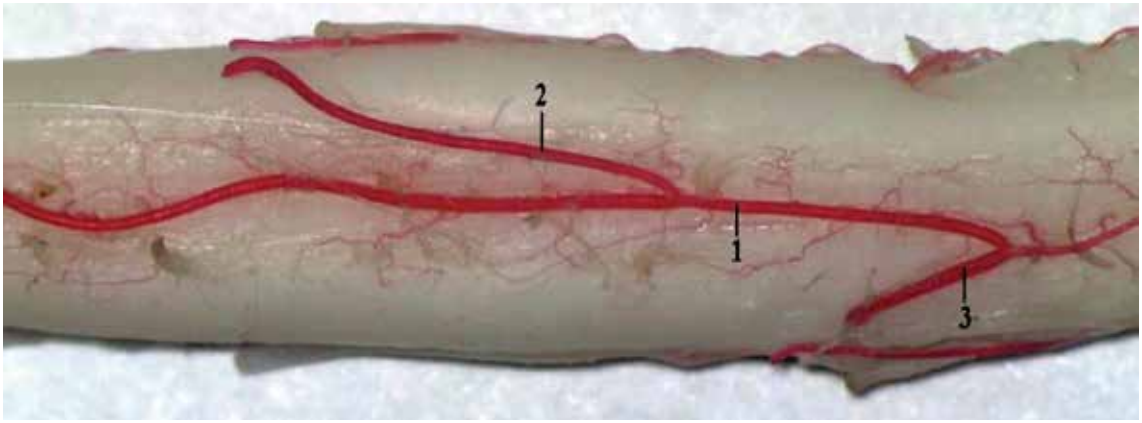

Fig. 2. Doubled artery of Adamkiewicz
(1) *arteria spinalis ventralis;* (2) right sided artery of Adamkiewicz; (3) left sided artery of Adamkiewicz
Ventral view. Magn ×8

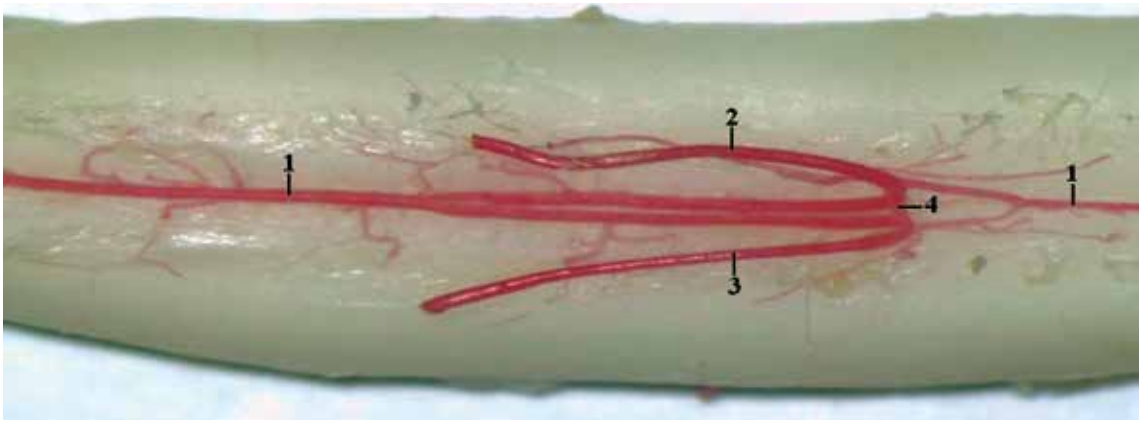

Fig. 3. Doubled artery of Adamkiewicz
(1) *arteria spinalis ventralis;* (2) right-sided artery of Adamkiewicz; (3) left-sided artery of Adamkiewicz; (4) communicating branch
Ventral view. Magn. ×12.5

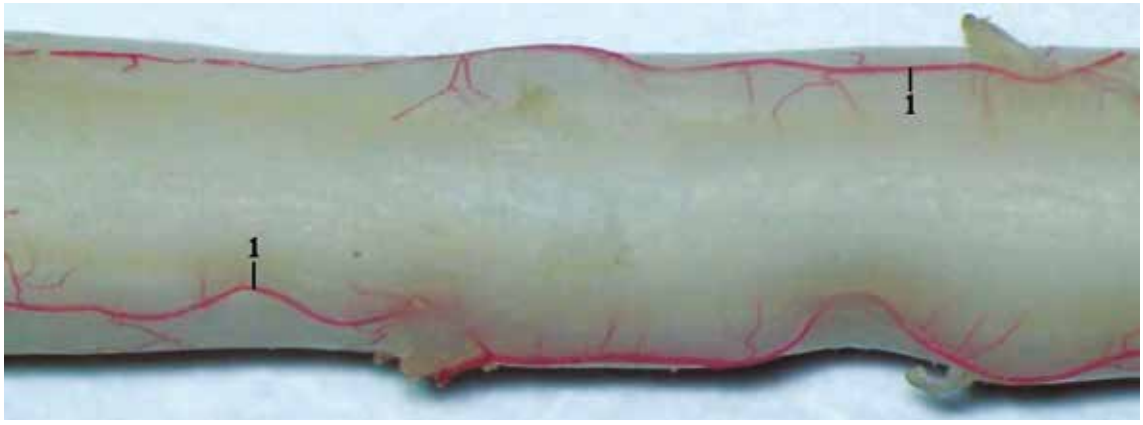

Fig. 4. Presence of two longitudinal dorsal spinal arteries
(1) dorsal spinal artery
Dorsal view. Magn. ×12.5

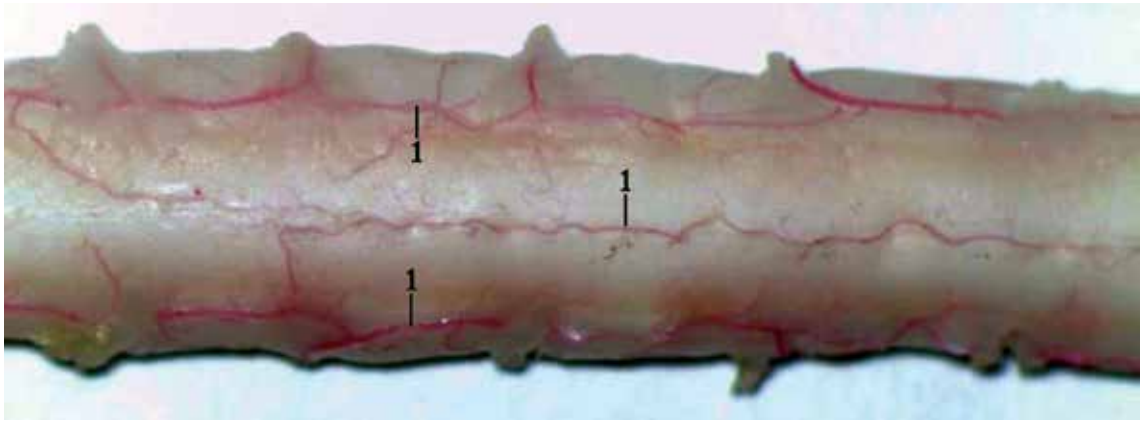

Fig. 5. Presence of three longitudinal dorsal spinal arteries
(1) dorsal spinal artery
Dorsal view. Magn. ×12.5

the dorsal and ventral branches. The ventral branches entered the ventral spinal artery. The occurrence of individual ventral branches are shown in Table 1. The ventral spinal artery ran subdurally in the ventral median fissure of the spinal cord. The presence of branches entering the ventral spinal artery in the thoracic region was observed in 69.5 % of the cases on the left side and in 30.5 % on the right side. In the lumbar region, the left-sided ventral branches were observed in 54.2 % of the cases and the right-sided in 45.8 % of the cases. Along the entire thoracic and lumbar spinal regions, we observed the left-sided branches in 63.8 % and the right-sided in 36.2 % of the cases, which is most likely related to the left-sided localization of the aorta.

Except for the relatively small and weak segmental spinal arteries, we found a bigger feeding artery arising from the *ramus spinalis* of the left fifth lumbar artery in 60 % of the cases (Fig. 1). In 30 % of the cases, the artery of Adamkiewicz was doubled with two different levels of origin. The right-sided artery originated from the *ramus spinalis* of fifth lumbar artery and the left-sided from the *ramus spinalis* of the fourth lumbar artery (Fig. 2). In 10 % of the cases, the artery of Adamkiewicz was originating from the *ramus spinalis* of the right and left fifth lumbar artery. These two arteries continued separately caudally on the ventral surface of the spinal cord. At the level the sixth lumbar artery, they fused together and continued caudally as *arteria spinalis ventralis*. They communicated together by means of a communicating branch at the level of the *ramus spinalis* of the fifth lumbar artery and sent cranially weak branches entering the *arteria spinalis ventralis* (Fig. 3). In all the cases, the artery of Adamkiewicz was entering the *arteria spinalis ventralis*.

On the dorsal surface, we found two irregular longitudinal dorsal spinal arteries lying in the *sulcus lateralis dorsalis,* bilaterally in 60 % of the cases (Fig. 4). They received the dorsal branches of the *rami spinales*. In 40 % of the cases, three irregular longitudinal dorsal spinal arteries receiving dorsal branches of *rami spinales* were present (Fig. 5). The third dorsal spinal artery was located in the *sulcus dorsalis*. The occurrence of individual dorsal branches is shown in Table 2. When the two irregular dorsal spinal arteries were present,

they were formed only by fusion of the small cranial and caudal branches arising from the dorsal branches. Among the dorsal branches observed in the thoracic region, 51.5 % of them were in the left side and 48.5 % in the right side. In the lumbar region, the observed dorsal branches were left-sided in 65.5 % of the cases and right-sided in 34.5 % of the cases. Along the entire thoracic and lumbar spinal regions, the branches were left-sided in 56.8 % of the cases and right-sided in 43.2 % of the cases. Based on our results, we can conclude that there is a high variability of the blood supply in the thoracolumbar part of the spinal cord in guinea pigs.

DISCUSSION

Dogs, rats, pigs, rabbits and mice have been used as experimental animals in the study of spinal cord ischemic damages. In the dogs, the high variability in the density of the arteries forming the spinal arterial ring and in the frequency of occurrence of the *rami spinales* have been described as arising from the radicular arteries [14]. The rat is one of the most frequently used experimental animals in the study of spinal cord injuries, therefore the blood supply of the rat spinal cord is probably the most profusely documented, but the results differ [3], [15], [16], [20]. Two dorsal spinal arteries are usually found in the rat [9], but in albino Wistar rats, they are less constantly observed [20]. The variations and the presence of extrasegmental arteries of the spinal cord blood supply have been described in the pig [17]. In the mouse, the spinal cord blood supply has been only partially described [8].

On the dorsal surface of the spinal cord, we found two to three irregular longitudinal dorsal spinal arteries (in human the posterior spinal arteries). In humans, the posterior spinal arteries are normally continuous cranial to caudal [4]. In dogs, four dorsal spinal arteries have been described on the dorsal surface of the spinal cord. They were divided into two pairs. Each pair was formed by a larger calibre lateral dorsal spinal artery and a thinner medial dorsal spinal artery [14]. Two much less constant dorsal spinal arteries forming irregular connections between each other were described in rats [20]. The number of dorsal spinal arteries in mice differs from study to study. Lang-Lazdunski et al. [8] found two dorsal spinal arteries but Bilgen and Al-Hafez [2] described only one single artery.

Our results indicate a high variability in the presence of dorsal and ventral branches supplying blood to the spinal cord. On the left side, they occurred in higher number. The segmental arteries of the thoracic and lumbar parts of spinal cord ensured a supply of the respective segments of the *arteria spinalis ventralis* and *arteriae spinals dorsales*. Segmental arteries in the lumbar part of spinal cord occurred irregularly, and their absence was noted more frequently than in the thoracic part, which allowed us to assume a higher risk of irreparable ischemic damage to the lumbar part of the spinal cord in the guinea pig.

Until now, works dealing with the study of the spinal cord blood supply in the guinea pig have only been published sporadically. Soutoul et al. [16] studied the spinal cord blood supply in several species, but the presence of the artery of Adamkiewicz, its level of origin, as well as the frequency of occurrence of spinal branches in the guinea pig, have not been described. Knox-Macaulay et al. [7] stated only the number of radicular arteries, in the thoracic part of spinal cord from five to seven and in the lumbar part was noticed a great variation in their number. *Arteria spinali sventralis* was described as a vessel sometimes interrupted in its course. We found this artery as an uninterrupted trunk. The dorsal spinal arteries were noticed as two smaller anastomotic chains of arteries, running in the *sulcus lateralis dorsalis*. In our study, the number of dorsal spinal arteries varied from two to three. In this study, the artery of Adamkiewicz was described as a doubled artery originating from the spinal branch of the third or fourth lumbar artery. We found the artery of Adamkiewicz as a single or doubled vessel with a different level of origin.

The artery of Adamkiewicz was present only in half of all the specimens of dogs [14]. In the rat, the artery of Adamkiewicz was described in all cases [3], [6], [16], [20] but many works doubt the presence of the artery of Adamkiewicz in rats [15], [19]. In the pig, the artery of Adamkiewicz has not been recorded [17], [18]. The presence of the artery of Adamkiewicz was declared also in the mouse [8]. In humans, the artery of Adamkiewicz was present in all cases [12].

CONCLUSIONS

The presence of the artery of Adamkiewicz and nearly regular segmental blood supply of the thoracolumbar part of spinal cord in all our studied animals are responsible for the use of guinea pigs as a simple model of ischemic damage to the thoracolumbar part of the spinal cord.

ACKNOWLEDGEMENTS

The present study was carried out within the framework of the project VEGA MŠ SR No. 1/0111/13 of the Slovak Ministry of Education.

REFERENCES

1. Alleyne, C. H., Cawley, C. M., Shengelaia, G. G., 1998: Microsurgical anatomy of the artery of Adamkiewicz and its segmental artery. *J. Neurosurg.,* 89, 791—795.

2. Bilgen, M., Al-Hafez, B., 2006: Comparison of spinal vasculature in mouse and rat: investigations using MR angiography. *Neuroanatomy*, 5, 12—16.

3. Brightman, M. W., 1956: Comparative anatomy of spinal cord vasculature. *Anat. Rec.*, 124, 264.

4. Cheshire, W. P., Santos, C. C., Massey, E. W., Howard, J. F., 1996: Spinal cord infarction: etiology and outcome. *Neurology*, 47, 321—330.

5. Duerstock, B. S., Borgens, R. B., 2002: Three-dimensional morphometry of spinal cord injury following polyethylene glycol treatment. *J. Exp. Biol.*, 205, 13—24.

6. Gouazé, A., Soutoul, J. H., Santini, J. J., Duprey, G., 1965: L´artére du renflement lombaire de la moelle chez quelques mammiferes. *Comptesrendus der Association des Anatomistes*, 49, 762—775.

7. Knox-Macaulay, H., Morrell, M. T., Potts, D. M., Preston, T. D., 1960: The arterial supply to the spinal cord of the guinea pig. *Acta. Anat.*, 40, 249–255.

8. Lang-Lazdunski, L., Matsushita, K., Hirt, L., Waeber, C., Vonsattel, J. P., Moskowitz, M. A., Dietrich, W. D., 2000: Spinal cord ischemia. Development of a model in the mouse. *Stroke*, 31, 208—213.

9. Lazorthes, G., Gouaze, A., Zadeh, J. O., Santini, J. J., Lazorthes, Y., Burdin, P., 1971: Arterial vascularization of the spinal cord: recent studies of the anastomotic substitution pathways. *J. Neurosurg.*, 35, 253—262.

10. Luo, J., Li, N., Robinson, J. P., Shi, R., 2002: The increase of reactive oxygen species and their inhibition in an isolated guinea pig spinal cord compression model. *Spinal Cord*, 40, 656—665.

11. McBride, J. M., Smith, D. T., Byrn, S. R., Borgens, R. B., Shi, R., 2007: 4-Aminopyridine derivatives enhance impulse conduction in guinea-pig spinal cord following traumatic injury. *Neuroscience*, 148, 44—52.

12. Milen, M. T., Bloom, D. A., Culligan, J., 1999: Albert Adamkiewicz (1850—1921) — his artery and its significance for the retroperitoneal surgeon. *World J. Urol.*, 17, 168—170.

13. Nijenhuis, R. J., Leiner, T., Cornips, E. M., 2004: Spinal cord feeding arteries at MR angiography for thoracoscopic spinal surgery: feasibility study and implications for surgical approach. *Radiology*, 233, 541—547.

14. Pais, D., Casal, D., Arantes, M., Casimiro, M., O'Neill, J. G., 2007: Spinal cord arteries in *Canis familiaris* and their variations: implications in experimental procedures. *Braz. J. Morphol. Sci.*, 24, 224—228.

15. Schievink, W. I., Luyendijk, W., Los, J. A., 1988: Does the artery of Adamkiewicz exist in the albino rat ? *J. Anat.*, 161, 95—101.

16. Soutoul, J. H., Gouaz'e, A., Castaing, J., 1964: Les arte' resde la moelleepiniere des animaux d' experimentation. III. — etude comparative durat, cobaye, lapin, chat, chien, orang-outang, chimpnaze, avec l'homme et lefoetus. *Pathol. Biol.*, 12, 950—962.

17. Strauch, J. T., Spielvogel, D., Lauten, A., Zhang, N., Shiang, H., Weisz, D., et al., 003: Importance of extrasegmental vessels for spinal cord blood supply in a chronic porcine model. *Eur. J. Cardiothorac. Surg.*, 24, 817—824.

18. Strauch, J. T., Lauten, A., Zhang, N., Wahlers, T., Griepp, R. B., 2007: Anatomy of spinal cord blood supply in the pig. *Ann. Thorac. Surg.*, 83, 2130—2134.

19. Tveten, L., 1976: Spinal cord vascularity IV. The spinal cord arteries in the rat. *Acta Radiol.*, 17, 385—398.

20. Woollam, D. H. M., Millen, J. W., 1955: The arterial supply of the spinal cord and its significance. *J. Neurol. Neurosurg. Psychiatry*, 18, 97—102.

Received July 15, 2014

FOLIA VETERINARIA, 58, 3: 185—188, 2014

ARRANGEMENT OF SEGMENTAL ARTERIES SUPPLYING THE THORACOLUMBAR SPINAL CORD IN GUINEA PIGS

Maženský, D.[1], Petrovová, E.[1], Supuka, P.[2]
Krešáková, L.[1], Supuková, A.[3]

[1]Department of Anatomy, Histology and Physiology
[2]Institute of Nutrition, Dietetics and Feed Production
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
[3]Institute of Experimental Medicine, Faculty of Medicine, UPJŠ Košice, Trieda SNP 1, 041 01 Košice
The Slovak Republic

mazenskyd@gmail.com

ABSTRACT

The aim of this study was to describe the anatomical arrangement of the segmental arteries arising from the descending aorta which supply the thoracolumbar spinal cord in guinea pigs. The study was carried out on twenty adult English self guinea pigs. We prepared corrosion casts of the arteries of the thoracolumbar spinal cord by using Batson's corrosion casting kit No. 17. There were present 12 intercostal arteries. These branches, which arose from the dorsal surface of the thoracic aorta were found in 8 pairs in 70 % of the cases, in 7 pairs in 20 % of the cases, and in 9 pairs in 10 % of the cases. The rest of the dorsal intercostal arteries branched from the *arteria intercostalis suprema*. The lumbar arteries were present in 7 pairs in all of the cases. In 60 % of the cases, the *arteriae lumbales* originated by means of a common trunk with a division in the right-left direction. In 40 % of the cases, both the right- and left-sided arteries originated independently at the same level. The nearly regular segmental blood supply of the thoracolumbar part of spinal cord in all of the guinea pigs studied, reveals why the guinea pig serves as a simple model for investigations involving ischemic damage to the thoracolumbar part of the spinal cord.

Key words: anatomy; arteria intercostalis dorsalis; arteria lumbalis; corrosion cast

INTRODUCTION

Laboratory animals are used in several experimental studies of spinal cord damage. The more detailed knowledge of the anatomy of the spinal cord blood supply with a focus on all of the possible variations can make a valuable contribution toward the eventual protection and repair of the spinal cord following traumatic injury.

Guinea pigs, as laboratory animals, are frequently used in studies of spinal cord ischemia [1], [7], [8]. The arterial supply of the thoracolumbar part of the spinal cord in guinea pigs has been described in only a few studies [6], [11].

The aim of this study was to describe the arterial arrangement in the thoracolumbar part of spinal cord in guinea pigs. The surgical procedures carried out in this region are associated with a high risk of ischemic damage.

MATERIALS AND METHODS

This study was carried out on 20 adult guinea pigs (age 220 days). We used English self guinea pigs of both sexes (female n = 10; male n = 10) with an average weight of 0.8—1 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 (FANTASIA). 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 death, the vascular network was perfused with a physiological solution. During the 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 a well perfused injection. Batson's corrosion casting kit No. 17 using a volume of 50 ml (Dione, Českе Budějovice, Czech Republic) was used as the casting medium. After polymerization, the 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 decision No. 2647/07-221/5.

RESULTS

The thoracic part of the spinal cord received oxygenated blood by means of the *rami spinales* which arose as branches from the *arteriae intercostales dorsales* (Fig. 1). Twelve intercostal arteries were present. These branches arose from the dorsal surface of the thoracic aorta and were found in 8 pairs in 70 % of the cases, in number of 7 pairs in 20 % of the cases and in 9 pairs in 10 % of the cases. The rest of the dorsal intercostal arteries branched from the *arteria intercostalis suprema*. In 70 % of the cases, the arteries originated by means of a common trunk. The common trunk was divided in the right-left direction in 60 % of the cases (Fig. 1) and in the cranio-caudal direction, in 40 % of the cases (Fig. 1). The formation of a common trunk showed a high degree of variability. It was formed by 2 dorsal intercostal arteries in 4 cases, by 3 arteries in 1 case, by 4 arteries in 1 case and by 5 arteries also in 1 case. In 30 % of the cases, both the right- and left-sided arteries originated independently at the same level.

The lumbar part of the spinal cord was supplied by *rami spinales* arising from the paired *arteriae lumbales* which originated from the dorsal surface of the abdominal aorta. They were present in 7 pairs in all the cases. The first 6 pairs arose from the abdominal aorta and the last one from the *arteria sacralis mediana*. In one case, the two last pairs originated from the *arteria sacralis mediana* and in one case, all 7 pairs originated from the abdominal aorta. In 60 % of the cases, the *arteriae lumbales* originated by means of a com-

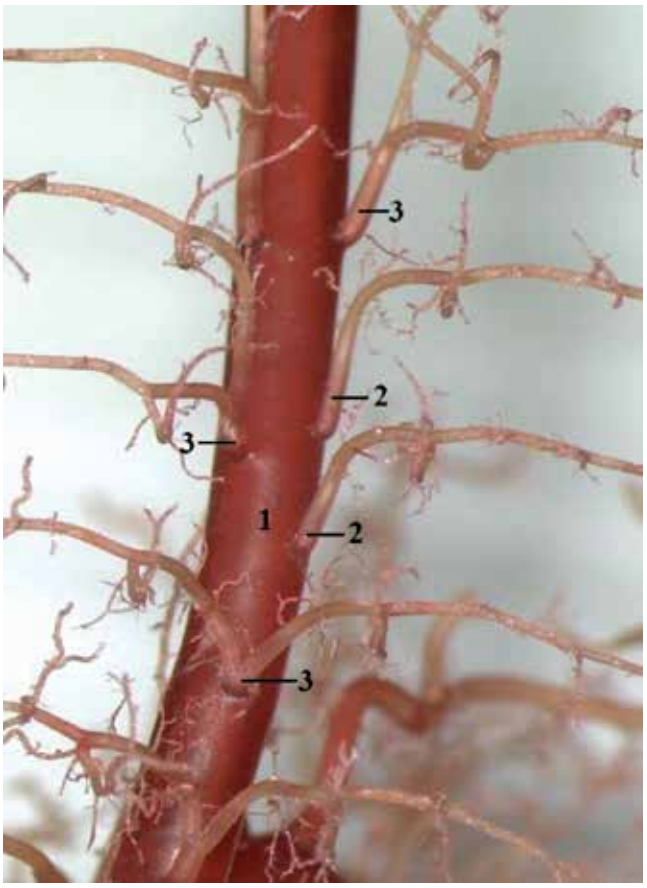

Fig. 1. Origin of *arteriae intercostales dorsales*

(1) *aorta thoracica*; (2) independent origin of *arteriae intercostales dorsales*; (3) origin of *arteriae intercostales dorsales* by means of a common trunk with division in cranio-caudal direction; (4) origin of *arteriae intercostales dorsales* by means of a common trunk with division in right-left direction. Dorsal view. Magn ×5

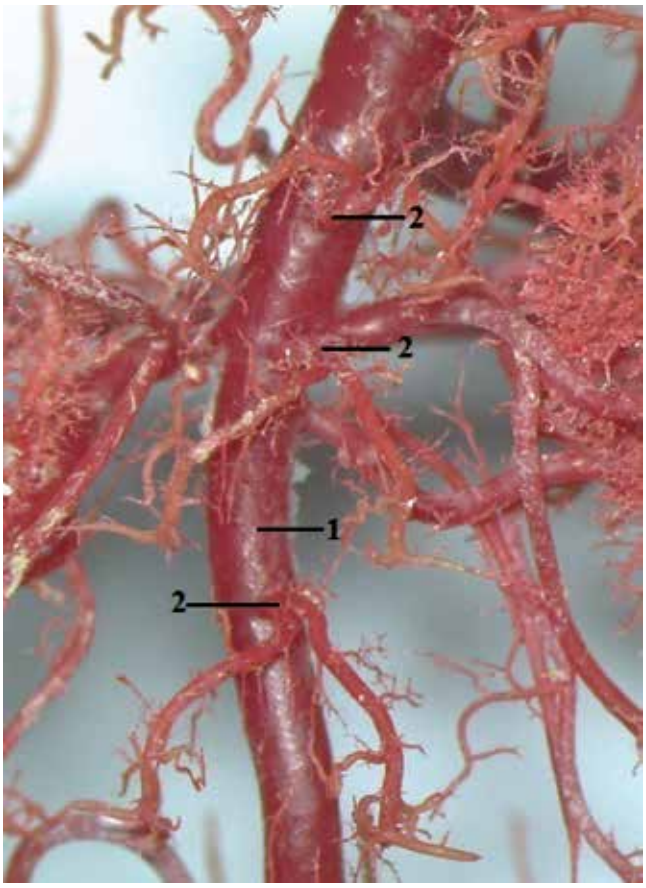

Fig. 2. Origin of *arteriae lumbales* by means of a common trunk

(1) *aorta abdominalis*; (2) common trunk of *arteriae lumbales* Dorsal view. Magn. ×5

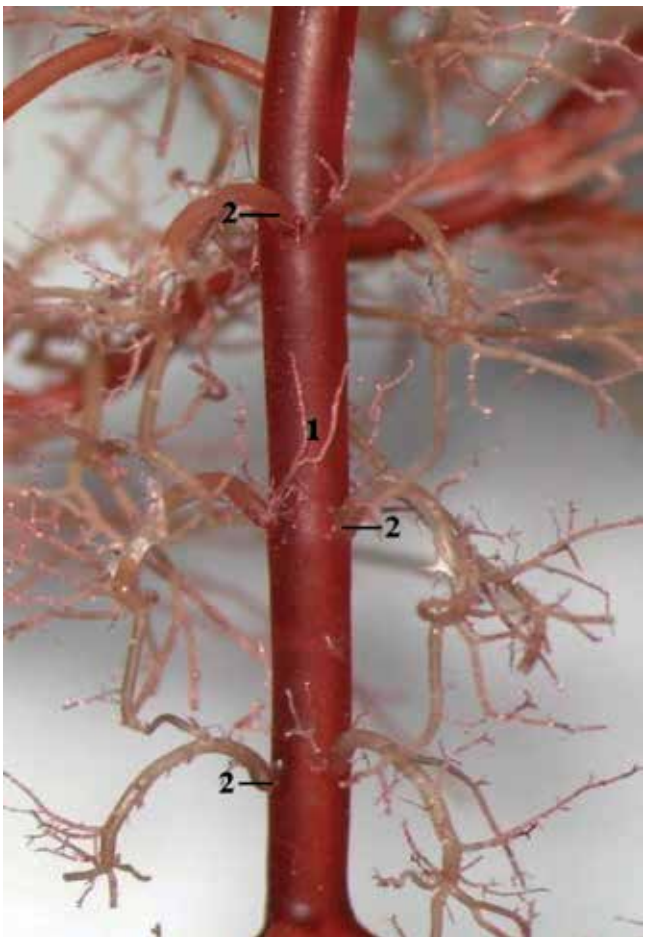

Fig. 3. Independent origin of *arteriae lumbales*.

(1) *aorta abdominalis*; (2) *arteria lumbalis*
Dorsal view. Magn. ×5

mon trunk with a division in the right-left direction (Fig. 2). In 40 % of the cases, both the right- and left-sided arteries originated independently at the same level (Fig. 3).

DISCUSSION

During operations to correct thoracoabdominal aneurysms, the arrangement of the origin of the segmental dorsal intercostal and lumbar arteries have a very important role [2]. The risk of spinal cord ischemia which can also lead to paraplegia, decreases by correctly performed re-implantation of the segmental arteries [5]. Shively and Stump [12] found that the number of dorsal intercostal arteries, which are supplied by the costocervical trunk and the highest intercostal artery, is not consistent from animal to animal. In this study, it varied from four to seven on each side, with only the first and occasionally the second one coming directly from the costocervical trunk, and the remainder from the highest intercostal artery. There are typically twelve pairs of dorsal intercostal arteries, and the balance are direct branches from the thoracic aorta. Dorsal intercostal arteries were described as paired segmental branches with independent origin arising from the thoracic aorta [9], [10]. Shively and Stump [13] described two types of origins of seven pairs of lumbar arteries from the abdominal aorta: the independent origin and the origin by means of a common trunk of arteries at the same level. Other authors found lumbar arteries as segmental paired branches arising from the dorsal surface of the abdominal aorta [9], [10].

Animal models, especially rodent models, are designed to help predict functional outcomes of neurological disorders and injuries. Many of the behavioural outcomes appear as parallel clinical symptoms which may be observed in human patients to a remarkable degree. Understanding the strengths and limitations of the models will allow more relevant analysis of the injury, behavioural sequel, and therapeutic approaches. Each aspect of a study should be planned before the experiment is begun [4]. The understanding of the vascular blood supply to the spinal cord is important to avoid spinal cord ischemia or infarction during surgical approaches to the spine [3].

CONCLUSIONS

Nearly regular segmental blood supplying of the thoracolumbar part of spinal cord in all of our studied animals are responsible for the use of guinea pigs as a simple model of ischemic damage to the thoracolumbar part of the spinal cord.

ACKNOWLEDGEMENTS

The present study was carried out within the framework of the project VEGA MŠ SR No. 1/0111/13 of the Slovak Ministry of Education.

REFERENCES

1. Duerstock, B. S., Borgens, R. B., 2002: Three-dimensional morphometry of spinal cord injury following polyethylene glycol treatment. *J. Exp. Biol.*, 205, 13—24.

2. Etz, D. C., Halstead, J. C., Spielvogel, D., Shahani, R., Lazala, R., Homann, T. M., et al., 2006: Thoracic and thoracoabdominal aneurysm repair: is reimplantation of spinal cord arteries a waste of time? *Ann. Thorac. Surg.*, 82, 1670—1677.

3. Gao, L., Wang, L., Su, B., Wang, P., Ye, J., Shen, H., 2013: The vascular supply to the spinal cord and its relationship to anterior spine surgical approaches. *Spine J.*, 13, 966—973.

4. Geissler, S. A., Schmidt, C. E., Schallert, T., 2013: Rodent models and behavioural outcomes of cervical spinal cord injury. *J. Spine*, doi: 10.4172/2165-7939.S4-001.

5. Grabitz, K., Sandmann, W., Stühmeier, K., Mainzer, B., Godehardt, E., Ohle, B., et al., 1996: The risk of ischemic spinal cord injury in patients undergoing graft replacement for thoracoabdominal aortic aneurysms. *J. Vasc. Surg.*, 23, 230—240.

6. Knox-Macaulay, H., Morrell, M. T., Potts, D. M., Preston, T. D., 1960: The arterial supply to the spinal cord of the guinea pig. *Acta. Anat*., 40, 249—255.

7. Luo, J., Li, N., Robinson, J. P., Shi, R., 2002: The increase of reactive oxygen species and their inhibition in an isolated guinea pig spinal cord compression model. *Spinal Cord*, 40, 656—665.

8. McBride, J. M., Smith, D. T., Byrn, S. R., Borgens, R. B., Shi, R., 2007: 4-aminopyridine derivatives enhance impulse conduction in guinea-pig spinal cord following traumatic injury. *Neuroscience*, 148, 44—52.

9. Nejedlý, K., 1965: *Biology and Systematic Anatomy of Laboratory Animals* (In Czech). SPN, Prague, 482—485.

10. Popesko, P., Rajtová, V., Horák, J., 1990: *Anatomic Atlas of Small Laboratory Animals* (In Slovak). Príroda, Bratislava, 184—188.

11. Soutoul, J. H., Gouaz'e, A., Castaing, J., 1964: Les arte'res de la moelleepiniere des animaux d'experimentation. III. – etude comparative durat, cobaye, lapin, chat, chien, orang-outang, chimpnaze, avec l'homme et lefoetus. *Pathol. Biol.*, 12, 950—962.

12. Shively, M. J., Stump, J. E., 1974: The systemic arterial pattern of the guinea pig: The head, thorax, and thoracic limb. *Am. J. Anat.*, 139, 269—284.

13. Shively, M. J., Stump, J. E., 1975: The systemic arterial pattern of the guinea pig: The abdomen. *Anat. Rec.*, 182, 355

Received July 15, 2014

FOLIA VETERINARIA, 58, 3: 189—192, 2014

THE RELATIONSHIP BETWEEN THE NUMBER AND ARRANGEMENT OF *AA. JEJUNALES* AND INTESTINAL LENGTH

Vdoviaková, K.[1], Petrovová, E.[1], Maloveská, M.[1]
Krešáková, L.[1], Elias, M., Z. J.[2]

[1]Department of Anatomy, Histology and Physiology, Institute of Anatomy
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic
[2]Eduardo Mondlane University, Faculty of Veterinary Medicine, Maputo
Mozambique

katarina.vdoviakova@uvlf.sk

ABSTRACT

The aim of this study was to describe in detail the anatomical arrangement and number of jejunal vessels and their relationship to the intestinal length in rats. The present study was carried out on 50 adult Wistar rats (*Rattus norvegicus f. domestica*). We measured the length of the jejunum after laparotomy and then we prepared corrosion casts of the arteries of the small intestine. We used Duracryl Dental®, PUR SP, and red latex as an injection medium. We observed that the *aa. jejunales* originated from the *a. mesenterica cranialis*. This artery is the thickest branch of the abdominal aorta. In 26 % of the cases we observed 14 *trunci jejunales*, and 4 *aa. jejunales* which originated separately from the *a. mesenterica cranialis*. In 26 % of the cases we detected 16 *trunci jejunales*, and 3 *aa. jejunales* which originated separately from the cranial mesenteric artery. In 24 % of the cases we found 18, and in another 24 % cases, 20 *trunci jejunales*. The results of our study showed that the number of jejunal trunks and jejunal arteries increased continually with the elongation of the rat intestine and body weight. Our results point to the importance of research of the circulatory system of the rat intestine for future experimental transplantation of digestive organs.

Key words: abdominal cavity; blood supply; laboratory rat; length of intestine; rat anatomy; small intestine

INTRODUCTION

The small intestine is the major site of the gastrointestinal tract for the absorption of nutrients and medicaments. From this point of view, the anatomical knowledge is necessary to understand the functions of the intestine and its blood supply in laboratory animals. These animals are the most appropriate for the research of anatomical, physiological and biochemical relations in the digestive system, which could be applied not only to domestic mammals, but also to man. The rats, mice, hamsters, guinea pigs, and rabbits are the best for determining the mechanism of drug absorption and bioavailability values from powder or solution formulations [5]. Nowadays, the laboratory rat is one of the most popular models for the surgery of the abdominal cavity, intestinal transplantation, or for the study of drug absorption [6]. In the massive small bowel resection, the length of the intestine to be excised and to be preserved, is an important factor.

In the measurement of the length of the preserved intestine, the measurement of the length of the intestine at the time of the operation may produce a large error when compared to the length of the intestine at normal times [4]. Therefore, we focused our study on the number and arrangement of the jejunal arteries, and a comparison between vessels and the length of the intestine in laboratory rats at normal times.

MATERIALS AND METHODS

We studied 50 one-year-old Wistar rats (*Rattus norvegicus f. domestica*); males and females, weighing 350—520 g, kept under standard breeding conditions. The rats were divided to 4 groups according to their body weight; group 1 (300—350 g); group 2 (351—400 g); group 3 (401—450 g); and group 4 (451—520 g).

Measurement of the length of the intestine

We performed laparotomies under ether anaesthesia. Then we opened the abdominal cavity and to avoid artificial traction of the intestine, a 1—0 silk thread was led along the antimesenteric side of the intestinal wall. The length of the jejunum was measured from the *flexura duodenojejunalis* to the ileum.

Preparation of the corrosion cast specimens of the arterial system

We euthanized the animals by ether and then dissected the left ventricle of the heart. We inserted a cannula into the aorta through the left ventricle. The cannula was supported by a ligature. A portion of the venous system had to be opened to ensure a good distribution of the perfusion medium. The right auricular appendage served for this purpose. The vessels were perfused with saline (0.9 % NaCl), (Mikrochem, Slovakia). An improved method for washing out the clotted blood from the vessels was used based on the addition of 0.05 % NaOH to the perfusion medium. The perfusion pressure was approximately 200—250 mm Hg (2.6—3.25 m H_2O). The success of the perfusion was indicated by a uniform fading of the tissues seen during the procedure. We mixed the injection media in stechiometric rates. In our study we used two injection media, Duracryl Dental ® (Spofa-Dental, Czech Republic), and PUR SP (Ústav polymérov, SAV, Slovakia). A suitable colour tone was achieved by adding 2—3 drops of red oil (red paint 0), (Mikrochem, Slovakia). After a proper mixing of all components, we injected this mass into the arterial system through the left ventricle of the heart. After the injection, maceration of the soft tissues was carried out in 2—4 % solution of KOH at 60—70 °C. The maceration took approximately 2—3 days. In some cases we applied red latex (Latex, Het Color, Czech Republic) into the arterial system. The number of jejunal arteries and jejunal trunks were counted. The results were listed in percentages. We used the latest edition of the Veterinary Anatomic Nomenclature throughout in this study [2].

RESULTS

Our investigations showed that the *aa. jejunales* originated from the *a. mesenterica cranialis.* This artery is the thickest branch of the abdominal aorta. The cranial mesenteric artery originates behind the celiac artery and supplies the intestine from the caudal part of duodenum to the *colon transversum.*

The aa. jejunales transport blood to the longest part of the small intestine — the *jejunum.* The *arteriae jejunales* ran parallel to the jejunal veins. In most cases, the common trunks originated from the cranial mesenteric artery for the jejunal arteries, these common trunks were named *trunci jejunales* (Fig. 1). In 26 % of the cases we observed 14 *trunci jejunales* and 4 *aa. jejunales* separately originated from the *a. mes-*

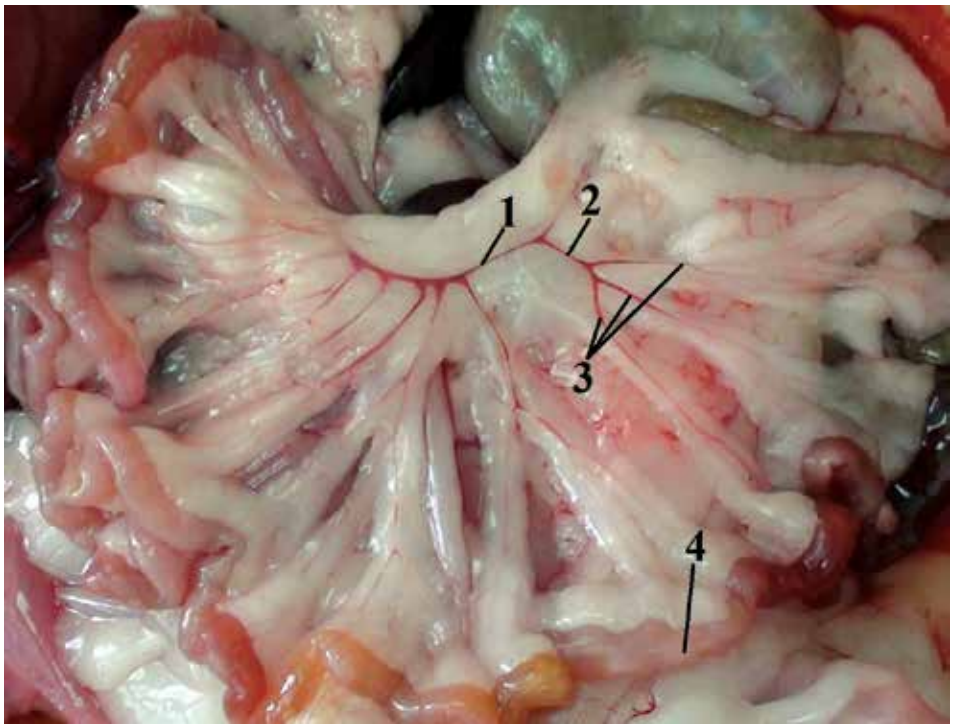

Fig. 1. The presence of *truncus jejunalis* of *aa. jejunales* (injection medium – red latex)
(1) *a. mesenterica cranialis;* (2) *truncus jejunalis;* (3) *aa. jejunales;* (4) *jejunum*

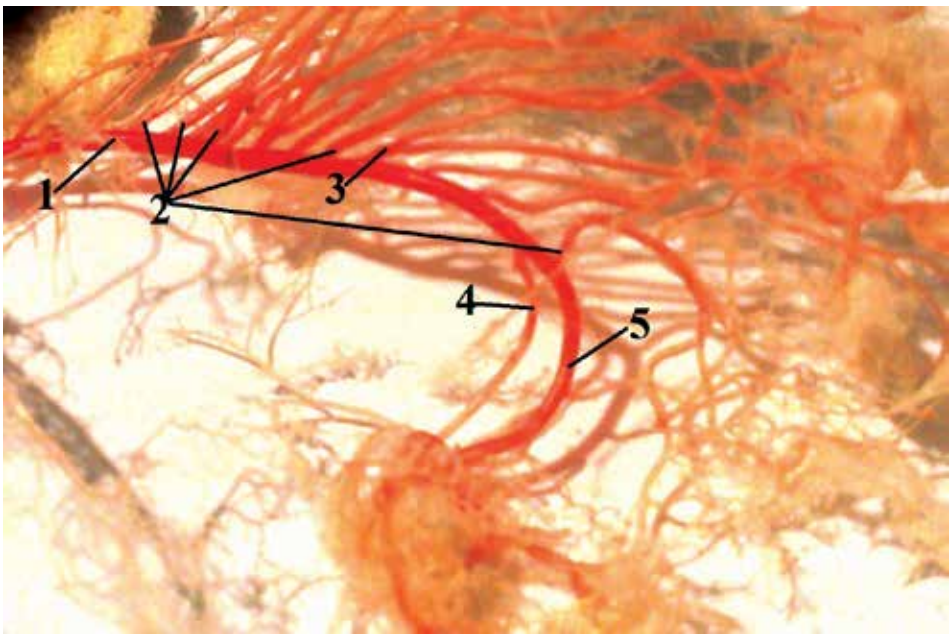

Fig. 2. The origin of *aa. jejunales* and terminal division of *a. mesenterica cranialis* (injection medium — PUR SP)
(1) *a. mesenterica cranialis;* (2) *aa. jejunales;* (3) *truncus jejunalis;* (4) *r. colicus;* (5) *a. ileocecalis,*

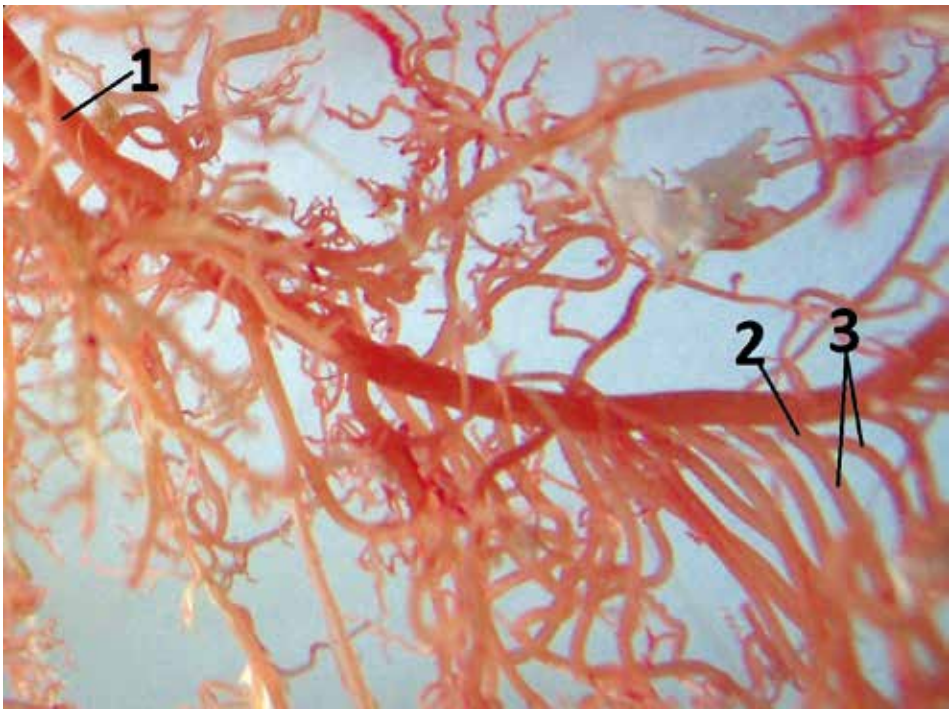

Fig. 3. The division of the *truncus jejunalis* into two *aa. jejunales* (injection medium — Duracryl Dental®)
(1) *a. mesenterica cranialis;* (2) *truncus jejunalis;* (3) *aa. jejunales*

enterica cranialis (Fig. 2). In 26% of the cases we observed 16 *trunci jejunales*, and 3 *aa. jejunales* which separately originated from the cranial mesenteric artery. In 24% of the cases we observed 18, and in another 24% of the cases, 20 *trunci jejunales.* In all cases, the *trunci jejunales* were divided into two (Fig. 3) or three jejunal arteries. These *aa. jejunales* were directed to the intestinal wall to form the jejunal arcades. No difference was noticed between sexes. The *trunci jejunales* were divided into jejunal arteries in the region about 5 mm away from the cranial mesenteric artery.

The results of our study also showed, that the number of jejunal trunks and jejunal arteries increased simultaneously

with the increasing length of the rat intestine (Tab. 1). The length of the jejunum increased with the increasing body weight of the animals. The length of the jejunum in group 4 was nearly 1.5-fold the length in the first group.

Table 1. Length of the intestine and the number of intestinal vessels in rats

Group	Number of rats	Body weight [g]	Length of intestine [mm]	Number of *trunci jejunales*
1	13	300—350	900—1000	14
2	13	351—400	1001—1200	16
3	12	401—450	1201—1300	18
4	12	451—520	1301—1350	20

DISCUSSION

In rats the *a. mesenterica cranialis* was the thickest branch of the abdominal aorta which originated behind the coeliac artery [10]. Some authors described the origin of the cranial mesenteric artery 3—5 mm caudally to the oigin of the *a. coeliaca* [1], [3]. The origin of the cranial mesenteric artery from the abdominal aorta in laboratory animals varied according to the species. In rabbits, it was an independent trunk arising from the abdominal aorta 1.5—2 cm caudally to the origin of the *a. celiaca*. *A. mesenterica cranialis* branched off two main trunks: *truncus jejunalis* and *truncus ileocecocolicus* in rabbits [8]. Other authors described that the cranial mesenteric artery arose from the ventral wall of the abdominal aorta at the level of the caudal border of the 2nd lumbar vertebra arch [11], or at the level between the first and second lumbar vertebrae [9].

According to our results the *aa. jejunales* always originated from the cranial mesenteric artery. Baláž et al. [1] and Hebel et al. [3] described the origin of the *aa. jejunales* from the *a. colica dextra* in rats. Some of the *aa. jejunales* arose directly from the trunk of the cranial mesenteric artery and in some cases it arose from the convexity of the arch of the *truncus jejunalis* in rabbits [8]. According to Uddin et al. [11], 18 to 20 of the jejunal arteries were given off from the cranial mesenteric artery, after the *a. ileocecocolica* had left it in rabbits. Other authors described that the jejunal arteries arose directly from the cranial convex part of the cranial mesenteric artery in rabbits. The last jejunal artery represented the termination of the cranial mesenteric artery [9].

In order to estimate the normal length of the preserved intestine after enterectomy, the number of arteriae jejunales was measured as an index, and the number was correlated to the normal length of the intestine. Chiba et al. [4] described similar research in rats. They studied the relationship between the number of *arteriae rectae* of the intestinal artery and the intestinal length. The detailed arrangement of the jejunal arteries and their number are more important during experimental transplantation of the intestine in rats [6]. The discontinuance of the blood flow to the intestinal arteries is significant at individual methods of transplantations and research of the ischaemia — reperfusion injury of the small intestine [7].

CONCLUSIONS

The results of our study points out that the research of the circulatory system of the intestine in rats is important for future experimental transplantation of digestive organs. Our study showed that the number of jejunal trunks and jejunal arteries increase continuously with the increasing length of the rat intestine and body weight.

ACKNOWLEDGEMENTS

The study was supported by the project of the Slovak Ministry of Education, grant VEGA 1/0111/13.

REFERENCES

1. Baláž, P., Mergental, H., 2006: *Transplantation Experiment* (In Czech). Galén, Praha, 25—29.

2. Danko, J., Šimon, F., Artimová, J., 2012: *Nomina Anatomica Veterinaria*. UVLF, Košice, 267.

3. Hebel, R., Stromberg, M. W., 1989: *Anatomy and Embryology of the Laboratory Rat*. Wőrthsee, BioMed, Verlag, Germany, 106—110.

4. Chiba, T., Boles, E. T. jr., 1984: Studies on the relationship between the number of arteriae rectae of intestinal artery and intestinal length. *Tohoku Journal of Experimental Medicine,* 143, 27—31.

5. Kararli, T. T., 1995: Comparison of the gastrointestinal anatomy, physiology and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug. Dispos.*, 16, 351—380.

6. Lopes, M. F. S. C., Cartucho, D. J. F., Cabrita, A. M. S., Patritio, J. A. B., 1998: Techniques of intestinal transplantation in rat. *Microsurg.*, 18, 424—429.

7. Mallick, I. H., Yang, W., Winslet, M. C., Seifalian, A. M., 2004: Ischemia — reperfusion injury of the intestine and protective strategies against injury. *Digest. Dis. Sci.,* 49, 9, 1359—1377.

8. Malinovský, L., Bednárová, Z., 1990: Variability of ramification of the *a. mesenterica cranialis* in the domestic rabbit (*Oryctolagus cuniculus* f. *domestica*). *Folia Morphol. (Prague),* 3, 283—289.

9. Mohamed, R. A. A., 2014: Arterial supply of the intestine of Baladi rabbit. *Inter. J. Vet. Sci.,* 2, 52—60.

10. Nejedlý, K., 1965: *Biology and Systematic Anatomy of Laboratory Animals* (In Czech). SPN, Prague, 328—332.

11. Uddin, M., Rahman, M. L., Alim, M. A., Ahasan, A. S. M. L., 2012: Anatomical study on origin, course and distribution of cranial and caudal mesenteric arteries in the White New Zealand rabbit (*Oryctolagus cuniculus*). *Int. J. Nat. Sci,* 1, 54—59.

Received August 2, 2014

FOLIA VETERINARIA, 58, 3: 193—196, 2014

PREVALENCE OF DIGITAL DERMATITIS IN DAIRY COWS WITH DIFFERENT BEDDING

Mudroň, P.

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

pmudron@hotmail.com

ABSTRACT

An experimental study was conducted to estimate the prevalence of digital dermatitis in dairy cows (Holstein-Friesian) housed on two different bedding systems: deep straw bedding (Farm A) and surface straw bedding (Farm B). On the farm with the deep straw bedding 397 cows, and on the farm with surface straw bedding 283 dairy cows were examined. In both bedding systems, two claw lesions dominated: digital dermatitis and pododermatitis. The prevalence of contagious digital dermatitis for cows housed in deep-bedded free-stalls was 22.5 % and for the cows housed in free-stalls with surface straw bedding it was 27.8 % ($P < 0.001$). The prevalence of the wall and sole pododermatitis was 14.1 % and 17.7 % on farm A and 16.4 % and 11.4 % ($P < 0.001$) on farm B. The prevalence of the sole ulcer was 11.2 % on farm A and 15.4 % on farm B ($P < 0.001$). Our data indicated that the most frequent digital lesions affected the skin structures of the dairy cows on both bedding systems, moreover, the surface straw bedding seems to be associated with a higher prevalence of digital dermatitis.

Key words: cattle; digital dermatitis; lameness; pododermatitis; stall bedding

INTRODUCTION

Claw diseases, especially injuries and infections of the feet, constitute one of the most serious and painful, yet least well-managed categories of diseases for the modern high yielding dairy cow. This problem has been recognised and studied for many years but is not getting any better. Approximately 20 to 25 % of intensively managed dairy cattle in North America are lame at any one time [3], [7], and recently reported lameness prevalences in U. K. dairy herds ranged from 0—80 % [1]. The most frequent causes of bovine lameness are digital dermatitis and pododermatitis [5]. The inflammatory character of the claw diseases have also been proved by increased concentrations of serum haptoglobin and amyloid A in lame heifers [24].

A high proportion of cows become lame within the first two months of their first lactation and relapse regularly. This indicates quite clearly that the conventional accepted practices for housing, feeding and managing of the modern Holstein-Friesian dairy cow lead to injury or otherwise affect the feet to a degree where 50 % of the animals will experience the chronic pain of lameness in any lactation [28].

The obvious consequences of lameness are: less time spent eating, less movement to bunks, subsequent weight and production losses, and failure to show heat. Extreme lameness with weight loss causes a negative energy balance and can cause anoestrus [14]. Assessing the effects of such diseases on milk yield is a difficult task [8]. The milk production before disease incidence can confound the effects of such disease on overall milk yields. Milk yields are higher before rather than after a lameness occurrence; high milk production has been shown to be associated with lameness [11]. Lameness prevention remains a significant priority for the dairy industry as consumer demand drives changes in housing and management to promote

improved wellbeing, and farmers strive to improve productivity. Providing a clean, dry, and comfortable surface for cows to rest on, is an important factor for the welfare of dairy cows, as they spend approximately 12 h per day resting [13]. Lameness prevalence appears to be greater in free-stall facilities compared with other management systems such as tie-stall housing [22]. Lameness was found to be less prevalent in herds using deep-bedded sand stalls than herds using mattresses [3], [7]. The stall surface has also been shown to affect the prevalence of hock lesions, which are indicative of inadequate lying surfaces [16]. Lesions were observed less frequently in cows housed in deep-bedded sand stalls than cows on mattresses [9] and severe lesions were less prevalent in sand beds than on mattresses [27]. Exposure to concrete walking surfaces in alleys and other changes such as the regrouping of cattle around the time of calving are potential differences typical of free-stall design and management that may be important factors elevating lameness risk [4]. Increased costs and reduced availability of other common bedding sources has prompted many dairy producers to search for more feasible alternatives such as sand or recycled manure solids. Although sand can be considered the ideal bedding source for dairy cows, not all producers are willing and able to convert to sand bedding, as it presents several challenges related to manure management [15].

The aim of this study was to estimate the prevalence of digital dermatitis in dairy cows housed on two different straw bedding systems: deep straw bedding and surface straw bedding.

MATERIALS AND METHODS

This observational study was conducted on two dairy farms. All the animals were housed in a free stall system. On the farm with the deep straw bedding (Farm A), 397 and on the farm with flat straw bedding (Farm B), 283 Holstein-Frisian dairy cows were examined. The deep straw bedding (Farm A) was removed once in three weeks and the surface (2—3 cm layer) straw bedding (Farm B) was replaced daily.

The mean annual milk yield on farms A and B was 7,100 and 8,000 kg, respectively. The dairy cows on both farms were fed TMR with maize and alfalfa silage as the main components. Claw corrections were performed twice a year by external professional claw trimmers.

All the dairy cows were examined in the trimming crush and the diagnoses were recorded. There was a differentiation between a typical sole ulcer and other types of pododermatitis. Solar pododermatitis is usually caused by traumatic contamination of the corium or by progress of the sole ulcers. Wall pododermatitis is mainly from traumatic origin (sharp constructions, sharp and rough concrete) but it can occasionally develop from digital dermatitis.

Statistical analysis was performed by running a Chi-squared test using the number of cows suffering from particular diseases.

RESULTS

In both bedding systems, two claw lesions dominated: digital dermatitis and pododermatitis. The prevalence of the contagious digital dermatitis on farm A and B was 22.5 % and 27.8 % ($P < 0.001$), respectively. The prevalence of the wall or sole pododermatitis was 14.1 % and 17.7 % on farm A and 16.4 % and 11.4 % ($P < 0.001$) on farm B, respectively. The prevalence of the sole ulcer was 11.2 % on farm A and 15.4 % on farm B ($P < 0.001$). There was no difference in the prevalence of other claw lesions between the farms.

No acute laminitis could be observed in the examined dairy cows, however, several cases of subclinical and chronic laminitis were found on both farms (Table 1). This table presents also the results from the Chi-squared test describing individual claw lesions on the deep straw bedding versus the surface straw bedding.

Table 1. Prevalence of digital dermatitis in dairy cows on different beddings

Variable	Deep straw bedding	Surface straw bedding
Number of dairy cows	397	283
Lesion count (100 %)	233	449
Digital dermatitis	22.5 %	27.8 %*
Interdigital dermatitis	10.8 %	6.19 %
Wall pododermatitis	14.1 %	16.4 %
Sole pododermatitis	17.7 %	11.4 %*
Sole ulcer	11.2 %	15.4 %*
Interdigital hyperplasia	17.9 %	16.5 %
Subclinical laminitis	3.59 %	3.11 %
Chronic laminitis	2.24 %	3.21 %

* — $P < 0.001$

DISCUSSION

Ninety-five percent of bovine lameness is based on claw lesions which are characterised by painful inflammatory processes. The remaining 5 % of lameness cases are due to other locomotor disorders, which include; diseases of joints, tendons, tendon sheets, muscles, and bones, or which may occur due to animals suffering from neurological disorders (central or peripheral nervous system).

Cows housed in deep-bedded free-stalls demonstrated a lower prevalence of claw lesions than cows housed in free-stalls with surface straw bedding. This difference was based on a higher occurrence of digital dermatitis and sole ulcer on the farm with surface straw bedding. Digital dermatitis represents a lesion that frequently affects more than one leg. The digital dermatitis prevalence was clearly different ($P < 0.001$) between the two different beddings. Bovine digital dermatitis is a common, worldwide, painful, infectious disease

of the feet of intensively managed cattle [20]. The cause of digital dermatitis is multifactorial, with an essential spirochetal bacterial component. Several cultural, phenotypic and molecular studies have demonstrated that the spirochetes belong to a diverse phylogenetic group of *Treponema* spp. [19]. Acute digital dermatitis lesions were reproduced experimentally in Holstein heifers, thus, a systematic method to determine the efficacy of interventions aimed at the control of acute digital dermatitis is now available [10]. A new hypothesis about participation of *Dichelobacter nodosus* in the development of skin lesions typical for digital dermatitis has been demonstrated [21]. The authors hypothesize that external noxious stimuli allow *D. nodosus* to break down the epidermal barrier creating a suitable environment for the secondary invaders (*Treponema* species) which gradually take over the infection site. A recent study has revealed that the two most frequent claw diseases in dairy cows were digital dermatitis and septic pododermatitis in the region of the white line [18].

The significant effects of stall surface on lameness prevalence have been reported [3], [7]. In these studies, the lameness prevalence was compared between herds with deep-bedded sand and mattress-based free-stalls. Cook [3] observed that the lameness prevalence in sand stalls was lower than the prevalence observed in non-sand stalls. Similarly, high producing Holstein cows had a lameness prevalence of 17.1 % in herds with sand-based free-stalls compared with 27.9 % in herds with mattresses [7]. It is interesting to note that the claw lesion prevalence for deep straw beds in the current study was much lower than the lesion prevalence observed with deep bedded manure solids. Easier spread of the digital dermatitis on manure solids can be responsible for this observation. Differences in lameness prevalence likely occur between deep-bedded and mattress based stalls due to a greater resting comfort in deep-bedded stalls. When provided, the choice between deep beds with either sand or sawdust bedding and mattresses with 2 to 3 kg of bedding, cows showed a preference for deep beds [25]. Several studies have shown cows prefer stalls with greater surface cushion and spend more time lying down and less time standing when stall surfaces provide a greater degree of comfort [13], [26]. However, the use of mattresses as a stall surface has been implicated as a risk factor for lameness in dairy cows [6]. Deep-bedded free-stalls likely provide greater comfort than mattresses with a small amounts of bedding.

The higher prevalence of the sole ulcer in dairy cows on the farm with surface straw bedding may be related to the varied and uneven pressure on the claw sole. Risk factors for increased lameness are: the presence of damaged concrete in yards; cows pushing each other or turning sharply near the parlour entrance or exit; cattle grazing pastures that are also grazed by sheep; the use of automatic scrapers; and delayed treatment of lame cows [2]. There are several measures to prevent a high lameness prevalence, such as; feeding, welfare, hoof trimming, and foot baths. Hoof trimming remains the most widely used method available to producers to prevent claw disorders from evolving from the subclinical to the clinical stage.

Studies have shown that long intervals between hoof trimmings, or a lack of routine hoof trimming, are associated with increased lameness [17], [23]. Professional trimming was found to be more effective on farms with no nutritional disorders and where refurbishment works were carried out. The greatest decrease in the prevalence of lameness was observed on farms which provided professional trimming, effective foot bathing, improved walking and resting surfaces and which treated severely lame cows between regular trimmings [12].

Our data indicate that the most frequent digital lesions are affecting skin structures of dairy cows on both bedding systems, moreover, the surface straw bedding seems to be associated with higher prevalences of digital dermatitis and sole ulcer.

ACKNOWLEDGEMENT

This study was supported by the Slovak Research and Development Agency under the contract No. APVV-0701-11.

REFERENCES

1. Archer, S., Bell, N., Huxley, J., 2010: Lameness in UK dairy cows: a review of the current status. *In Practice*, 32, 492—504.

2. Barker, Z. E., Leach, K. A., Whay, H. R., Bell, N. J., Main, D. C. J., 2010: Assessment of lameness prevalence and associated risk factors in dairy herds in England and Wales. *J. Dairy Sci.*, 93, 932—941.

3. Cook, N. B., 2003: Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface. *J. Am. Vet. Med. Assoc.*, 223, 1324—1328.

4. Cook, N. B., Nordlund, K. V., 2009: The influence of the environment on dairy behaviour, claw health and herd lameness dynamics. *Vet. J.*, 179, 360—369.

5. DeFrain, M., Socha, T., Tomlinson, D. J., 2013: Analysis of foot health records from 17 confinement dairies. *J. Dairy Sci.*, 96, 7329—7339.

6. Dippel, S., Dolezal, M., Brenninkmeyer, C., Brinkmann, J., March, S., Knierim, U., Winckler, C., 2009: Risk factors for lameness in freestall-housed dairy cows across two breeds, farming systems, and countries. *J. Dairy Sci.*, 92, 5476—5486.

7. Espejo, L. A., Endres, M. I., Salfer, J. A., 2006: Prevalence of lameness in high-producing Holstein cows housed in freestall barns in Minnesota. *J. Dairy Sci.*, 89, 3052—3058.

8. Ettema, J. F., Capion, N., Hill, A. E., 2007: The association of hoof lesions at claw tri

9. Fulwider, W. K., Grandin, T., Garrick, D. J., Engle, T. E., Lamm, W. D., Dalsted, N. L., Rollin, B. E., 2007: Influence of freestall base on tarsal joint lesions and hygiene in dairy cows. *J. Dairy Sci.*, 90, 3559—3566.

10. Gomez, A., Cook, N. B., Bernardoni, N. D., Rieman, J., Dusick, A. F., Hartshorn, R., et al., 2012: An experimental infection model to induce digital dermatitis infection in cattle. *J. Dairy Sci.*, 95, 1821—1830.

11. Green, L. E., Hedges, V. J., Schukken, Y. H., Blowey, R. W., Packington, A. J., 2002: The impact of clinical lameness on milk yield of dairy cows. *J. Dairy Sci*., 85, 2250—2256.

12. Gudaj, R., Brydl, E., Lehoczky, J., Komlósi, I., 2013: Different management methods on prevalence of lameness in 25 Holstein-Friesian herds in Hungary. *Acta Veterinaria (Beograd)*, 63, 405—420.

13. Haley, D. B., de Passille, A. M., Rushen, J., 2001: Assessing cow comfort: Effects of two floor types and two tie stall designs on the behaviour of lactating dairy cows. *Appl. Anim. Behav. Sci.*, 71, 105—117.

14. Hillman, R., Gilbert, R. O., 2008: Reproductive diseases. In *Rebhun's Diseases of dairy Cattle,* St. Louis, Elsevier, 438—439.

15. Husfeldt, A. W., Endres, M. I., 2012: Association between stall surface and some animal welfare measurements in freestall dairy herds using recycled manure solids for bedding. *J. Dairy Sci.*, 95, 5626—5634.

16. Huxley, J., Whay, H. R., 2006: Cow based assessments. Part 2: Rising restrictions and injuries associated with the lying surface. *UK Vet. (Kiev)*, 11, 1—6.

17. Manske, T., Hultgren, J., Bergsten, C., 2002: The effect of claw trimming on the hoof health of Swedish dairy cattle. *Prev. Vet. Med.*, 54, 113—129.

18. Milosavljevič, P., Savič-Stevanovič, V., 2013: Frequency of some acropodium diseases in dairy cows in Serbia. *Acta Veterinaria (Beograd)*, 63, 247—254.

19. Nordhoff, M., Moter, A., Schrank, K., Wieler, L. H., 2008: High prevalence of treponemes in bovine digital dermatitis – a molecular epidemiology. *Vet. Microbiol.*, 131, 293—300.

20. Olechnowicz, J., Jaskowski, J. M., Antosik, P., Bukowska, D., Urbaniak, B., 2010: Claw diseases and lameness in polish Holstein-Friesian dairy cows. *Bull. Vet. Inst. Pulawy*, 54, 93—99.

21. Rasmussen, M., Capion, N., Klitgaard, K., Rogdo, T., Fjeldaas, T., Boye, M., Jensen, T. K., 2012: Bovine digital dermatitis: Possible pathogenic consortium consisting of Dichelobacter nodosus and multiple Treponema species. *Vet. Microbiol.*, 160, 151—161.

22. Sogstad, A. M., Fjeldaas, T., Osteras, O., Plym Forshell, K., 2005: Prevalence of claw lesions in Norwegian dairy cattle housed in tie stalls and free stalls. *Prev. Vet. Med.*, 70, 191—209.

23. Somers, J. G. C. J., Frankena, K., Noordhuizen-Stassen, E. N., Metz, J. H. M., 2005: Risk factors for interdigital dermatitis and heel erosion in dairy cows kept in cubicle housing in The Netherlands. *Prev. Vet. Med.*, 71, 23—34.

24. Tóthová, Cs., Nagy, O., Seidel, H., Paulíková, I., Kováč, G., 2011: The influence of hoof diseases on the concentrations of some acute phase proteins and other variables of the protein profile in heifers. *Acta Veterinaria (Beograd)*, 61, 141—150.

25. Tucker, C. B., Weary, D. M., Fraser, D., 2003: Effect of three types of free-stall surfaces on preferences and stall usage by dairy cows. *J. Dairy Sci.*, 86, 521—529.

26. Tucker, C. B., Weary, D. M., 2004: Bedding on geotextile mattresses: How much is needed to improve cow comfort? *J. Dairy Sci.*, 87, 2889–2895.

27. Weary, D. M., Taszkun, I., 2000: Hock lesions and free-stall design. *J. Dairy Sci.*, 83, 697—702.

28. Webster, J., 2002: Effect of environment and management on the development of claw and leg diseases. In *Recent Development and Perspectives in Bovine Medicine,* Hannover, Germany, Hildesheimer Druck- und Verlags-GmbH, 248—256.

Received September 24, 2014

FOLIA VETERINARIA, 58, 3: 197—200, 2014

MACROSCOPIC CHARACTERISTICS AND VARIABILITY OF SELECTED LYMPHATIC STRUCTURES IN THE RABBIT

**Maloveská, M.[1], Krešáková, L.[1], Petrovová, E.[1]
Vdoviaková, K.[1], Boldižár, M.[2], Elias, M. Z. J.[3]**

**[1]Department of Anatomy, Histology and Physiology
[2]Equine Clinic
University of VeterinaryMedicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic
[3]Faculty of Veterinary Medicine, Eduardo Mondlane University, Maputo
Mozambique**

marcela.maloveska@gmail.com

ABSTRACT

The lymphatic system can be anatomically divided into the cellular component, that is, the lymphoreticular or lymphatic tissue of which all lymphatic organs are composed, and the actual lymphatic vascular system. The aim of our study was to provide a detailed description of the two largest vascular components of the lymphatic system — the *ductus thoracicus* and the *cisterna chyli.* For visualizing these structures we used ink of various colours, as well as the X-ray method employing contrast media. The anatomical variability was presented in varied shapes, sizes, and placement of the *cisterna chyli,* whereas for the *ductus thoracicus* we focused upon its course, its non-uniformity and the location of its opening into the venous system. In 70 % of the cases the chyle cistern was a plexiform structure located at the roof of the abdominal cavity and extending into the thoracic cavity, spread between the 2nd—3rd lumbar and the 11th–12th thoracic vertebra. The thoracic duct in all rabbits starts to form in the thoracic cavity and leads to the dorsolateral right surface of the thoracic aorta, ventrally from the *v. azygos dextra* up to the level of the 4th – 5th rib, where it disappeared from the right side. It entered uniformly in the area of connection of the *v. subclavia sinistr*a with *v. jugularis externa sinistra into the venous system*. An enlargement was not recorded. In 20 % of the cases, the *ductus thoracicus* was located left of the *thoracic aorta.*

Key words: anatomical description; cisterna chyli; rabbit; thoracic duct; variations

INTRODUCTION

The rabbit has established a dominate place in the number of experimental and research activities in the areas of biology, physiology and medicine. Despite this, the detailed information concerning the topography of the *ductus thoracicus* (DT) and *cisterna chyli* (CC) in the rabbit are rare. Results gained from the macroscopic observation of the thoracic duct and cisterna chyli with the use of various spraying materials enables us to use the rabbit as an experimental model in thoracic surgery for lymph circulation disorders.

Cisterna chyli represents a sac-like, often non-uniform and indented formation located at the top of the abdominal cavity, which after crossing into the thoracic cavity narrows and continues cranially as the *ductus thoracicus* [14]. Disorders of the lymphatic circulation in the thoracic cavity are usually the result of inherited malformation, infectious disease, malignancies, and last, but not least, trauma. Lymphatic vessels, as opposed to blood vessels, are not easily visible and damage can occur during surgery of the oesophagus, lungs, heart, or the large vessels [16]. The escape of lymph into the thoracic cavity causes chylothorax, which has been described in veterinary medicine as well as human medicine [13]. Chylothorax belongs among the group of pleural effusions with its typical biochemical indicators. Possible leakage does not appear as an immediate result and clinical symptoms appear only after the failure of the compensatory mechanisms [15]. One of the manners of therapy for chylothorax is ligation of the thoracic duct with access through the thoracic wall [12], or percutaneous embolization of the *ductus thoracicus* [6]. A perfect knowledge of surgical anatomy is important at the stage of preparing the surgical field.

The aim of our study was to provide a detailed description of the two largest formations of the vascular components of the lymphatic system — the *ductus thoracicus* and the *cisterna chyli*.

MATERIALS AND METHODS

The experiments were carried out on ten rabbits (age 13—15 weeks). We used New Zealand white male rabbits (breed HY+) 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). Drinking water was available to all animals *ad libitum*. Euthanasia was performed by embutramide (T-61, in a dosage of 0.3 ml.kg^{-1} i. v.). Into the easily-accessed popliteal lymph nodes *(lnn. poplitei)* of both pelvic limbs, we slowly injected ink (Drawing ink, KOH-I-NOOR HARDMUTH, CR) for the purpose of visualizing the lumbar trunks, which then coloured the chyle cisternas as well. The abdominal cavity was opened by surgical incision on the *linea alba* and two transversal incisions were done behind the costal arch with the intention of maximizing the access to the roof of the abdominal cavity. The DT was observed after the opening of the thoracic cavity. The arrangement, course and variability of the lymphatic structures were observed macroscopically and documented using a digital camera (CANON –POWER SHOT SX 40 HS) and surgical microscope (Leica M 320). When using the X-ray, we proceeded in the same way, but for better optical inspection of the procedure, we mixed the contrast substance (Uro-

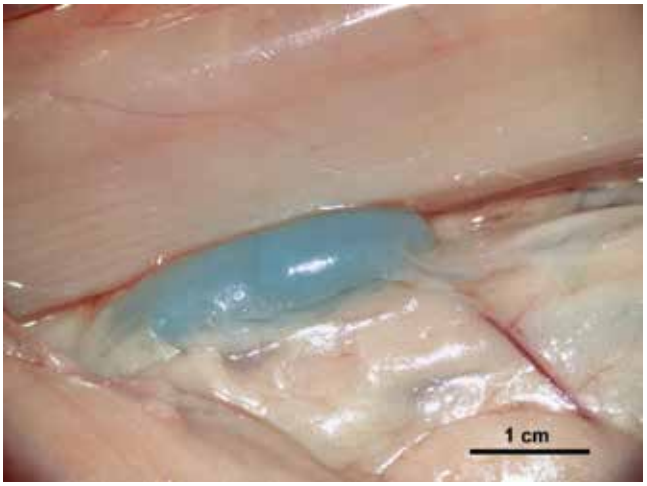

Fig. 1. *Cisterna chyli* as an elliptical, sac-like structure
Magn. ×3.2

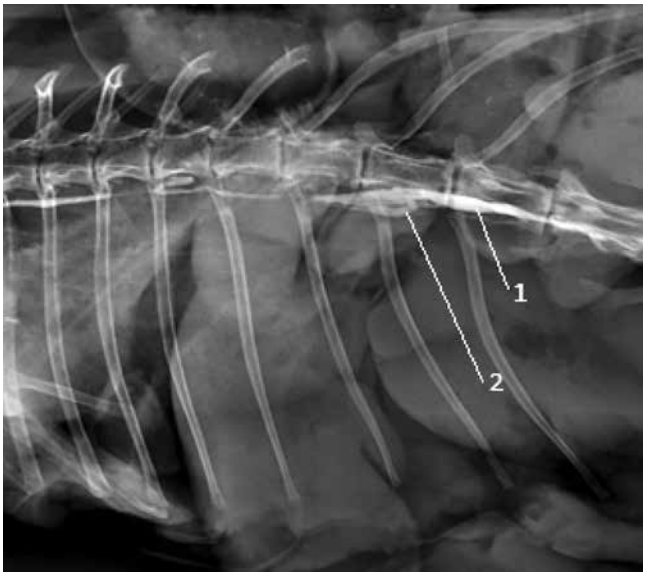

Fig. 2. Partial duplication of ductus thoracicus immediately upon exiting *cisterna chyli*
1 — *cisterna chyli*; 2 — *ductus thoracicus*
X-ray

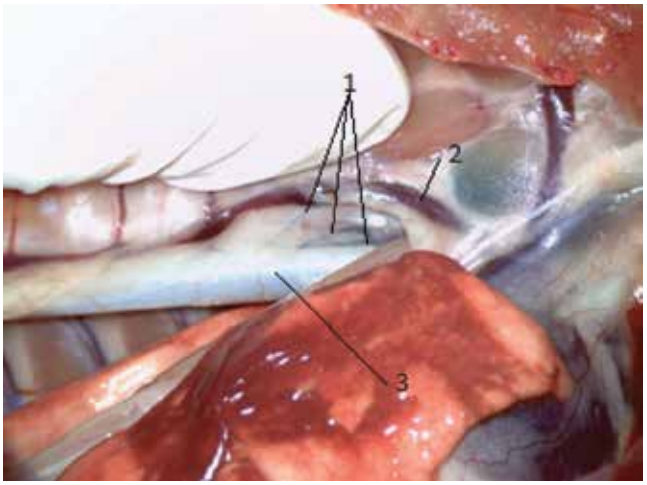

Fig. 3. Network course of *ductus thoracicus* upon passage to the left surface of *aorta thoracica*
1 — *ductus thoracicus*; 2 — *v. azygos dextra*; 3 — *aorta thoracica*
Macroscopic image

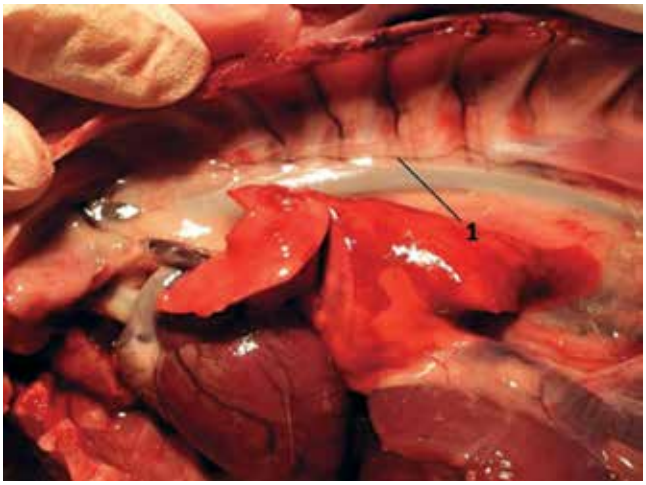

Fig. 4. Leftward positioning of *ductus thoracicus*
1 — *ductus thoracicus*
Macroscopic image

grafin 76 % sol. inj. Bayer Pharma AG, Germany, at a dose of 20 ml per rabbit) beforehand with the ink. X-ray images were obtained by means of an instrument DRTECH FDX D 810 (Drtech Corp., Corea). The anatomical nomenclature presented corresponds to the NAV [5].

RESULTS AND DISCUSSION

In rabbits, the *Cisterna chyli* begins to form at the level of the second or third lumbar vertebra. It is at the same level where the *a. renalis sinistra is* located. It passes the diaphragm through the *hiatus aorticus* together with the aorta, where we observed a slightly constricted chyle cistern. After entering into the thoracic cavity it expands again and from the penultimate intercostal space, it continues cranially as the *ductus thoracicus*. Its shape varies considerably. As a uniform, elliptical, sac-like structure, the *cisterna chyli* is recorded in only 20 % of the cases (Fig. 1). In 10 %, it was cranially bifurcated from the *crura diaphragmatis* and then united with the formation of the thoracic duct. In 70 % of the cases, we observed more of a non-uniform, plexiform and fusiform shape of the CC. The dimensions vary considerably in relation to nourishment and the current state of intestinal filling. Its length can range from 2 to 5 cm and the width is hard to measure because of its segmentation. We observed that it caudally receives two lumbar lymphatic trunks, in 90 % and in 10 %, three *trunci lumbales.* The unpaired lymphatic trunks (which deliver lymph from the organs of the digestive system) enter ventrally into the chyle cistern at the level of the *glandula adrenalis dextra. The ductus thoracicus* emerges, according to our observation, in the thoracic cavity, where CC suddenly reduces its diameter at approximately the level of the 11th thoracic vertebra. It runs cranially along the dorsal side of the thoracic aorta with a slight shift to the right. The thoracic duct runs close to the *v. azygos dextra*. At the location where the *aorta thoracica* connects with the oesophagus, the DT moves left from the mid-level of the chest and is no longer visible from the rig

Located at the *arcus aortae* (second rib), lays the thoracic duct more ventral in comparison with its caudal parts. At this location the DT loses connection with the aorta. The DT leaves beneath the left *m. longus colli* and enters the venous system at the level of the *v. subclavia sinistr*a connecting with the *v. jugularis externa sinistra* (approximately 1—2 cm caudally from the left clavicle). Not even in one case, did we observe enlargement of the terminal DT parts. However, the variability was recorded in the subsequent endpoints. After the exiting of the DT from the CC, there appeared in 20 % of the cases, a partial doubling of the thoracic duct (Fig. 2). In 30 %, there were created parallel running DT branches for a short sections (on the same side) and in 10 % of the cases, we observed the network course of the thoracic duct at the location of its passage to the left surface of the *v. azygos dextra,* which subsequently adjusted the connections of all branches (Fig. 3). In two individuals, we observed the DT course on the left side of the *aorta thoracica* (Fig. 4), something not excluded by some authors.

The topographical anatomy and variability of the DT and CC has been documented for some types of domestic mammals [18], [9] e. g., dogs [7], [11], [8] and cats [10]. The information concerning the anatomical description of these two structures in the rabbit is relatively scarce in comparison with the other types of farm and pet animals. This was described partly in the publications of Craigie [4], Nejedlý [17], Barone [1] and in the anatomical atlases of Popesko [19] and Barone [2]. In the available literature, we found only one more recent study [3] concerning the macroscopic research of the DT and the CC in the rabbit.

CONCLUSION

The results presented in this paper show that the shape of the ciserna chyli was flexiform and segmented in most cases and an enlargement of the *ductus thoracicus* was not recorded. Despite the fact that the results of our study are mostly of a descriptive character, it should facilitate the use of the rabbit as an experimental model for thoracic surgery for the treatment of lymphatic circulation disorders. The studies carried out on laboratory animals for the purpose of developing new, less invasive therapeutic methods require not only precise knowledge of anatomical proportions, but also reporting and documentation of anatomical variations.

ACKNOWLEDGEMENT

The study was carried out within the framework of the project ITMS No. 26220120066 "Centre of excellence for biomedical technologies", which is supported by the Operational Programme "Research and Development", financed through the European Regional Development Fund.

REFERENCES

1. Barone, R., 1996: *Anatomie Compareé des Mammiféres domestiques.* Vigot Fréres, Paris, 854—855.

2. Barone, R., Pavaux, C., Blin, P. C., Cuo, P., 1973: *Atlas D' Anatomie du Lapin*. Masson et cie. Paris, 99—102.

3. Besoluk, K., Tipirdamaz, S., Yalcin, H., Eken, E., 2001: Macroanatomic investigations on the cisterna chyli and thoracic duct of the White New Zealand rabbit. *Vet. Bil. Derg*., 17, 2, 51—56.

4. Craige, E. H., 1948: *Bensley's Practical Anatomy of the Rabbit*. 8th edn., Toronto, University of Toronto Press. Toronto, 327, 337.

5. Danko, J., Šimon, F., Artimová, J., 2011: *Nomina Anatomica Veterinaria*. UVLF Košice, 267 pp.

6. Davies, H. E., Rosenstengel, A., Gary Lee, Y. C., 2011: The decline in the importance of surgery in diseases of the pleura (In Slovak). *Current Opinions in Pulmonary Medicine/CS,* 8, 49—56.

7. De Freitas, V., Pieffer C. R., Zorzetto N. L., Seullner G., 1981: Über die Topographie des Ductus thoracicus beim Hund. *Anat. Anz.,* 149, 451—454.

8. DurasGomercic, M., Gomercic, T., Skrtic, D., Galov, A., Lucic, H., Vukovic S., 2009: The accessory thoracic duct in a dog. *Veterinarski Arhiv,* 79, 157—165.

9. DurasGomercic, M., TrbojevicVukicevic, T., Gomercic, T., Galov, A., Fruk, T., Gomercic, H., 2010: The cisterna chyli and thoracic duct in pigs *(Sus scrofa domestica). Veterinary Medicine*, 55, 30—34.

10. Eken, E., Besoluk, K., Tipirdamaz, S., Gezici, M., Bahar, S., 2002: Thoracic duct in cats *(Feliscatus). Revue Méd. Vét.,* 153, 717—721.

11. Enwiller, T. M., Radlinsky, M. G., Mason, D. E., Roush, J. K., 2003: Popliteal and mesenteric lymph node injection with methylene blue for coloration of the thoracic duct in dogs. *Vet. Surg.,* 32, 359—364.

12. Fossum, T. W., 2011: New development and mini-invasive techniques in thoracic surgery, cardiosurgery and endovascular interventions in small animals (In Czech). In *Proceedings of the seminar "Endoscopy and Mini-invazive Interventioms"*. FVL VFU and LF MU Brno. 5—21.

13. Kumar, K. S., Ramesh, P., 2007: Chylothorax associated with lung lobe torsion in a dog – a case report. *Veterinarski Arhiv*, 77, 561–566.

14. Lešník, F., Danko, J., 2005: *Medicinal Lymphology* (In Slovak). Hajko and Hajková, Bratislava, 57.

15. Mallick, A., Bodenham, A. R., 2003: Disorders of the lymph circulation: the irrelevance to anaesthesia and intensive care. *Br. J. Anaesth*., 91, 265—72.

16. Manďák, J., Habal, P., Štětina, M., Harrer, J., 2011: Chylothorax — a rare complication after cardiac surgery (A case report). *Acta Medica,* 54, 37—39.

17. Nejedlý, K., 1965: *Biology and Systematic Anatomy of Laboratory Animals* (In Czech). State Pedagogical Publ. House, Prague. 473—474.

18. Nickel, R., Schummer, A., Seiferle, E., 1981: *The Circulatory System, the Skin, and the Cutaneous Organs of the Domestic Mammals.* Verlag Paul Parey Berlin, Hamburg, 269 pp.

19. Popesko, P., 1992: *Anatomy of Farm Animals* (In Slovak). Príroda, Bratislava, 454 pp.

20. Popesko, P., Rajtová, V., Horák, J., 1990: *Anatomical Atlas of Small Laboratory Animals* (In Slovak). Príroda, Bratislava, 67—78.

Received, November 4, 2014