FOLIA VETERINARIA

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

ISSN 0015-5748
eISSN 2453-7837

2

LXI • 2017

FOLIA VETERINARIA is a scientific journal issued by the University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia.
The journal is published quaterly in English (numbers 1—4) and distributed worldwide.

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Electronic Publisher: De Gruyter Open, Bogumila Zuga 32A str. 01-811
Warsaw, Poland

ISSN 2453-7837 on-line
ISSN 0015-5748 print
EV 3485/09

Publisher's identification number: IČO 00397474

June 2017

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
SLOVAKIA

Folia Veterinaria
Vol. 61, 2017

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2017

FOLIA VETERINARIA, 61, 2, 2017

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OPEN

DOI: 10.1515/fv-2017-0011

FOLIA VETERINARIA, 61, 2: 5—10, 2017

DETECTION OF *TOXOPLASMA GONDII* ANTIBODIES IN FARMED TURKEYS (*MELEAGRIS GALLOPAVO*)

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ABSTRACT

Several seroprevalence studies have been conducted on the natural infections of *Toxoplasma gondii* in domestic chickens around the world but only a few have published data on turkeys. The purpose of this study was to investigate the level of exposure of farmed Nigerian turkeys to *T. gondii* infection. Sera obtained from 320 turkeys reared intensively in 3 states of southwest Nigeria were screened for *T. gondii* antibodies using a modified agglutination test. Antibodies were detected in 4.1 % (13/320) of the turkeys with titres of 1 : 20 in 7 turkeys, 1 : 40 in 5 and 1 : 80 in 1, while none was seropositive at 1 : 160 or 1 : 320. The seroprevalence of *T. gondii* was comparable among turkeys regardless of their breed, age, location and management system (P > 0.05). None of the variables were significantly associated with *T. gondii* antibodies by multivariate logistic regression. This first report of *T. gondii* infections in Nigerian turkeys recommends that turkey meat and its products be adequately processed before consumption.

Key words: meat; Nigeria; *Toxoplasma gondii*; turkey; zoonosis

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite infecting warm-blooded animals including birds [6, 7]. Birds are important intermediate hosts of *T. gondii* considering the fact that they feed directly from the ground and also serve as vital sources of infection for cats, humans, and other animals. *T. gondii* tissue cysts have been detected in internal organs, muscles and even in the eggs of these birds [3, 13, 14].

Turkeys are an important source of meat worldwide and are mainly reared on the free-range system either in backyard operations or on a large commercial scale. They are susceptible to *T. gondii* infection and can serve as its intermediate hosts [8, 19, 22]. Edible tissues of turkeys have been shown to harbour *T. gondii* cysts, hence, raw or undercooked turkey meat is a potential risk for parasitic transmission to humans [5].

The prevalence of *T. gondii* infections in free-range chickens has been considered as one of the best indicators of soil contamination with *T. gondii* oocysts, because of the chicken's habit of scavenging for food on the ground [3]. This, by extension, could apply to other birds such as sparrows, pigeons, ducks and turkeys, since they also scavenge for food, thus, they might play some significant role in the epidemiology of *T. gondii* infections. Serosurveillance for *T. gondii* infection in chickens has been conducted extensively [3], with only a few studies available on turkeys. Worldwide, reports on the seroprevalence of *T. gondii* in turkeys revealed rates ranging from ≤ 1 % to 80 % and varying from one place to another [11, 15, 16].

In Nigeria, turkey farming is rapidly growing at a production estimate of about 1.5—2 million tons per year through intensification and production and development of large breeds [18]. However, to our knowledge, there is, so far, no available study on the seroprevalence of *T. gondii* infections in Nigerian turkeys. This indifference regarding the studying of the level of exposure of turkeys to *T. gondii* infection and the possibility of humans acquiring the infection through consumption of turkey meat, could be because the disease does not produce clinical symptoms in turkeys and also does not appear to affect their productivity. However, infected turkeys could become a public health risk for humans. This current study was therefore aimed at investigating the level of exposure of farmed Nigerian turkeys to *T. gondii* infection in order to provide baseline data for assessing the possible risk of human infections through turkey meat consumption.

MATERIALS AND METHODS

Sample collection

The sampling sites for this study were Ondo, Osun and Oyo states of southwest Nigeria (Fig. 1). Blood samples were randomly collected from 320 intensively reared turkeys (294 on the floor, 26 in cages) located on farms in Iwo (7°39' N, 4°9' E), Osogbo (7°48' N, 4°37' E), Ibadan (7°22' N, 3°58' E), and Akure (7°15' N, 5°5' E). Sera were obtained from individual samples and stored at –20 °C until processed for serology.

Modified agglutination test

The sera obtained from the turkeys were tested for the presence of antibodies (IgG) to *T. gondii* using formalin-killed, whole *T. gondii* tachyzoites (RH strain) and two-fold dilutions of each serum from 1:20 to 1:640 in a modified agglutination test (MAT) as previously described [3]. Briefly, the sera were diluted with phosphate buffered saline (PBS, pH 7.2). The tachyzoites were diluted with antigen diluting buffer (alkaline borate buffer at pH 8.7, containing 0.4 % bovine serum albumin and 0.2 % sodium azide) to which Evans blue dye solution (as an indicator) and 2-mercaptoethanol were added. Positive and negative control sera were incorporated in each plate with the same dilutions as the test sera. Sera with MAT titres of 1 : 40 or higher with visible agglutination of the formalin-fixed parasite at the base of the U-bottom microtitre plate were considered positive, while the formation of a button or pellet was indicative of a negative sample.

Statistical Analysis

The data obtained from this study were analysed using SPSS 20 statistical software (SPSS Inc., Chicago, IL, USA). Univariate analysis was done to evaluate the strength of the relationship between outcome variables and the explanatory variables. The independent effects of variables were assessed using a multivariate logistic regression model to determine the predictors' effect on the response variable. All analyses were based on the 5 % level of significance.

RESULTS

The serological analysis showed that out of the 320 sera screened, antibodies to *T. gondii* were detected in 13 (4.1 %) of the turkeys with a titre of 1 : 20 in 7, 1 : 40 in 5 and 1 : 80 in 1, while none were seropositive at 1 : 160 or 1 : 320 (Table 1). The seroprevalence in adult turkeys (2.2 %, 4/179) was lower than in growers (6.4 %, 9/141). None of the male turkeys tested positive, while 5.7 % (13/228) of the females had antibodies to *T. gondii*. The seropositivity was higher in turkeys raised on the floor 4.1 % (12/294) compared to those in the cages 3.8 % (1/26). Both exotic and local breeds had similar seroprevalence rates (4.5 %), while none were positive among cross-bred turkeys. The bivariate analysis showed that among other variables (age, breed, management system and location), only the sex of the turkeys was significantly associated with *T. gondii* infections (Table 2). However, on fitting the data to a multivariate logistic re-

Table 1. Seropositivity and antibody titre of *T. gondii* in turkeys from Ondo, Osun and Oyo states of Nigeria

	Titre of antibodies					
	Positive [%]	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**
Breed						
Exotic	7 (4.5)	4	2	1	0	0
Local	6 (3.9)	3	3	0	0	0
Cross	0 (0)	0	0	0	0	0
Sex						
Male	0 (0)	0	0	0	0	0
Female	13 (5.7)	7	5	1	0	0
Age						
Adult	4 (2.2)	2	2	0	0	0
Grower	9 (6.4)	5	3	1	0	0
Total	**13 (4.1)**	**7**	**5**	**1**	**0**	**0**

gression model (Table not shown), it was detected that sex was also not significantly associated with *T. gondii* infections but was rather a confounder.

DISCUSSION

Turkeys are susceptible to clinical toxoplasmosis [12, 19] and can harbour cysts of *T. gondii* in their tissues, thereby posing a potential public health risk to individuals who consume their meat raw or undercooked. This study was therefore conducted to determine the exposure of turkeys farmed for food in 3 southwestern states of Nigeria to *T. gondii* infections. The seroprevalence obtained in this study was lower than the 59.5 % reported in Egypt [10], but comparable to the 10 % obtained in the USA [19]. Although there are very few studies available on the seroprevalence of *T. gondii* in naturally infected turkeys that

Table 2. Analysis of risk factors associated with *T. gondii* infection in turkeys from Ondo, Osun and Oyo states of Nigeria

Variable	**Test Result**		**Unadjusted OR (95 % CI)**	**X^2**	**P-value**
	Toxoplasma Negative [%]	**Toxoplasma Positive** [%]			
Sex					
Male	92(100)	0 (0)	1.1 (1.03—1.10)	5.468	0.023*
Female	215 (94.3)	13 (5.7)			
Age					
Grower	132 (93.6)	9 (6.4)	0.3 (0.1—1.11)	3.482FT	0.086
Adult	175 (97.8)	4 (2.2)			
Management					
Floor	282 (95.9)	12 (4.1)	0.9 (0.11—7.5)	0.003	1.000
Cage	25 (96.2)	1 (3.8)			
Bred with other avian species in the same pen					
Yes	281 (96.2)	11 (3.8)	2.0 (0.4— 0.34)	0.747FT	0.317
No	26 (92.9)	2 (7.1)			
Breed					
Exotic	148 (95.5)	7 (4.5)	–	1.454	0.483
Local	128 (95.5)	6 (4.5)	–		
Cross	31 (100)	0 (0)	–		

OR — Odd Ratio; CI — Confidence Interval; FT — Fisher Test; * — Significant ($P < 0.05$)

Fig. 1. Map showing the sampling sites of turkeys in Southwestern Nigeria

could have served as comparison, this study (which to the best of our understanding is the first report on *T. gondii* infection in Nigerian turkeys) showed that turkeys in Nigeria, as in other countries of the world, are exposed to *T. gondii*. The low seroprevalence and the detection of a low titre of antibodies in the screened turkeys compared with our previous report on Nigerian domestic chickens using MAT [1, 2], suggest that turkeys reared intensively on farms have lower exposure to *T. gondii* than free-range chickens which obviously have more access to the contaminated environments. Although, chickens are known to be one of the resistant hosts for developing clinical toxoplasmosis [7, 13], studies are needed to compare the resistance of turkeys and domestic chickens so as to determine the best species to rear in places with high exposure to infective sources for *T. gondii*.

The analysis of variables in this study showed that the seroprevalence of *T. gondii* infections were comparable among turkeys regardless of their sex, breed, age, location, and management system with no statistically significant association found with *T. gondii* infections ($P > 0.05$), particularly after a multivariate logistic regression analysis. However, it is worth noting that the detection of higher seroprevalence in adults rather than growing turkeys, as observed in this present studies, has also been reported in chickens in our previous study [2]. This could be due to more active feeding. On the contrary, some studies have suggested that repeated exposure of adults to sources of *T. gondii* infected birds during their longer lifetime could lead to higher prevalences in adults [9, 17]. Similarly, the higher seroprevalence obtained for floor-raised turkeys than those reared in cages may be associated with more access to litter that might have been contaminated with *T. gondii* oocysts than birds fed with drinkers and feeders. Studies are needed to determine whether other variables, like gender, breed and location of farms are potential predictors of *T. gondii* infection in turkeys.

It is interesting to know that despite the absence of domestic cats in the farms studied, both caged and floor-raised turkeys had at least a sample testing positive for *T. gondii* antibodies. This suggests the presence of other sources of infection on the farms. *T. gondii* oocysts could have been introduced to these farms by feral cats or through other sources like winds, earth-worms, coprophagous insects, rain or surface water, and feeds [21]. Feral cats that prey on rodents that are usually present on poultry farms have been suggested to play a more important role in the epidemiology of *T. gondii* infection than domestic cats [4]. Hence, the

absence of domestic cats is not a predictor of the absence of *T. gondii* infections.

Although the presence of antibodies to *T. gondii* in the turkeys in this study is simply an indication of exposure to the parasite and does not necessarily imply infectivity to humans, certain experimental studies have demonstrated that the tissues of seropositive turkeys can induce *T. gondii* infection in mice [20]. Furthermore, in experimental studies set up to mimic natural *T. gondii* infection in turkeys, the presence of viable cysts of the parasite was demonstrated in edible tissues including the drumstick muscle, thigh, breast, heart and liver [5]. Hence, raw or undercooked turkeys and their products such as sausages and cured turkey ham are potential sources of *T. gondii* infection for humans.

In conclusion, the present study reports a low prevalence of *T. gondii* infection in farmed turkeys in Nigeria and suggests that consumption of turkey meat is a likely source of acquiring toxoplasmosis. We recommend that all poultry meat and their products be adequately processed before human consumption as a preventive measure to avoid the potential risk of *T. gondii* infection.

ACKNOWLEDGEMENT

We thank our students who were involved in the field work during sample collection. The authors declare that there is no conflict of interests.

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Received June 1, 2016
Accepted March 15, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0012

FOLIA VETERINARIA, 61, 2: 11—16, 2017

DETERMINATION OF CAPSAICIN CONTENT AND PUNGENCY LEVEL OF DIFFERENT FRESH AND DRIED CHILLI PEPPERS

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ABSTRACT

One of the traditional plants that have so many pharmacological effects is chilli fruit (*Capsicum* sp.) that belong to the family Solanaceae. Around the world, five varieties of *Capsicum* are known, i. e., *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, and *C. pubescens*. Chilli peppers are known for causing the sensation of heat or burning when consumed. The heat sensation is incited by the type and the amount of a group of capsaicinoids; the alkaloids found only in chilli pepper pods. A widely used heat measurement of chilli peppers is the SHU (Scoville Heating Unit). This measurement is the highest dilution of a chilli pepper extract at which heat can be detected by a taste panel. Nowadays, the Scoville organoleptic test has been largely replaced by chromatographic methods which are considered to be more reliable and accurate. The HPLC (High Pressure Liquid Chromatography) method was used for the determination of capsaicin content in various fresh and dried peppers from the genera *C. chinense*. Currently, based on the results of HPLC, the hottest pepper has been Bhut Jolokia, followed by Habanero Red Savina and Habanero Yellow etc. The content of capsaicin in dried chillies is 7—10 times higher compared to fresh ones.

Key words: Bhut Jolokia; capsaicin; chilli peppers; Habanero; HPLC

INTRODUCTION

For a long time hot pepper fruit has been known all over the world as a delicious spice with a characteristic smell and taste. It is used for preparing spicy sauces and also it is very popular in Mexican and Asian cuisines. The value of hot pepper consists in its sensorial attributes, such as colour, spiciness and flavour [15]. Chilli peppers and their isolated constituents including capsaicinoids are appreciated because of their beneficial therapeutic effects, including antioxidants, anti-inflammatory, anticancer, antimicrobial and positive immunomodulatory effects.

Capsaicinoids are derivates of benzylamin. Differences within their structure depend mainly on their acyl moi-

eties, and three structural elements are involved: first of all, the length of the acyl chain (C8—C13), then the way it terminates (linear, iso or anteiso-series), and the presence or absence of unsaturation at the ω-3 (capsaicin type) or ω-4 carbon atom (homocapsaicin type I and II) [6]. Capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-nonenamide), is an active component of the red pepper. The level of the capsaicin in a seasoned pepper is around 0.025 %, and in the hot pepper around 0.25 % (9). Capsaicin represents 69 % in the group of capsacinoids; dihydrocapsacinoids with 22 %; nordihydrocapsacinoids with 7 %; and homocapsaicin and homohydrocapsaicin represents only 1 % in the group of capsaicinoids. Capsaicin and dihydrocapsaicin is approximately twice as pungent as nordihydrocapsaicin and homocapsaicin and they are responsible for the hotness of the pepper. The pungency of capsaicinoids and pepper containing preparations can be expressed in Scoville Heat Units (SHU) and the human palate can detect it even diluted in a 1 : 17 000 000 ratio. A widely used heat measurement of chilli peppers is the SHU [16]. This measurement is the highest dilution of a chilli pepper extract at which heat can be detected by a taste panel. Nowadays, however, the Scoville organoleptic test has been largely replaced by chromatographic methods which are considered to be more reliable and accurate [13].

The capsaicinoids have evolved in chilli peppers as a defence mechanism against mammalian predators; nevertheless, this trait is an important fruit quality attribute and one of the most important reasons chilli peppers are consumed. It is an extraordinarily versatile agent, and its use is ranging, in the fields from pharmaceutical purposes and nutrition (seasoning) to chemical weapons. It has been used as an analgesic against arthritis pain and inflammation [5]. It has also been reported to show anticancer effect [12] and to be active against neurogenic inflammation (burning and stinging of hands, mouth and eyes) [17]. The latter property is the basis for the use of capsaicin in defensive pepper sprays. Capsaicin has also been reported to show protective effects against high cholesterol levels and obesity [10]. Capsaicin and other members of the capsaicinoids group produce a large number of physiological and pharmacological effects on the gastrointestinal tract, the cardiovascular and respiratory systems, as well as the sensory and thermoregulation systems.

The amount of capsaicinoids in a chilli pepper pod is dependent on the genetic makeup of the plant and the environment where it is grown [21]. The amount of capsaicin in a given variety can vary depending on the light intensity and temperature at which the plant is grown, the age of the fruit, and the position of the fruit on the plant. Chilli peppers must be harvested at an appropriate degree of development in accordance with the criteria proper to the variety and the area in which they are grown.

MATERIALS AND METHODS

Six Habanero chilli varieties (Habanero Red Savina (HRS), Habanero Yellow (HY), Habanero Maya Red (HMR), Habanero Tasmania (HT), Habanero Paper Latern (HPL), Habanero Red (HR)), and a Bhut Jolokia variety (BJ) have been used in our experiments. The experiments have been started by buying seeds, then sowing, germination, transplanting gradually and care of mature plants, including adaptation of climatic conditions and fertilization in the greenhouse of the University of Veterinary Medicine and Pharmacy in Košice. After harvesting the ripe fruits collected from at least three plants, they were stored in a refrigerator at 0—4 °C for a maximum of one week. Samples of chilli pepper pods (6 pieces of each variety were used in the experiments) were dried within one week after harvest, when sufficient number of pods had been collected, in the stage of maturity in a laboratory oven with ventilation. Before drying, the chilli peppers were cut into halves or quarters (depending on the size) to speed up drying and to prevent undesired changes (moulds). Chilli peppers were dried along with the placenta and seeds. Drying was carried out in two stages, at 40 ± 5 °C in the first phase of 24 hours, and in the second phase for 12—24 hours, depending upon the water content. After drying, the chilli peppers were stored in a sealed glass container in a dry, dark place until analysis (not more than one month). The water content was determined in individually dried chilli peppers.

HPLC analysis of capsaicin

HPLC analysis of capsaicin content consisted of sample preparation, extraction and liquid chromatographical analysis.

Sample extraction: fresh and dried material were cut into pieces (all of the pods were mixed and homogenized together after the removal of the placenta and seeds). The extractions were carried out with ethanol at a ratio of 1 : 10,

sonication at the lasts 30 minutes, and then 4 hours of maceration with an extraction efficiency of 90 %.

HPLC analysis: column Ascentis Express RP-Amide 2.7 µm, 100 × 2.1 mm, gradient acetonitrile: 0.2 % HCOOH, 0 minutes 30 : 70, after 10 minutes 71 : 29, flow rate 0.5 ml.min, injection volume 1 µl, temperature 40 °C, and UV detection at 254 nm and 280 nm. Capsaicin content was determined based on a calibration curve and SHU units were determined by calculation.

According to the commonly accepted Scoville organoleptic test, the spicy strength of the samples are calculated by converting the capsaicin content expressed in grams of capsaicin per gram of pepper. This conversion to Scoville heat units is done by multiplying the capsaicin content in the pepper by the coefficient corresponding to the heat value for pure capsaicin and corrected for sample extraction efficiency according to the formula: 18 × µg.g.

RESULTS

The capsaicin content in fresh and dried samples of each chilli pepper was determined on the basis of compliance with the standard of capsaicin using HPLC. Fig. 1 shows a chromatogram of the standard capsaicin determination by HPLC using the optimized conditions described above. The highest peak in the chromatogram represents the standard of capsaicin at a wavelengths of 254 nm and 280 nm.

Table 1. Weight of fresh and dried chilli peppers and weight loss after drying

Chilli peppers	Weight of fresh peppers 6 pcs/Ø [g]	Weight of dried peppers 6 pcs/Ø [g]	Weight loss [%]
BJ	24.84/4.14	3.35/0.56	86.47
HRS	25.74/4.29	3.52/0.59	86.47
HY	29.88/4.98	3.42/0.57	88.15
HMR	46.74/7.79	6.75/1.13	85.49
HT	22.62/3.77	2.71/0.45	88.06
HPL	27.48/4.58	3.13/0.52	88.65
HR	49.26/8.21	7.25/1.21	85.26

BJ — Bhut Jolokia; HRS — Habanero Red Savina; HY — Habanero Yellow; HMR — Habanero Maya Red; HT —Habanero Tasmania; HPL — Habanero Paper Latern; HR —Habanero Red; Ø — arithmetic mean

Table 2. Capsaicin concentration in fresh and dried chilli peppers

Chilli peppers	Capsaicin in fresh chilli peppers [µg.g^{-1}]	Capsaicin in dried chilli peppers [µg.g^{-1}]
BJ	3 041	25 944
HRS	967	8 215
HY	522	6 284
HMR	275	2 677
HT	273	2 619
HPL	252	2 477
HR	327	1 128

BJ — Bhut Jolokia; HRS — Habanero Red Savina; HY — Habanero Yellow; HMR — Habanero Maya Red; HT — Habanero Tasmania; HPL — Habanero Paper Latern; HR — Habanero Red

Table 3. Calculated SHU in fresh and dried chilli peppers after correction for extraction yield

Chilli peppers	SHU in fresh chilli peppers [18 × µg.g^{-1}]	SHU in dried chilli peppers [18 × µg.g]
BJ	54 738	466 992
HRS	17 406	147 870
HY	9 396	113 112
HMR	4 950	48 186
HT	4 914	47 142
HPL	4 536	44 586
HR	5 886	20 304

BJ — Bhut Jolokia; HRS — Habanero Red Savina; HY — Habanero Yellow; HMR — Habanero Maya Red; HT — Habanero Tasmania; HPL — Habanero Paper Latern; HR — Habanero Red

The absorbance for standards of capsaicin have been used in preparation of the calibration curve. The results for the concentration of capsaicin in the analysed samples were calculated using the equation: y = 0.0009 x (µg).

Table 1 shows the weights of fresh and dried peppers (g) calculated as an average of six pods and the weight loss after drying (%). Habanero Red (HR) had the highest weight of fresh peppers and the lowest mass was found in Habanero Tasmania (HT). After drying, the weight of fresh chilli peppers decreased from 85.26 % to 88.65 %.

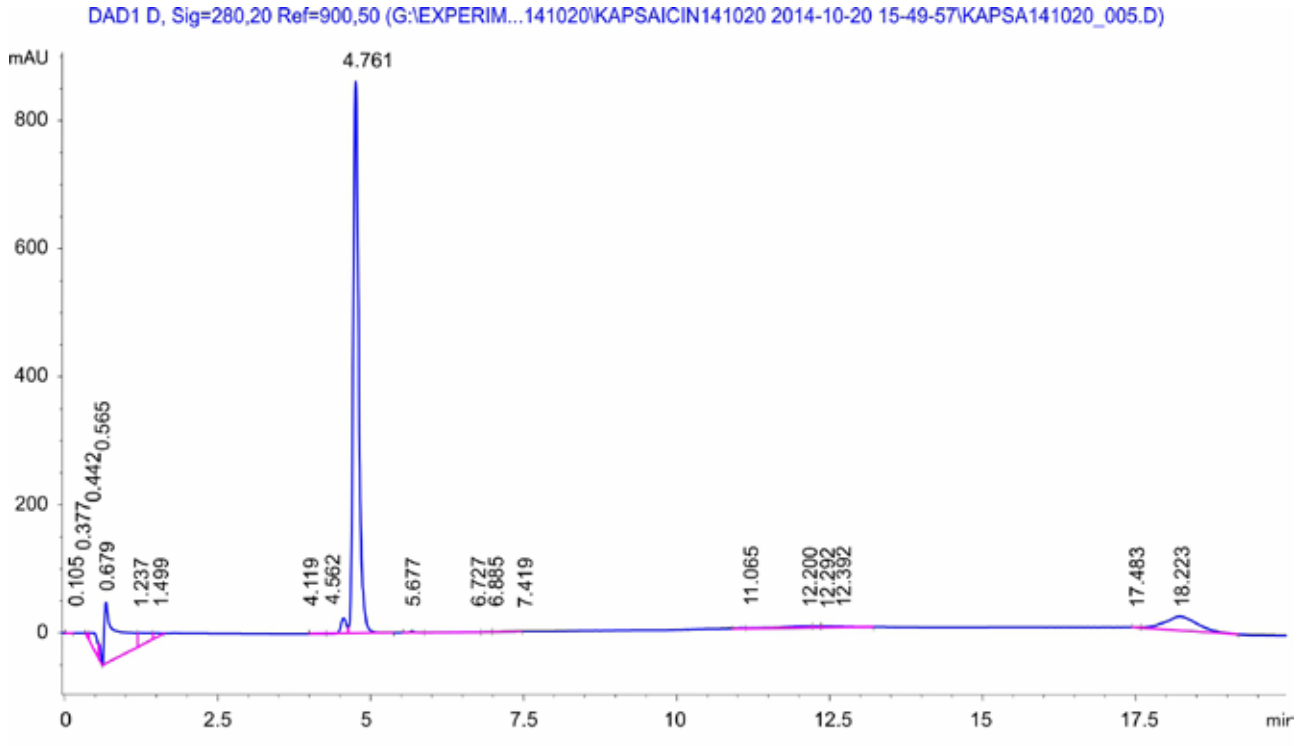

Fig. 1. Chromatogram of capsaicin standard determination using HPLC

In Table 2, fresh and dried chilli peppers are ranked according to capsaicin content expressed as $\mu g.g^{-1}$. The capsaicin content increased by drying 4—10 fold compared to fresh chilli peppers.

In Table 3, fresh and dried chilli peppers are ranked according to capsaicin content expressed as SHU units ($18 \times \mu g.g^{-1}$), calculated from capsaicin concentration given in Table 2 and corrected for the extraction yield (90 %). The content of capsaicin increased by drying 5 to 10 times compared with fresh chilli peppers.

DISCUSSION

In our study, we compared various Habanero varieties with the Bhut Jolokia variety. The results can be compared with the following study undertaken to compare the heat levels of Habanero Red Savina and Bhut Jolokia in a replicated field trial, i. e., establish whether Bhut Jolokia truly has a higher heat level than Habanero Red Savina. Once the fruit had matured on the plants in the field, a single harvest of 25 random mature fruits from at least 10 plants in each replication was combined. After harvest, the sample was dried and ground. The extraction of the capsaicinoids and the estimation of capsaicinoid amounts followed the high performance liquid chromatography (HPLC) procedures for the short run method as described by Collins et al. [4]. The HPLC data were converted from parts per million to SHU by multiplying the parts per million by 16 [3]. The environment is known to affect the heat level of chilli pepper cultivars [8]. Having a replicated field trial with standard control cultivars allowed for a better comparison of heat levels among cultivars. The results of the analysis for Bhut Jolokia indicated that it possessed an extremely high heat level, 1 001 304 SHUs, whereas Habanero Red Savina recorded a heat level of 248 556 SHUs. Independent tests confirmed this high level of heat for Bhut Jolokia with 927 199 SHUs and 879 953 SHUs from Southwest Bio-Laboratories and Ag-Biotech, respectively [3]. These results of Bhut Jolokia heat level have been higher compared to our results. The aim of the next study was to determine the content of capsaicin and dihydrocapsaicin in *Capsicum* samples collected from city markets in Riyadh (Saudi Arabia), calculate their pungency in Scoville heat units (SHU) and evaluate the average daily intake of capsaicin for the population of Riyadh. The samples consisted of hot chil-

lies, red chillies, green chillies, green peppers, red peppers and yellow peppers. The extraction of capsaicinoids was done using ethanol as a solvent, while high HPLC was used for separation, identification and quantitation of the components. The limit of detection (LOD) of the method was 0.09 and 0.10 μg.g^{-1} for capsaicin and dihydrocapsaicin, respectively, while the limit of quantification (LOQ) was 0.30 and 0.36 μg.g for capsaicin and dihydrocapsaicin, respectively. Hot chillies showed the highest concentration of capsaicin (4249 ± 190.3 μg.g) and the highest pungency level (67 984 SHU) comparable with our results for Bhut Jolokia [1].

Capsaicinoids are mainly ingested as naturally occurring pungency-producing components of *Capsicum* spices (chilli, cayenne pepper, red pepper). Their concentrations typically range from 100 μg.g in chilli pepper to 2500 μg.g in red pepper [14]. Pepper varieties from *Capsicum* annuum, *C. frutescens* and *C. chinense* were found to contain 220—20 000 μg total capsaicinoids.g dry weight [18]. In another study, cayenne pepper samples had mean capsaicin and dihydrocapsaicin contents of 1320 and 830 μg.g dry weight, respectively [11].

Capsaicinoids are synthesized exclusively in the epidermal cells of the placenta of *Capsicum* fruits and are accumulated in blisters along the epidermis. Their biosynthesis begins approximately 20 days postanthesis, with a number of enzymes being involved in the biosynthetic pathway. The degree of pungency depends on the *Capsicum* species and cultivars, and the capsaicin and dihydrocapsaicin contents can be affected by different factors such as the developmental stage of the fruit and the environmental growth conditions [7]. The biosynthesis of capsaicinoids occurs in the placenta, where the specialised epidermal cells accumulate in vacuoles and excrete on the inner surface of the seed and pericarp; therefore, the capsaicinoids should accumulate preferentially in the placenta rather than in the pericarp. Recently, similar studies indicated that capsaicin is mostly located in vesicles or vacuole like sub-cellular organelles of epidermal cells of the placenta in the pod. The highest concentrations of capsaicin was found in the ovary and in the lower flesh (tip) and the lowest content of capsaicin can be found in the seeds. The gland on the placenta of the fruit produces the capsaicinoids. The seeds are not the source of pungency but they occasionally absorb capsaicin because they are in close proximity to the placenta. No other plant part produces capsaicinoids. The majority, about 89 %, of the capsaicin is associated with the placental partition of the fruit and nearly 5—6 % in the pericarp and the seed. The composition of capsaicin may vary among different varieties of the same species and with fruit of a single variety [2]. A likely explanation for our finding is that the presence of capsaicinoids in the pericarp suggests that capsaicinoids are translocated from the placenta to the pericarp tissue via the cell walls of the epidermal layer of the placenta. The removing of the placenta and, less likely, environmental factors are obviously the reasons why the capsaicin concentration in our chillies is lower.

Currently, hot air drying is popular for drying chilli due to a relatively short drying time, uniform heating and more hygienic characteristics. The temperature ranges from 45 to 70 °C (approximately 10 % moisture content), and this reduces drying time to less than 20 hrs. This temperature range gives the maximum colour values and minimizes the loss of volatile oils and discolouration. The initial average moisture content of fresh chilli is 85.15 %. The average moisture contents of all dried chilli is 11 %. Furthermore, the capsaicin content and hotness of dried chilli are higher than in the fresh chilli sample ($P \leq 0.05$) [19]. Comparing the capsaicin content in fresh and dried chilli peppers in our study, in dried chilli peppers the content was 4 to 10 times higher than in fresh ones. This was caused by the dehydration of the chilli matrix and improved extractability of capsaicin by cell disruption during the thermal process. On the other hand, there is a study by Topuz and Ozdemir [20] who reported that sun-dried Turkish paprika chilli, which was processed for 5—7 days, lost 24.6 % of the capsaicin content (approximately 12—14 % moisture content). Oven-dried Turkish paprika chilli, which was dehydrated at 70 °C for 90 min, lost 21.5 % of the capsaicin content. This was due to the temperature, time and drying methods.

CONCLUSIONS

From a practical point of view, the planting of six varieties of Habanero chilli peppers and Bhut Jolokia was successfully completed. After harvesting the fresh, and then also drying the chilli peppers, they were analysed by HPLC to determine the content of capsaicin. Based on the results, the most pungent chilli pepper is Bhut Jolokia, which has several times higher content of capsaicin (54 738 SHU) in fresh, and also dried fruits (466 992 SHU) compared to the

Habanero varieties, in turn was followed in order by, Red Savina, Yellow, Maya Red, Tasmania, Paper Latern, and Red. They were found to have lower values in the content of capsaicin, in contrast to the values reported by other studies. The pungency can be influenced by the weather conditions such as heat and it increases with the maturity of the fruit. The great impact was also post harvesting processing such as removing of the seeds and placenta when capsaicin content is decreased rapidly.

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Received January 17, 2017

Accepted April 7, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0013

FOLIA VETERINARIA, 61, 2: 17—21, 2017

OCCURRENCE OF *MALASSEZIA* YEASTS IN DERMATOLOGICALLY DISEASED DOGS

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ABSTRACT

The *Malassezia* genus is represented by several lipophilic yeasts, normally present on the skin of many warm-blooded vertebrates, including humans. The aim of this study was to investigate the occurrence of *Malassezia* yeasts in dogs with skin lesions (dermatitis, interdigital dermatitis and inflammation of anal sacs) and otitis externa. The presence of *Malassezia* spp. was investigated in a group of 300 dogs exhibiting clinical manifestations. The isolates of *Malassezia* were identified by using phenotypic (biochemical-physiological and morphological characteristics) and genotypic methods (PCR, RFLP-AluI, BanI and MspA1I) which allowed their precise identification. *Malassezia* yeasts were isolated from 84 specimens obtained from 76 positive dogs. *M. pachydermatis* was the most frequently isolated species (79 isolates) in this study. *M. furfur* was identified in four dogs and *M. nana* in one dog. The prevalence of isolated *Malassezia* spp. was 25.3 % in dogs with skin lesions; from which 36.0 % were dogs suffering from otitis externa, 24.5 % from dogs having dermatitis, 16.4 % from dogs with interdigital dermatitis and 14.3 % from dogs having inflammation of the anal sacs. A higher prevalence of *Malassezia* spp. was observed in animals with pendulous ears in comparison with dogs having erect ears.

Key words: dermatitis; diagnostics; dogs; *Malassezia*; otitis externa

INTRODUCTION

Canine otitis externa and dermatitis are frequently encountered diseases in a veterinary practice. The etiology of canine otitis externa is complex and involves many factors which can be classified as: predisposing, primary and perpetuating. Microorganisms, both bacteria and yeasts, are considered perpetuating factors [2]. The associated microflora of otitic ears include most often bacteria (Staphylococcus spp., Pseudomonas aeruginosa, *Proteus* spp., *Streptococcus* spp.) and yeasts (*Malassezia* spp., *Candida* spp. and other) [18]. *Malassezia* yeasts also play an important

role in the development of seborrheic, atopic and allergic dermatitis in dogs. Yeasts are known to be more frequently found in areas where there is an ample amount of sebum secretions [6]. These lipophilic yeasts may be isolated from normal ear canals and also healthy skin, but if environmental conditions are suitable, the otitis externa or dermatitis can be created by these pathogens [9]. So far, 16 species of *Malassezia* are known: *M. dermatis*, *M. japonica*, *M. obtusa*, *M. restricta*, *M. yamatoensis*, *M. furfur*, *M. globosa*, *M. slooffiae*, *M. sympodialis*, *M. pachydermatis*, *M. caprae*, *M. equina*, *M. cuniculi*, *M. nana*, *M. brasiliensis* sp. nov and *M. psittaci* sp. nov. [3]. Up to now, only *M. pachydermatis* [1, 4], *M. furfur* [10, 11, 13] and *M. obtusa* in participation with *M. furfur* [10] have been identified in dogs. The preliminary diagnosis of *Malassezia* dermatitis and otitis is suggested by the typical clinical findings, such as erythema, greasiness, alopecia, lichenification, variable hyperpigmentation and marked pruritus, and by the identification of yeasts in specimens obtained from affected areas, which demonstrate the lack of response to treatment with antibiotics, corticosteroids and immunotherapy [20]. The definitive diagnosis is based upon yeast identification composed of phenotypic and genotypic methods. The phenotypic methods (biochemical and morphological characteristics) are time-consuming and are subject to variable interpretation. Genotypic identification (fingerprinting methods, DNA sequence analysis and restriction analysis of PCR amplicons) is necessary for the exact diagnosis [29].

The aim of this study was to identify *Malassezia* yeasts in dermatologically diseased dogs and to determine their prevalence.

MATERIALS AND METHODS

The survey was carried out on 300 dogs with skin lesions, otitis externa or inflammation of the anal sacs (174 male and 126 female). The ages of the animals ranged from 8 weeks to 14 years. The samples were collected from affected body sites (external ear canals, interdigital areas, cutaneous lesions, and anal sacs) of dogs with clinical manifestation, by using sterile cotton swabs (Fungi-Quick, Dispolab, SR). The samplings were acquired before employing any antimicrobial therapy, particularly from the affected areas with symptoms, such as seborrhea, erythema, alopecia, scaly plaques or pruritic lesions. The secretions of the anal sacs were sampled by using a lavage with 0.9 % sodium chloride solution. The samples were inoculated on Sabouraud dextrose agar with chloramphenicol (SCA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India), Modified Leeming & Notman agar medium (MLNA) [19] and Modified *Candida*-Chrom agar (HIT) with Tween 40 [16] and incubated at 32 °C for 7 days. The preliminary identification of yeasts was based on both the macroscopic appearance of the colonies and the microscopic cell morphology. Each sample was stained by Gram and examined by microscopy for the presence of the typical *Malassezia* yeast cells. More detailed identification was performed according to Kaneko et al. [17]. DNA was recovered from solitary colonies grown on MLNA at 32 °C for four days.

All phenotypically positive samples recognized as *Malassezia* yeast cells were investigated by PCR-RFLP [14]. The Internal Transcribed Spacer 2 region (ITS2) was amplified by PCR using the ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [30] (Life Technologies, California, USA). PCR was modified according to Gaitanis et al. [15] and performed in a total volume of 50 µl. The reaction mixture consisted of 1×concetrated PCR Buffer (Life Technologies, California, USA), 3 mmol.l^{-1} $MgCl_2$ (Life Technologies, California, USA), 15 µmol of each primer (Life Technologies, California, USA), 0.1 mmol of dNTPs (Thermo Fisher Scientific, Massachusetts, USA), 2.5 U of Taq polymerase (Life Technologies, California, USA) and 2 µl of template DNA. Five µl of the PCR products were analyzed by electrophoresis in 1.5 % agarose gel at 120 V for 1 h; the gel was stained (GelRed, Biotium Inc., California, USA) and visualized under UV light. Lengths of the amplified DNA fragments were verified using GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA) and ran simultaneously. The restriction endonucleases AluI, BanI and MspA1I (New England Biolabs, Massachusetts, USA) were used for digestion of the PCR products at 37 °C for 3 h [16] in the amount of 10 U. Restriction fragments were analyzed in 3 % GelRed stained agarose gel at 120 V for 2 h and were visualized by UV light. Lengths of the amplified DNA fragments were verified using Thermo Scientific GeneRuler Low Range DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA). Reference strains of *Malassezia* spp. (M. cuniculi CBS 11721, *M. pachydermatis* CBS 1879, *M. furfur* CBS 4162, *M. slooffiae* CBS 7956, *M. globosa* CBS 7874, *M. nana* CBS 9557, *M. sympodialis* CBS 8334,

M. equina CBS 9969, *M. caprae* CBS 10434) (CBS-KNAW Fungal Biodiversity Centre Utrecht, Netherland) were used as a positive control.

RESULTS

Of the 300 animals examined, *Malassezia* yeasts were detected in 76 dogs by cultivation and microscopical methods (Table 1). From these positive animals, a total of 84 isolates were collected, of which 79 were identified as *M. pachydermatis* (six dogs had atopic dermatitis and also otitis externa with the occurrence of *M. pachydermatis*). Four isolates were identified as *M. furfur* (two *M. furfur* samples were isolates in association with *M. pachydermatis*) and one as *M. nana*. All phenotypically positive samples were confirmed by PCR-RFLP, obtaining the same results. In our group of dogs with clinical manifestations of disease, the prevalence of *Malassezia* spp. was 25.3 %. The highest prevalence of *Malassezia* was determined in dogs with otitis externa (36.0 %), followed by dermatitis (24.5 %), interdigital dermatitis (16.4 %) and infected anal sacs (14.3 %). The Malasezia yeasts occurred most frequently in the group of dogs with pendulous ears (32.8 %) in comparison to dogs with erect ears (14.2 %). No differences related to gender or age were observed.

Table 1. Prevalence of *Malassezia* species in diseased dogs

	Total number of dogs	Number of positive dogs	Prevalence [%]
Clinical status			
Diseased	300	76	25.3
Sex			
Male	174	44	25.3
Female	126	32	25.4
Age			
< 1 year	54	13	24.1
1—10 years	220	58	26.4
> 10 years	26	5	19.2
Type of ears			
Pendulous	180	59	32.8
Erect	120	17	14.2
Diagnosis			
Otitis externa	125	45	36.0
Dermatitis	106	26	24.5
Interdigital dermatitis	55	9	16.4
Inflammation of anal sacs	14	2	14.3

DISCUSSION

Due to the variability of the phenotypic methods for the precise identification of the pathogen, we used a genotypic method based on PCR-RFLP. This study demonstrated that RFLP applying restriction enzymes, which is one of several molecular techniques used for identification and classification of *Malassezia* yeasts, is a quick and accurate method.

From the microbiological perspective, *Malassezia* is considered to be the primary fungal pathogen in dogs [7]. Our results indicated that the prevalence of *Malassezia* spp. in diseased dogs was 25.3 %. On the other hand, Nardoni et al. [23] described a prevalence of 67.6 % in dermatologically diseased dogs.

Ears are susceptible to unusual local conditions — high humidity, presence of cerumen and less air circulation — which constitute an ideal environment for *Malassezia* growth. Otitis externa is not a life threatening disease but can be frustrating for both patients and owners. We detected a 36.0 % prevalence of *Malassezia* species in the external ear canals of dogs with otitis externa. Cafarchia et al. [5] isolated *Malassezia* yeasts from 57.3 % of the dogs with otitis and from 28 % of the dogs without otitis externa. Nardoni et al. [22] detected *Malassezia* spp. in 63.4 % of the dogs with otitis. In contrast to this, Sarierler and Kirkan [27], in their study of 234 dogs with otitis externa, found *M. pachydermatis* only in 5.12 % of the samples and Campbell et al. [7] in 17 % of normal and diseased canine ears. According to the authors, the incidence of *Malassezia* varies and also different species of these yeasts were found. In our study, we identified 79 *M. pachydermatis* isolates, four *M. furfur* isolates and one *M. nana* from all positive samples. Similarly, Cafarchia et al. [6] reported in both, healthy dogs and in dogs with cutaneous lesions, a higher prevalence of *M. pachydermatis* than lipid dependent *Malassezia* species. Duarte et al. [12] identified

only one atypical strain of *M. furfur* isolated from a dog. Also, other results suggest that in the external ear canals of 57 dogs with chronic otitis externa, lipid-dependent *Malassezia* species were isolated in only three dogs. These species were identified as *M. furfur* and *M. obtuse*, but showed atypical assimilation patterns [10]. Nardoni et al. [22] isolated only *M. pachydermatis* in their study of 41 dogs.

In our study, we recorded the difference in occurrence of *Malassezia* yeasts in pendulous ears compared to erect ears. We suspected that the type of ears (pendulous or erect) may influences the prevalence of *Malassezia* occurrence. Kumar et al. [18] reported that the percentage of dogs with long pendulous ears and otitis externa was similar to the percentage of dogs with erect ears and medium hair on the ears, but Cafarchia et al. [5] reported that dogs with pendulous ears showed a higher incidence of infection than dogs with erect ears.

The most frequent conditions that may contribute to *Malassezia* overgrowth on skin are: hormonal imbalance, keratinization defects, excessive production of sebum, bacterial infections and hypersensitivity processes [28]. Other important causes of *Malassezia* occurrence in our patients were also dermatitis (24.5 %) and interdigital dermatitis (16.4 %). Nardoni et al. [22] considered the interdigital space to be the place with the highest *Malassezia* prevalence, but it was not confirmed in our results.

Our results show that 14.3 % of dogs with inflammation of the anal sacs were positive for *M. pachydermatis*. Cytological quantification of *Malassezia* in the anal sac contents of healthy dogs revealed a low occurrence of yeasts, with only 12.5 % in the examined dogs and 10 % of the anal sacs, demonstrating the presence of *Malassezia* yeasts [25].

Some authors described a predisposition of *Malassezia* infection to be dependent upon the age of dogs [8, 21]. We did not find a difference between the occurrence of *Malassezia* spp. and the age of the diseased dogs, which was similar to the results of Plant et al. [26] and Nobre et al. [24].

CONCLUSIONS

In conclusion, all isolated *Malassezia* species in our study were identified both phenotypically and genotypically as *M. pachydermatis*, except for four isolates that were identified as *M. furfur* and one isolate, which was identified as *M. nana*. No other species of *Malassezia* was found. The highest prevalence of *Malassezia* was detected in dogs with otitis externa. Our study confirmed that *Malassezia* remains the most prevalent yeast found in the dog and that the occurrence of other species is infrequent.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-15-0377.

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Received February 8, 2017
Accepted April 7, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0014

FOLIA VETERINARIA, 61, 2: 22—26, 2017

ORIGIN LEVEL OF THE VENTRAL BRANCHES OF THE ABDOMINAL AORTA IN THE RABBIT AND EUROPEAN HARE

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ABSTRACT

The aim of this research was to describe the level of origin of the branches originating from the ventral surface of the abdominal aorta in the rabbit and hare. The study was carried out on ten adult rabbits and ten adult European hares using the corrosion cast technique. After euthanasia, the vascular network was perfused with saline. Batson's corrosion casting kit No. 17 was used as a casting medium. After polymerization of the medium, the maceration was carried out in a KOH solution. We found variable levels of the origin of the celiac, cranial mesenteric and caudal mesenteric arteries in both species. In the rabbit, the celiac artery originated in the majority of cases at the cranial end of the first lumbar vertebra and in the hare at the middle part of the vertebral body of the same vertebra. The cranial mesenteric artery in the rabbit originated predominantly at the level of the first lumbar vertebra and in the hare at the level of the second lumbar vertebra. In the rabbit, the caudal mesenteric artery originated mainly at the level of the sixth lumbar vertebra and in the hare, at the level of the fifth lumbar vertebra. We concluded that there were higher variabilities of the origins of the ventral branches of the abdominal aorta in domesticated rabbit in comparison with the European hare.

Key words: caudal mesenteric artery; celiac artery; cranial mesenteric artery; European hare; rabbit

INTRODUCTION

The European hare belongs to the most frequently seen wild mammal in the territories of the Slovak Republic and neighbouring countries. Despite this fact, the literature dealing with the anatomy of this species is rather rare, except for some studies dealing with the anatomy of its arterial system [4, 5].

The arterial pattern including the arrangement, origin, course and variations of almost all arteries, have been the objects of different studies. The branches arising from the ventral surface of the abdominal aorta have been studied in such experimental animals as, a dog [1], cat [7], rabbit [2] and guinea pig [10].

The aim of this paper was to compare the variations in the level of origin of the celiac, cranial mesenteric and caudal mesenteric arteries in the domesticated rabbit and European hare.

MATERIALS AND METHODS

This study was carried out on 10 adult European hares (*Lepus Europaeus*, L. 1758, age 140 days) and on 10 adult rabbits (*Oryctolagus cuniculus f. domestic*a, L. 1758, age 140 days). We used hares (obtained from ISFA APRC, Nitra, Slovakia) of both sexes (female n = 5; male n = 5) with a weight range of 2.5—3.2 kg and New Zealand White rabbits (obtained from HYLAPA s. r. o., Prešov, Slovakia) of both sexes (female n = 5; male n = 5) in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Kosice, Slovakia. The animals were kept in cages under standard conditions (temperature 15—20 °C, relative humidity 45 %, 12-hour light period), and fed with a granular feed mixture (O-10 NORM TYP, Spišské kŕmne zmesi, Spišské Vlachy, Slovakia). The drinking water was available to all animals *ad libitum*. Thirty minutes before the animals were sacrificed by intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}) the animals were injected intravenously with heparin (50 000 IU.kg^{-1}). Immediately after euthanasia, the vascular network was perfused with a physiological solution. During manual injection through the ascending aorta, the right atrium of the heart was opened with an aim to lower the pressure in the vessels in order to ensure an optimal injection distribution; 50 ml of Batson's corrosion casting kit No. 17 (Dione, České Budějovice, Czechia) was used as the casting medium. The maceration was carried out in a 2—4 % KOH solution for a period of 5 days at 60—70 °C. This study was carried out under the authority decision No. 2647/07-221/5.

RESULTS

The unpaired celiac artery arose from the ventral surface of the abdominal aorta and supplied blood to the stomach, spleen, liver, pancreas and partially to the duodenum. In the rabbit, its origin was located at the level of the cranial end of the twelfth thoracic vertebra in 20 % of the cases; in the middle of the vertebral body of the thirteenth thoracic vertebra in 30 % of the cases (Fig. 1); and at the level of the first lumbar vertebra in 50 % of the cases. When originating at the level of the first lumbar vertebra, it was located at its cranial end in 30 % of the cases; and in the middle part of the vertebral body in 20 % of the cases. In the hare, the celiac artery originated at the level of the first lumbar vertebra in all the cases (Fig. 2); at the cranial end of this vertebra in 10 % of the cases; in the middle part of the vertebral body in 50 % of the cases; and at the caudal end in 40 % of the cases.

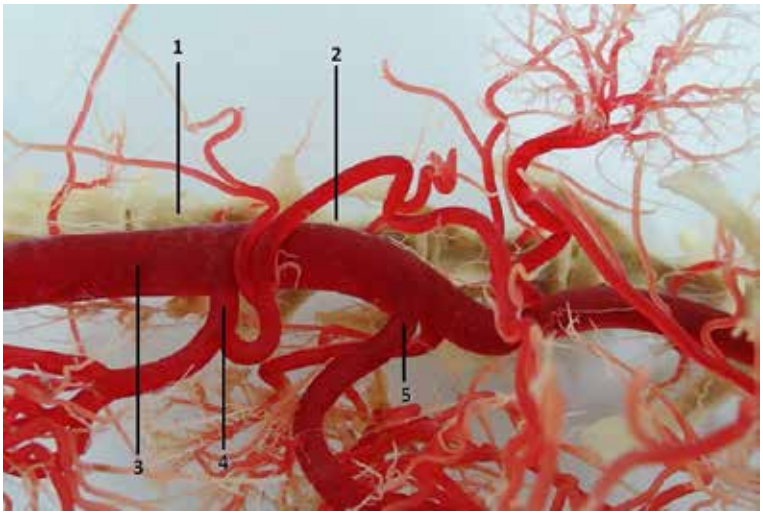

Fig. 1. Origin of the celiac artery at the level of the 13th thoracic vertebra and the origin of the cranial mesenteric artery at the level of the 1st lumbar vertebra in the rabbit
1 — 13th thoracic vertebra, 2 — 1st lumbar vertebra, 3 — abdominal aorta, 4 — celiac artery 5 — cranial mesenteric artery. Macroscopic image, ventrolateral view

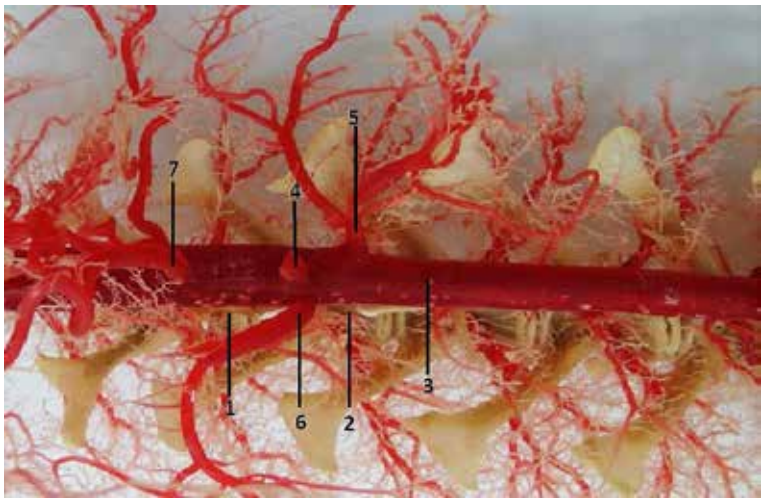

Fig. 2. Origin of the celiac artery at the level of the 13th thoracic vertebra and the origin of the cranial mesenteric artery at the level of the 1st lumbar vertebra in the hare
1 – 1st lumbar vertebra, 2 – 2nd lumbar vertebra, 3 – abdominal aorta, 4 – cranial mesenteric artery, 5 – left renal artery, 6– right renal artery, 7 – celiac artery. Macroscopic image, ventral view

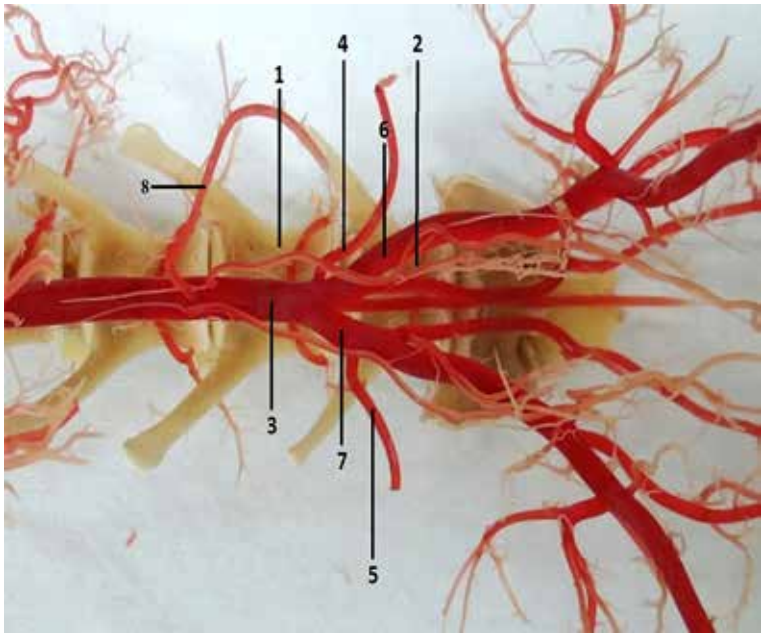

Fig. 3. Origin of the caudal mesenteric artery at the level of the 5th lumbar vertebra in the rabbit
1 – 6th lumbar vertebra, 2 – 7th lumbar vertebra, 3 – abdominal aorta,
4 – left deep circumflex iliac artery, 5 – right deep circumflex iliac artery, 6 – left common iliac artery, 7 – right common iliac artery. Macroscopic image, ventral view

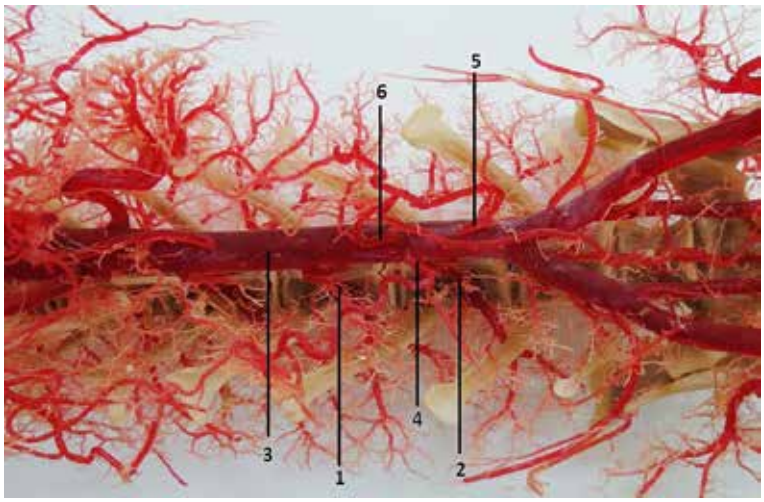

Fig. 4. Origin of the caudal mesenteric artery at the level of the 5th lumbar vertebra in the hare
1 – 4th lumbar vertebra, 2 – 5th lumbar vertebra, 3 – abdominal aorta, 4 – left ovarian artery, 5 – right ovarian artery
6 – caudal mesenteric artery. Macroscopic image, ventral view

The cranial mesenteric artery gave off branches supplying the pancreas, small intestine, cecum and ascending colon. It originated caudally to the origin of the celiac artery. In the rabbit, this artery originated at the level of the first lumbar vertebra in 60 % of the cases; in 20 % of the cases in the middle part of the vertebral body; and in 40 % of the cases at its caudal end (Fig. 1). In the remaining 40 % of the cases, the origin of this vessel was located at the level of the second lumbar vertebra and specifically as follows: at its cranial end in 20 % of the cases; and at the middle part of the vertebral body in 20 % of the cases. In the hare, the origin of this vessel was at the level of the second lumbar vertebra in 90 % of the cases (Fig. 2); at its cranial end in 30 % of the cases; at the middle part of the vertebral body in 50 % of the cases; and at its caudal end, in 10 % of the cases. At the level of the first lumbar vertebra, the cranial mesenteric artery originated in 10 % of the cases.

The third ventrally directed branch of the abdominal aorta was the caudal mesenteric artery supplying the descending colon and rectum. Its origin in the rabbit was at the caudal end of the fifth lumbar vertebra in 40 % of the cases (Fig. 3); and at the level of the cranial end of the sixth lumbar vertebra in 60 % of the cases. In the hare, the origin was positioned at the level of the middle part of the vertebral body of the fourth lumbar vertebra in 10 % of the cases; and in 90 % of the cases, at the level of the fifth lumbar vertebra (Fig. 4) as follows: at its cranial end in 30 % of the cases; in the middle part of the vertebral body in 20 % of the cases; and at its caudal end, in 40 % of the cases.

DISCUSSION

The detailed knowledge of the vascular anatomy plays an important role in the management of several pathologic conditions and treatments of various diseases. They must be considered not only in experimental models but also in surgical practice in laboratory and domesticated animals [8, 11].

The celiac artery in the rabbit was described as the first unpaired ventrally directed branch also in other studies [2, 9]. In the study of Abidu et al. [2], the place of origin of the celiac artery was very variable; in 40 % of the cases between the thirteenth thoracic and first lumbar vertebra; in 36.7 % of the cases between the twelfth and thirteenth thoracic vertebra; in 20 % of the cases at the level of thirteenth thoracic vertebra; and in 3.3 % of the cases at the level of the first lumbar vertebra. In 50 % of the cases, we found the origin at the level of the first lumbar vertebra, and in the rest of the cases, cranially to this vertebra. The comparison of the level of origin of the celiac artery between the rabbit and hare, showed a more caudal origin at the level of the first lumbar vertebra in the hare.

In our study, the second ventrally directed branch arising from the ventral surface of the abdominal aorta

in the rabbit was the cranial mesenteric artery. This observation correlated with the findings of Ahasan et al. and Popesko et al. [3, 9]. It originated at the caudal end of the second lumbar vertebra [12]. On the corrosion casts, we found the origin at the level of the first lumbar vertebra in 60 % of the cases and at the level of the second lumbar vertebra in 40 % of the cases. The origin of the cranial mesenteric artery in the hare was generally caudal to the origin in the rabbit. In 90 % of the cases, it arose at the level of the second lumbar vertebra.

In the rabbit, the origin of the caudal mesenteric artery was described at the level of the sixth lumbar vertebra [12]. In our study, it was at the caudal end of the fifth lumbar vertebra in 40 % of the cases and at the level of the cranial end of the sixth lumbar vertebra in 60 % of the cases. In the hare, the origin was positioned more cranially; at the level of the fifth lumbar vertebra in 90 % of the cases; and at the level of the middle part of the vertebral body of the fourth lumbar vertebra in 10 % of the cases.

The detailed description of the arterial system in the hare is still lacking in the literature. We believe that a better understanding of the differences between familiar species in domesticated and wild species will also increase the anatomical knowledge. Such knowledge is highly significant not only for comparative studies across species but also in everyday veterinary practices [6].

CONCLUSIONS

The results of this study indicated a relatively high variability in levels of the origin of the celiac, cranial mesenteric and caudal mesenteric arteries in the rabbit, with lesser occurrence in the hare. These findings are possibly associated with the different ways of life.

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Received February 21,2017
Accepted April 11, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0015

FOLIA VETERINARIA, 61, 2: 27—34, 2017

A HISTOPATHOLOGICAL STUDY OF ISCHEMIC AND COMPRESSIVE PARAPLEGIA IN DOGS

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ABSTRACT

It is well known that neuronal death, clinically manifested as paresis or plegia, is the end result of many pathological events affecting the central nervous system. However, several aspects of pathophysiological mechanisms involved in the development of tetra- or paraplegia caused by spinal cord traumatic or ischemic damage are only insufficiently understood and their histopathological manifestations remain poorly documented. That is why the authors decided to report on light-microscopic changes observed in 30 µm thick spinal cord sections cut from L3—S1 segments processed by the Nauta staining method in a group of 6 dogs with ischemic paraplegia induced by 30 min of a high thoracic aorta occlusion, and in a different group of 6 dogs with traumatic paraplegia induced by 5 min spinal cord compression with 200 g metallic rod. Both experimental groups (ischemic and compression) of spinal cord injuries (SCI) comprised the same number of mongrel dogs of both sexes, weighing 18—25 kg. In addition, each of the experimental groups had 3 normal dogs that served as controls. All experimental procedures were accomplished under general anaesthesia induced by pentobarbital and maintained by a mixture of halothane and oxygen. Following the 72 hour survival period, all 18 animals were euthanized by transcardial perfusion with 3,000 ml of saline and fixed by 3,000 ml of 10 % neutral formaldehyde during deep pentobarbital anaesthesia. The histopathological manifestation of neural tissue damage caused by ischemia or compression was similar. The light-microscopic images in both groups were characterised by argyrophilia and the swelling of grey matter neurons. However, in the dogs with traumatic SCIs, the changes only reached about 750 µm cranially and caudally from the necrotic epicentre. These findings indicated that the events taking part in secondary spinal cord injury mechanisms are similar in both, ischemic as well as in traumatic SCI.

Key words: compression; histopathology; ischemia-reperfusion; spinal cord injury;

INTRODUCTION

Spinal cord injuries, whether caused by external traumatic forces or ischemic events belong to the most serious healthcare problems in both, human and veterinary health care practices [1, 4, 6, 8, 18]. Due to their devastating effects on motor, sensitive and autonomic neural functions, SCI have attracted the interest of the people (from ancient physicians and philosophers, to modern scientists, veterinarians, as well as laymen and the public) for a long time [2, 13, 14, 15]. Despite an increasing amount of information accumulated during the last several decades, many aspects of the pathophysiological mechanisms involved in the development of tetra- or paraplegia have only been partially elucidated and their neurohistological manifestations remain incompletely documented [7, 14, 18, 21]. This situation inspired the authors to study light-microscopic changes characteristic for spinal cord damage induced by two different types of insults to the spinal cord, i. e. ischemia-reperfusion and compression injuries, in two different canine experimental models.

MATERIALS AND METHODS

The experimental protocols were prepared with respects to the instructions of the Animal Protection Act of the Slovak Republic No. 15/1995 [5] and approved by the Ethical Commission of the Institute of Neurobiology, Slovak Academy of Sciences in Košice, Slovakia.

Eighteen healthy adult mongrel dogs of both sexes, free of heart worm disease, weighing 18—25 kg, divided into four groups, were used in this study:

1. Non-ischaemic sham controls (n = 3). Animals underwent a left-sided thoracotomy only.
2. Non-compressive sham controls (n = 3). The standard L4–L5 laminectomy was performed.
3. Ischemic injuries of the spinal cord (n = 6). The descending aorta just distal to the origin of the left subclavian artery was occluded by a tourniquet for 30 min.
4. Compression SCI (n = 6). The spinal cord was traumatised by a metallic rod ended with a small knob weighing 200 g lowered onto the exposed spinal dural sac containing the spinal cord through the L4—L5 laminectomy for 5 min.

The experimental procedures were carried out under general anaesthesia induced by Pentobarbital (pentobarbitalum natricum) administered intravenously in a 30 $mg.kg^{-1}.10^{-1}$ dose. Then the dogs were intubated with an endotracheal cannula (diameter 8—12 mm) and placed on a volume-cycled ventilator. The anaesthesia was further maintained by a mixture of oxygen and Halothane. Also, the continuous direct monitoring of the arterial blood pressure in the radial artery, EKG, and arterial blood gases were carried out. The rate of ventilation was adjusted to maintain arterial pO_2 between 80—100 mm Hg and pCO_2 at about 38 mm Hg, i. e., normal canine levels of pO_2 and pCO_2 [19]. The arterial blood pressure (which increased in every dog after a high thoracic aorta occlusion) was kept at pre-surgical levels by a continuous infusion of nitroprusside in saline.

The operations were performed under sterile conditions and basic surgical principles were strictly observed. Antibiotics (1,500,000 u. i. of *benzylpenicillinum procainum* and 1.5 g of streptomycin sulphate) were administered intramuscularly during the surgery to every dog. After completing the surgical procedures, the dogs were placed into separate disinfected compartments, covered with warm blankets, and followed until they completely awakened from the narcosis. Then, they were offered drinking water *ad libitum* and food granules. Suppression of postoperative pain was ensured by the intramuscular administration of a strong analgesic/opioid (Tramal, i. e. *tramadoli hydrochloridum* 50 mg) in 6 hour intervals for 3 days.

After a 72 hour survival period, all dogs (experimental as well as controls) were deeply anaesthetized by pentobarbital (50 $mg.kg^{-1}.10^{-1}$ of body weight i. v.), then transcardially perfused with 3,000 ml of saline and fixed by the same volume of 10 % neutral formaldehyde. Spinal cord specimens comprised of L3—S1 segments were removed, 24 hours post-fixation, cut into 30 µm thick sections and processed by silver compounds according to a protocol of Nauta staining methods for light-microscopic observations [12].

RESULTS

All 18 dogs survived the experimental procedures without surgical complications. All six control dogs revealed no neurological deficit. On the other hand, all of the experimental animals awakened from anaesthesia with fully

developed paraplegia; their clinical picture was characterized by a complete palsy of their pelvic limbs. Parts of the spinal cord located in the epicentre of the compression injury in members of this experimental group (6 dogs), were crushed; the appearance of the neural tissue resembled an engorged haemorrhagic pulp. The spinal cord in the vicinity of the trauma was oedematous and haemorrhagic, but macroscopic changes did not exceed 14 mm cranially and caudally from the epicentre. The L3—S1 segments of the spinal cords removed from the vertebral canals of members of the ischemic group (6 dogs) were pale and oedematous, but not haemorrhagic. The macroscopic changes were not restricted to the epicentre and 1—2 adjacent segments only, but affected the whole specimen.

Light-microscopic observations

No visible histopathological changes developed in the specimens prepared from the lumbosacral spinal cord segments of the sham controls. The identity of neurons in semi-thick sections (30 μm) of the L3—S1 spinal cord segments of sham controls processed according to the Nauta staining method was hardly distinguishable. The perikarya were only minimally impregnated and their dendritic ramifications were completely Nauta-negative. The cytoplasm of middle-sized and large neural cells (especially the large motoneurons in Rexed's lamina IX) appeared as pale shadows, almost identical with the background [17]. The localisation of neural cell somata was facilitated by large light nuclei and darkly stained nucleoli (Fig. 1).

The histopathological changes of the spinal cord tissue damaged by 30 min of ischemia and 72 hour reperfusion on the one hand, and 5 min compression by 200 g metallic rod on the other hand, were similar with only insignificant differences. The histopathological images were characterised by argyrophilia of the neural cells, as well as swelling and boutonic enlargement of presynaptic parts of their axons encircled by clear halos (Figs. 2—4). The majority of boutons was localised in the central part of spinal cord grey matter, i.e. Rexed's laminae V—VII [17]. The above changes were diffusely spread throughout the spinal cord grey matter in both experimental groups, but in the dogs with compressive paraplegia, they did not exceed 750 μm cranially and caudally from the necrotic epicentre.

Fig. 1. Microphotograph of a specimen from L5 spinal cord segment of a control dog. Large motoneurons with darker nucleoli are Nauta-negative and appear as pale shadows, almost identical with surrounding neuropil. Magn. ×180

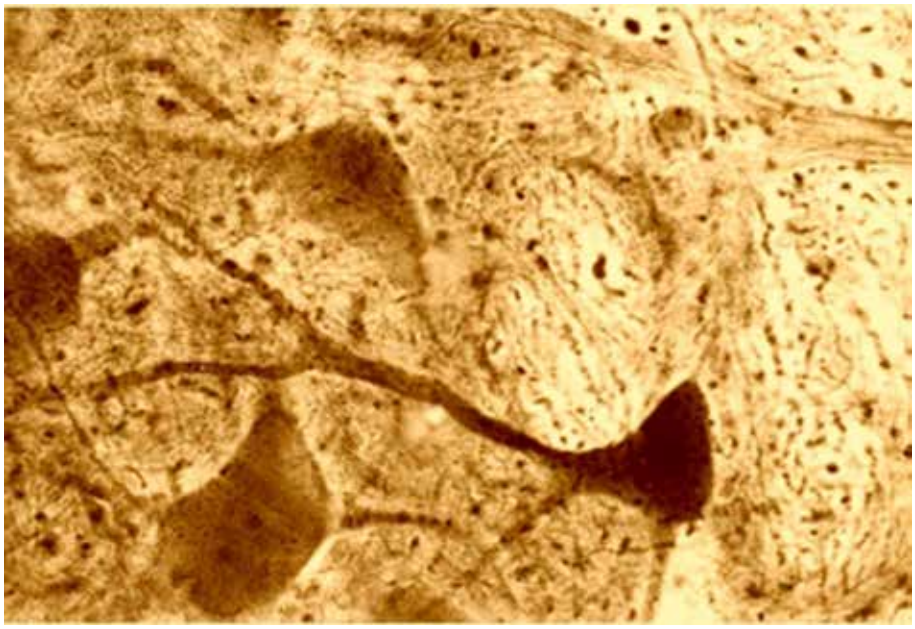

Fig. 2. Microphotograph of a specimen from L5 spinal cord segment of a dog after 30 min ischemia and 72 hour survival. Oedema and enhanced argyrophilia of interneurons in zona intermedia. Magn, ×320

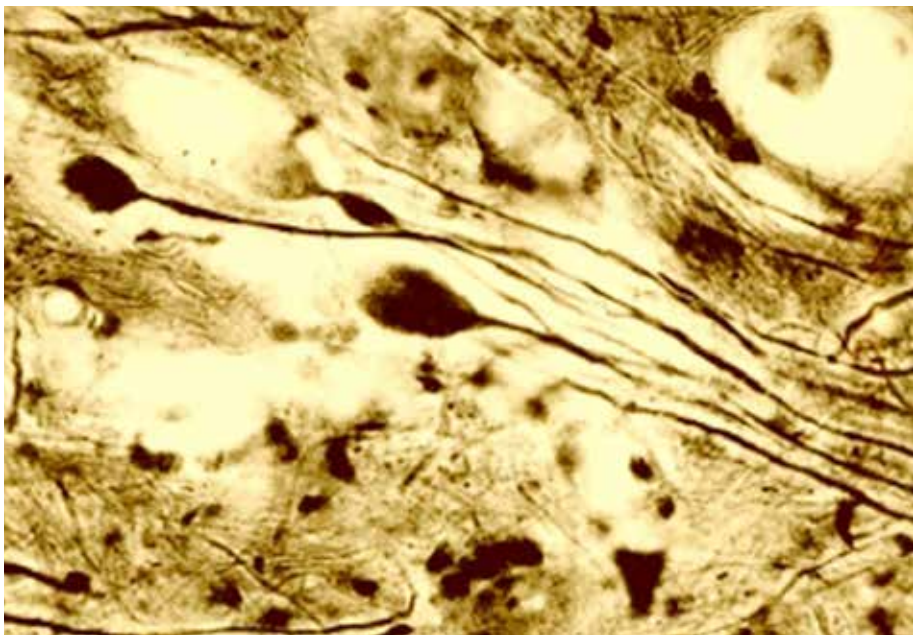

Fig. 3. Microphotograph of a specimen from L5 spinal cord segment of an animal after 30 min ischemia and 72 hour survival. Enlarged terminal parts of axons (boutons) in the 6th Rexed's lamina of the dorsal horn. Magn. ×150

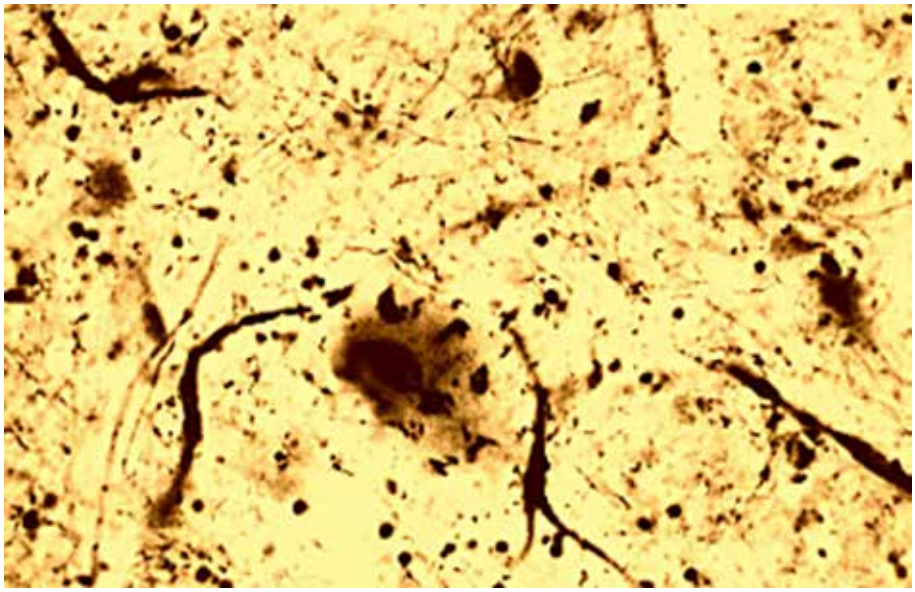

Fig. 4. Microphotograph of a specimen from the L3 spinal cord segment of an animal following the 5 min of compression trauma and 72 hour survival. Neuropil is Nauta-positive, motoneurons in anterior horns are oedematous, and within their cytoplasm are argyrophilic accumulations of irregular shape and density. Magn. ×120

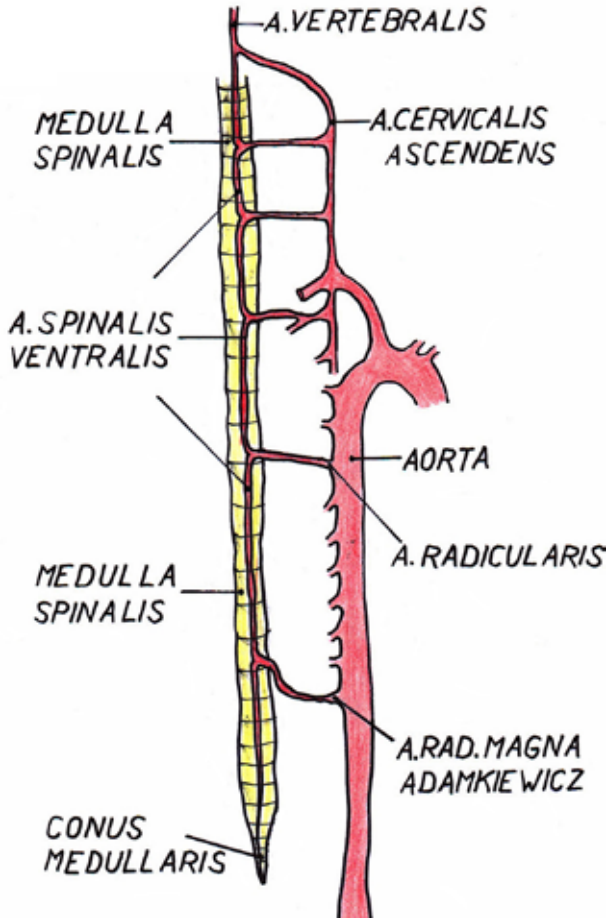

Fig. 5. Schematic drawing of arteries supplying the spinal cord modified after Lazorthes et al. [9]

DISCUSSION

The terms tetraplegia and paraplegia refer in mammals to the permanent deficit of motor functions of the trunk, all four extremities and tail (tetraplegia) or pelvic limbs and tail muscles palsy (paraplegia) accompanied by a loss of: voluntary control over the urinary bladder and anal sphincters´ functions, sexual incompetence as well as a complete anaesthesia of the affected body area [6, 7, 8]. A very similar neurological deficit is characteristic for spinal cord lesions in humans [1, 18, 21]. Trauma to the vertebral column and spinal cord plays the main role in the development of the clinical syndrome; less frequently it is caused by an interruption of spinal cord blood flow [8, 11, 13, 18].

The human spinal cord is supplied by 1 ventral and 2 dorsal spinal arteries, which extend longitudinally in a variable fashion [9, 22]. Cranially these arteries originate from the vertebral arteries and anastomose at the level of the *conus medullaris*. At many levels they receive blood supplies from the radicular arteries, which enter the vertebral canal along the nerve roots. The anterior spinal artery gives rise to the central arteries, entering the spinal cord to supply the ventral horns and ventral parts of the lateral columns. Each radicular artery supplies a separate functional region of the spine, particularly via the ventral spinal artery. The first region extends from C1 to the Th3 segment and is supplied at the C3 level from the vertebral arteries. The second region extends from Th3 until the Th7 and receives a branch from the intercostal artery (originating directly from the aorta) at the Th7 level. The third region, supplied by only one branch, arteria radicularis magna (Adamkiewicz artery) originating from an intercostal or lumbar artery between Th9, L1, L2, extends from Th8 to the *conus medullari*s (Fig. 5). This arrangement of the spinal cord blood supply explains why even a temporary cross-clamping of the thoracic aorta can cause necrosis of the medullary tissue and lead to the development of ischemia-reperfusion paraplegia, characterized by bilateral motor deficit with complete sensory deficit due to the so called transverse medullary infarct [22].

The temporary cross-clamping is a condition sine qua non in the aortic or principal vessels operations [1, 11, 16]. However, awakened from anaesthesia after an uneventful, technically straightforward procedure with an irreversible paraplegia, means a catastrophe for a patient and a nightmare for the vascular surgeon. The risk of this complication is about 10 % in elective thoracoabdominal aortic reconstruction and significantly increases in patients struck with a rupture of an atherosclerotic aneurysm of the vessel [1, 11]. The reason is that haemorrhagic shock causing a rapid decrease of blood pressure which, in combination with preexisting latent insufficiencies of the spinal cord vessels and their supplying arteries, is an extremely malignant combination [8, 13, 16].

The experimental model of spinal cord ischemia-reperfusion injury used in our study was developed with an aim to imitate the situation in humans as much as possible. Originally we considered the utilisation of small laboratory animals in our experiments [10]. We had both, rabbits, as well as rats at our disposal. However, interventions in the thoracic cavity of rabbits or rats require a special apparatus for maintaining artificial ventilation and an endotracheal intubation which is so technically difficult, that a cannula often can be introduced into trachea through a tracheotomy only. Moreover, in rabbits another problem appears; the spinal cord lesion leads to a complete urinary retention, which causes an early death of the animal [10]. On the other hand, administration of general anaesthesia to larger animals is simpler; the surgical situation in a thoracic cavity resembles the situation in humans, and as a result are more reliable. That is why minipigs, dogs or cats are used more and more frequently in experimental studies nowadays [16, 20]. Having at our disposal the necessary equipment for intrathoracic and intraspinal procedures in dogs, as well as sufficient experience with operations on them, we decided to use these animals again [19].

The pathophysiological consequences of spinal cord damage are explained by the theory of primary and secondary injury mechanisms. Primary SCI result from the direct mechanical insults to neural tissues and spinal cord (especially grey matter) vascular network [15, 21]. Four basic types of primary SCI and their combinations can occur. They include: the laceration of the spinal cord (caused by shearing and traction), compression of the cord (by an intraspinal haematoma, herniated intervertebral disc, dislocated vertebra or its fragments in a case of vertebral fracture or luxation), concussion of the spine (e. g. when the victim was hit by an automobile, gunshot or falling from a height), and lack of blood supply, i. e. ischaemia [3, 4, 6, 7, 11]. The secondary mechanism in SCI develops as an expansion of the primary damage [14]. Regardless of the type of insult, which started the cascade of events leading to disintegration of spinal cord tissue (neurons and sup-

portive glial cells), and destruction of connections between brain — spinal cord and spinal cord — spinal cord neurons, the secondary mechanism in SCI develop as an expansion of primary damage [13, 14, 15]. The vast majority of investigators comply with the theory of a multi-step process, including: persistent depolarization of cell membranes, energy depletion from repolarization, deregulation of intracellular calcium, formation of reactive oxygen species, lipid peroxidation, loss of cellular ionic gradients, oedema formation, nuclear DNA fragmentation, rupture of cell membranes and inflammatory reaction in both, traumatic and ischemic SCIs [3, 8, 14, 18, 21]. An important finding of our study was the ability of the Nauta staining method to visualize and localize damaged spinal cord neurons when their somata and dendrites became argyrophilic due to an ischemia-reperfusion or a compression injury. The unusual type of synaptic degeneration, characterized by a massive occurrence of enlarged boutons encircled by clear halos, could be a light-microscopic manifestation of ionic shifts and membrane depolarization processes, which are considered an integral part of a cascade leading to neuronal death in SCI. The density and laminar distribution of boutons correlated with the distribution of small argyrophilic neurons localized in the deep dorsal horn layers. The results of our experiments demonstrated the high vulnerability of these spinal cord grey matter cells. With regard to the fact, that many small neurons and interneurons form synapses with axons of descending supraspinal tracts, the damage induced by ischemia-reperfusion and compression SCI very probably play an important role in the gradual transformation of post-ischemic or compressive paraplegia from a flaccid paralysis to a spastic palsy [14, 21, 22].

More than a decade ago, spinal cord traumatic or ischemia-reperfusion lesions resulted in confinement of the patients to wheelchairs and struggles with different medical complications for the rest of their life [1, 6, 7, 8, 9, 13, 21]. The treatment armamentarium was limited and the provision of care for individuals with SCI was met with frustration in the majority of cases. Progress in neuroscience research has made the idea, that SCI will eventually be treatable, more plausible [3, 14, 16, 18].

The authors believe that the histopathological changes observed in the spinal cord specimens of dogs of both experimental groups, expressed the consequences of compromised spinal microcirculation. It means that ischemia plays an important role in the pathophysiology of both, traumatic and ischemia-reperfusion SCI. Therapeutic measures need to take this observation into consideration.

CONCLUSIONS

The comparison of neurohistopathological changes observed in specimens prepared from L3—S1 spinal cord segments of dogs with ischemic and compressive paraplegia stained by the Nauta method indicated that metabolic events leading to cell death in these different experimental models are similar and certain ischemic components also participate in development of traumatic spinal cord lesions.

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Received January 11, 2017
Accepted April 18, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0016

FOLIA VETERINARIA, 61, 2: 35—43, 2017

THE INFLUENCE OF SHORT DURATION EXERCISE ON THE CONCENTRATION OF C-REACTIVE PROTEIN AND SELECTED HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN THE BLOOD OF GERMAN SHEPHERD DOGS

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ABSTRACT

Short-term intensive exercise may be associated with many short-lasting metabolic changes. These changes depend on the duration and intensity of the exercise. The aim of our study was to determine potential changes in C-reactive protein (CRP) and selected haematological and biochemical parameters in clinically healthy German shepherd dogs before short duration high-intensity exercise and 6 hours after the exercise. During the study, the dogs were subjected to the following defensive training: detaining a figurant running away from a dog (2×) and detaining with a counterattack (2×). The running distance was 200 m at a mean speed of 28 km.h^{-1}. The investigation of haematological parameters revealed a significant decrease in the mean values of platelet haematocrit (PCT) 6 hours after the training. Significant differences in the levels determined before and after exercise were observed also for phosphorus (P) and for iron (Fe). At the same time, we observed a significant increase in the mean calcium (Ca) level and a significant decrease in sodium (Na) and chlorides (Cl). The activity of aspartate aminotransferase (AST) significantly increased after exercise. Concentrations of acute phase C-reactive proteins were increased 6 hours after exercise in comparison to those before exercise but the difference was insignificant. Although the concentration of CRP was increased insignificantly after the exercise, when interpreting concentrations of this nonspecific indicator of inflammation, one should keep in mind that intensive exercise may also affect its concentrations.

Key words: dog; exercise; C-reactive protein; blood parameters

INTRODUCTION

Intensive physical exercise can be associated with a number of short-term metabolic changes, including changes in glucose homeostasis and disturbances of electrolyte and acid-base balance [21]. These changes depend on several factors, such as intensity and length of the challenge, condition of the animal, thermoregulation and at-

mospheric conditions [16]. Many authors have described organ system changes during exercise and investigated the time needed for returning the exercise-altered parameters back to the quiescent level. Changes in serum concentrations of biochemical parameters and acute phase proteins were described in sled dogs [2]. Acute phase response (APR) is a rapid inherent protective mechanism of the organism developing before stimulation of specific immune response that limits damage to tissues. APR supports restoration of homeostasis. The APR triggering stimuli include: trauma, infection, stress, neoplasia or inflammation [5, 12]. APR is characterised by a number of various systemic influences, such as: fever, leukocytes, changes in the concentrations of hormones and mineral elements, and metabolic changes [7]. APR is also associated with changes in the level of acute phase proteins (APP) [3]. C-reactive protein (CRP) is the most sensitive acute phase protein in dogs [11]. In these animals, the increase in CRP is more rapid than in humans. After the stimulation, increased levels of CRP can be detected for the first time after 4 hours and maximum levels of this parameter are detected after approximately 24 hours. Contrary to this, the increase in CRP in humans is generally detected 6 hours after the stimulus and its maximum levels are reached after 48 hours. The half-time of CRP break-down is relatively short and it occurs after about 6—8 hours [4, 13, 14]. Some biological factors may affect the level of APP in dogs, such as: breed, pregnancy, nutrition status, environment, some medicines, and physical exercise [3, 9]. Increase in the concentrations of CRP in dogs observed during systemic inflammatory response is difficult to distinguish from that induced by intensive physical activity [22]. Although greyhound and sled dog races are the best known dog sports, dog athletes can compete in a range of other sports.

The aim of our study was to investigate the potential changes in haematological and biochemical parameters in German shepherd dogs involved in sport and defence training.

MATERIALS AND METHODS

We investigated a group of 10 healthy German shepherd dogs (9 bitches and 1 male, 2—6 years old). Before the investigation, all dogs were examined clinically, were afebrile and exhibited normal quiescent values of respiration rates and pulse. Samples of blood were collected from vena cephalica antebrachii before a short duration high-intensity exercise and 6 hours after the exercise. The dogs were not exposed to physical exertion for 48 hours before the first blood sampling. The relevant biochemical and haematological parameters were determined in the blood of the dogs.

During the study, the dogs were subjected to the following defensive training: detaining a fugitive running away from a dog (2×) and detaining with a counterattack (2×). The running distance was 200 m at a mean speed of 28 $km.h^{-1}$.

The haematological examinations included the determination of the following parameters: erythrocytes (RBC), leukocytes (WBC), concentration of haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean concentration of haemoglobin in erythrocytes (MCHC), distribution curve of erythrocytes (RDW), reticulocytes (RETIC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), thrombocytes (platelets — PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet haematocrit (PCT). The haematological parameters were determined employing an automatic haematological analyser ABX Micros ABC Vet (Horiba Medical, France).

The biochemical examinations involved: determination of C-reactive protein (CRP), calcium (Ca), magnesium (Mg), phosphorus (P), sodium (Na), potassium (K), chlorides (Cl), iron (Fe), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), pancreatic amylase (pAMS), glucose (GLU), total cholesterol (TCHOL), triglycerides(TG), total bilirubin (TBil), albumin (ALB), creatinine (CREAT) and urea (UREA).

The C-reactive protein was determined by enzymatic immuno-analysis using commercial ELISA tests (Tridelta Development, Ireland). Serum samples were diluted 1 : 500. The intensity of the colour reaction was evaluated spectrophotometrically at 450 nm by means of an automatic photometer Opsys MR (Dynex Technologies, USA), with 630 nm as a reference value. Other biochemical parameters were determined employing a biochemical analyser Cobas c111 (Roche, Switzerland).

All statistical and database operations were performed with the help of a personal computer using a table proces-

sor Excel (Microsoft, USA) and the following tests: Grubbs test, Bartlett test, and one and two factor analysis of variance ANOVA. The level of statistical significance was set to $P < 0.05$.

RESULTS

The investigation of haematological parameters showed a significant decrease in the mean values of PCT 6 hours after the training (from 0.32 ± 0.08 % to 0.25 ± 0.06 %; Table 2, Fig. 1). Significant differences ($P < 0.05$) in the levels determined before and after exercise were observed also for phosphorus, which increased from 1.13 ± 0.19 to 1.38 ± 0.10 mmol.l^{-1} (Table 2, Fig. 4), and for iron which decreased from 21.41 ± 10.38 to 19.37 ± 5.19 mmol.l^{-1} (Table 2, Fig. 5). At the same time, we observed a significant increase ($P < 0.05$) in the mean calcium level from 1.87 ± 0.17 to 2.16 ± 0.12 mmol.l^{-1} (Table 2, Fig. 3) and a significant decrease ($P < 0.05$) in sodium from 140.97 ± 1.69 to 137.12 ± 1.76 mmol.l^{-1}) (Table 2) and chlorides from 110.75 ± 2.51 to 107.78 ± 2.25 mmol.l^{-1} (Table 2). The activity of AST significantly increased from 0.40 ± 0.08 to 0.48 ± 0.09 μkat.l^{-1} (Table 2, Fig. 6). The concentrations of the acute phase C-reactive proteins were increased 6 hours after exercise (45.00 ± 13.37) in comparison to those before exercise (40.69 ± 16.48 mg.l^{-1}), but the difference was insignificant (Table 1, Fig. 2). The results of other parameters presented in Tables 1 and 2 showed no significant differences in the other concentrations or activities.

Table 1. Haematological parameters and concentrations of C-reactive protein in the blood of German shepherd dogs before and after exercise

Parameter		Before exercise		After exercise	
		n =10		n =10	
		x	±SD	x	±SD
Haematological parameters	HGB [g.dl^{-1}]	14.40	1.49	14.06	1.69
	RBC [T.l^{-1}]	6.45	0.73	6.31	0.88
	WBC [G.l^{-1}]	11.87	3.73	12.23	2.81
	MCV [f.l^{-1}]	66.77	2.58	63.64	2.77
	HCT [l.l^{-1}]	43.19	4.86	41.52	5.71
	MCH [pg]	22.38	1.15	22.33	1.13
	MCHC [g.dl^{-1}]	33.53	0.83	33.93	0.94
	RDW [%]	18.81	1.56	16.03	1.83
	RETIC [K.μl^{-1}]	32.37	21.50	30.32	19.66
	NEU [×10^9.l^{-1}]	6.61	1.35	7.96	2.11
	LYM [×10^9.l^{-1}]	2.06	0.45	2.26	0.70
	MONO [×10^9.l^{-1}]	1.00	0.50	1.02	0.40
	EOS [×10^9.l^{-1}]	1.00	0.30	0.96	0.40
	BASO [×10^9.l^{-1}]	0.04	0.02	0.04	0.03
	PLT [K.μl^{-1}]	218.00	110.81	196.00	73.47
	MPV [fl]	11.67	0.76	11.78	0.66
	PDW [fl]	15.50	1.94	15.91	1.64
	PCT [%]	0.32	0.08*	0.25	0.06*
Specific protein	**CRP** [mg.l^{-1}]	**40.69**	**16.48**	**45.00**	**13.37**

* — Significant at $P < 0.05$

HGB — haemoglobin; RBC — erythrocytes; WBC — leukocytes; MCV — mean corpuscular volume; HCT — haematocrit; MCH — mean corpuscular haemoglobin; MCHC — mean concentration of haemoglobin in erythrocytes; RDW — distribution curve of erythrocytes; RETIC — reticulocytes; NEU — neutrophils; LYM — lymphocytes; MONO — monocytes; EOS — eosinophils; BASO — basophils; PLT — thrombocytes (platelets); MPV — mean platelet volume; PDW — platelet distribution width; PCT — platelet haematocrit; CRP — C-reactive protein

Table 2. Concentration of electrolytes and minerals and enzyme activity in the blood of German shepherd dogsbefore and after exercise

Parameter		Before exercise		After exercise	
		n =10		n =10	
		x	±SD	x	±SD
Electrolytes and minerals	Ca [mmol.l^{-1}]	1.87*	0.17	2.16*	0.12
	Mg [mmo.l^{-1}]	0.66	0.08	0.67	0.07
	P [mmol.l^{-1}]	1.13*	0.19*	1.38*	0.10*
	Na [mmol.l^{-1}]	140.97*	1.69	137.12*	1.76
	K [mmol.l^{-1}]	5.53	0.85	5.05	0.29
	Cl [mmol.l^{-1}]	110.75*	2.51	107.78*	2.25
	Fe [µmol.l^{-1}]	21.41	10.38*	19.37	5.19*
Enzyme activity	AST [µkat.l^{-1}]	0.40*	0.08	0.48*	0.09
	ALT [µkat.l^{-1}]	0.77	0.93	0.67	0.74
	GGT [µkat.l^{-1}]	0.10	0.04	0.12	0.03
	ALP [µkat.l^{-1}]	0.75	0.33	0.70	0.32
	pAMS [µkat/l^{-1}]	7.83	1.85	7.71	1.78
	LDH [µkat.l^{-1}]	0.91	0.43	1.16	0.84

* — Significant at $P < 0.05$
AST — aspartate aminotransferase; ALT — alanine aminotransferase; GGT — gamma-glutamyl transferase
ALP — alkaline phosphatase; pAMS — pancreatic amylase; LDH — lactate dehydrogenase

Table 3. Concentration of metabolites and lipids in the blood of German shepherd dogs after exercise

Parameter		Before exercise		After exercise	
		n =10		n =10	
		x	±SD	x	±SD
Metabolites	ALB [g.l^{-1}]	29.56	2.91	29.74	3.01
	CREAT [µmol.l^{-1}]	51.38	8.06	51.10	8.80
	UREA [mmol/l^{-1}]	3.65	1.35	3.45	1.21
	TBIL [µmol.l^{-1}]	1.63	0.68	1.58	0.49
	GLU [mmol.l^{-1}]	3.31	0.30	3.18	0.29
Lipids	TCHOL [mmol.l^{-1}]	3.93	0.71	3.91	0.61
	TG [mmol.l^{-1}]	0.41	0.14	0.39	0.11

ALB — albumin; CREAT — creatinine;UREA — urea; TBIL — total bilirubin
GLU — glucose; TCHOL — total cholesterol; TG — triglycerides

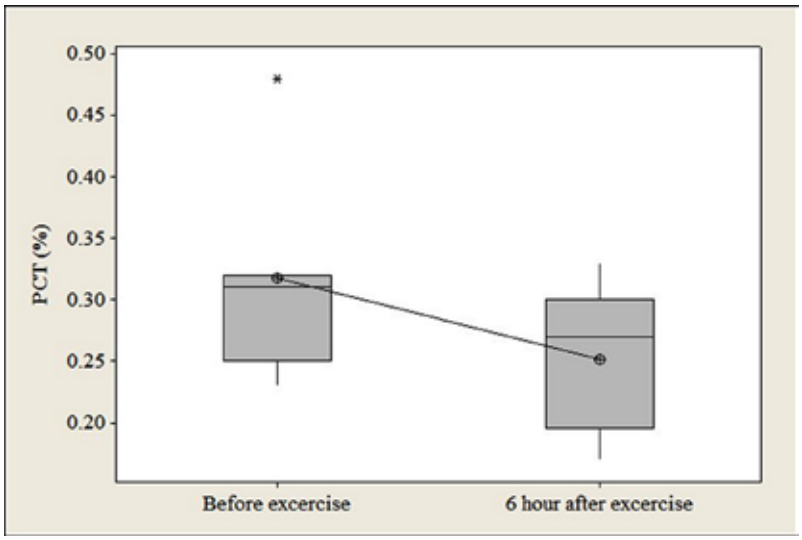

Fig. 1. Changes in platelet haematocrit (PCT) in the blood of German shepherd dogs 6 hours after exercise, * — (P < 0.05)

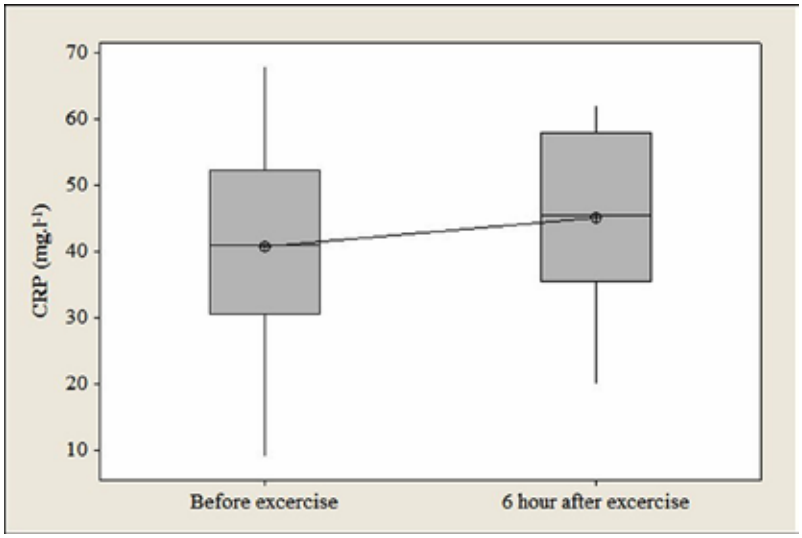

Fig. 2. Changes in concentration of c-reactive protein (CRP) in the blood of German shepherd dogs 6 hours after exercise

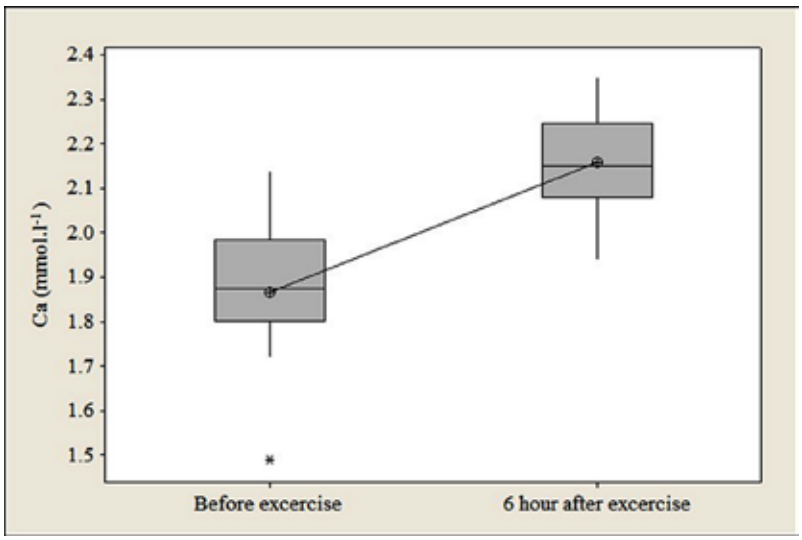

Fig. 3. Changes in the concentration of calcium in the blood of German shepherd dogs 6 hours after exercise

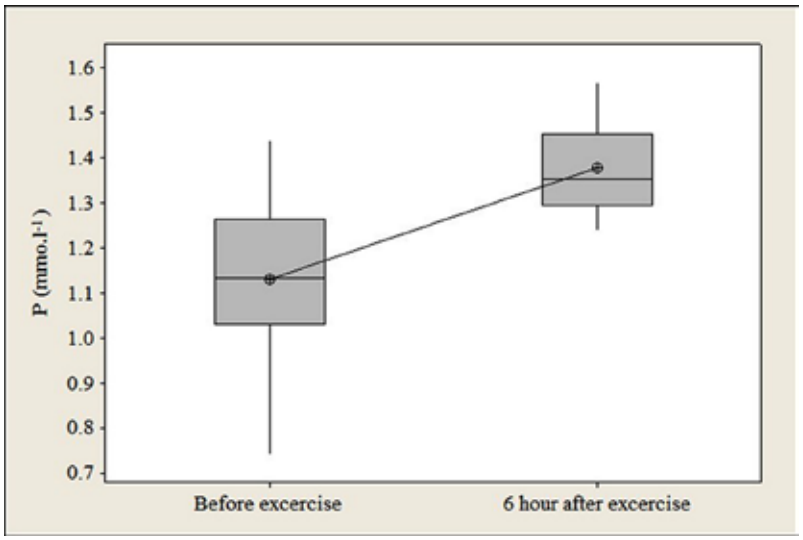

Fig. 4. Changes in concentration of phosphorus in the blood of German shepherd dogs 6 hours after exercise

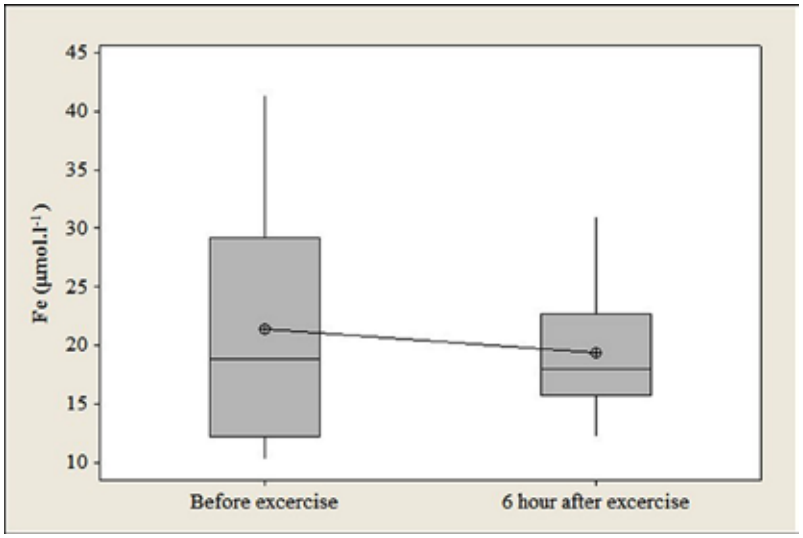

Fig. 5. Changes in concentration of iron in the blood of German Shepherd dogs 6 hours after exercise

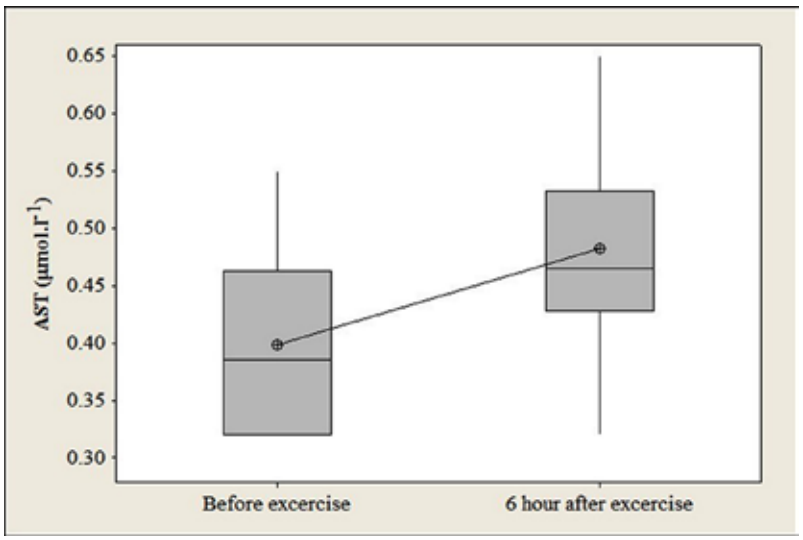

Fig. 6. Changes in the activity of aspartate aminotransferase (AST) in the blood of German shepherd dogs 6 hours after exercise

DISCUSSION

High-intensity exercise results in many short-term metabolic changes including altered glucose homeostasis and electrolyte and acid-base imbalance. The degree of such changes is affected by the intensity and duration of physical activity [22]. Casela et al. described changes in CRP in hunting dogs after 3 hours of physical activity during hunts. These dogs exhibited significantly increased CRP levels [2]. Such changes agree with those reported by other authors who pointed to the role of the acute phase proteins not only in association with inflammatory processes, but also in situations not related to inflammation, when the animals are exposed to stress factors.

In our study we detected increased levels of CRP 6 hours after exercise (Table 1, Fig. 2), however, the increase was insignificant. It should be mentioned that the examination of blood samples before exercise showed that haematological and biochemical parameters in the clinically healthy dogs were within the reference range except for CRP which exceeded the reference limit (CRP < 20 mg.l^{-1}). This may be ascribed to the regular training of these dogs. The levels of CRP determined after high-intensity exercise were higher than those observed at inflammatory processes associated with infectious diseases. Wakshlag et al. reported that despite increased levels of CRP in dogs, these animals did not show an increase in IL-6 compared to the basal level [21]. A study in people demonstrated that after 25 km runs in 4 subsequent days, the CRP levels were increased on days 3 and 5 but no changes were observed in components of C3 and C4 complements [8]. Studies in humans also found a decrease in HGB and HCT in athletes in comparison with untrained persons, between periods of rest and training seasons and between beginning and completion of races [8, 19]. In athletes during training, particularly for endurance races, a decrease in HCT was described due to increase in plasma volume referred to as "sport anaemia". This increase in plasma volume in trained individuals is most likely caused by aldosterone-dependent renal reabsorption of sodium and retention of water. This occurs due to an increase in antidiuretic hormone as a compensatory mechanism of fluid losses during physical challenge [10]. A decrease in HGB and HCT was described also in sled dogs during training and racing [6, 15]. Our study also showed a decrease in the levels of HGB and HCT after exercise (Table 1). Angle et al., Rovira et al. and Tharwat et al., failed to observe changes in haematological parameters in dogs during exercise [1, 17, 20]. The slight decrease in RBC observed in our study was insignificant (Table 1). A different way of training, type and exercise duration could also affect the results of RBC. It is important to consider also the time of blood sampling as the altered haematological parameters may return to their initial level as soon as 5—10 minutes after termination of the physical activity [1]. Leukocytosis appears to be a physiological response of dogs to physical challenge. Such response was described in sled dogs after short races although no changes in WBC were reported after agility races. It was assumed that an increase in WBC after exercise is caused by mobilisation of WBC subpopulations from peripheral reserves. These changes arise due to activation of soluble molecules released as a result of damage to muscle mass, hormonal changes (particularly influence of catecholamines) and oxidation stress. Rovira et al. reported increased levels of WBC in horses associated with mobilisation of lymphocyte-rich blood from the spleen [17]. The moderate increase in WBC observed in our study agreed with the results of Rovira at al. who observed physiological, blood and endocrine parameters in search and rescue-trained dogs (20 min training) (Table 1) [17]. Angle et al. reported significantly higher concentrations of Na, K, Ca and ALB in samples collected from sled dogs before short duration high-intensity exercise [1]. The observations by Rovira at al. indicated a decrease in sodium at 5 and 15 minutes after exercise. After 30 minutes, the levels of Na returned back to those before exercise and, at the same time, a gradual decrease in Cl was observed in these dogs [17]. Our study showed a decrease in K, Na and Cl after exercise but contrary to the above shifts, Ca and ALB were increased at 6 hours after exercise. The mean concentrations of Fe significantly differed at individual samplings before and after the exercise. The concentration of Fe before exercise (21.41 ± 10.38 μmol.l^{-1}) slightly exceeded the upper reference limit for this element (Fe 16—21 μmol.l^{-1}), but six hours after exercise it was decreased to 19.37 ± 5.19 μmol.l^{-1} (Table 2, Fig. 5). Wakshlag et al. reported only a slight decrease in the concentration of Fe (15 %) in dogs after exercise compared to changes in this parameter in humans (50 %), however, the diet of the dogs was rich in Fe [21]. Normally, most of the catabolism of damaged RBC takes place in the spleen and liver where endothelial phagocytosis of RBC occurs. Iron from the cells is incorporated in ferritin. A portion of RBC is eliminated

also in vessels during circulation. HGB released during haemolysis is subsequently bound in a complex with haptoglobin. Iron from this haemolysis does not bind to ferritin but is released to plasma in the form of ferrous ions. During exercise, oversaturation of haptoglobin develops, which prevents additional binding of haemoglobin. When the haemoglobin-binding capacity of haptoglobin is exceeded, haemoglobin is released through glomeruli into the urine and haemoglobinuria may develop [18].

CONCLUSIONS

Our results indicate that high-intensity short duration exercise induced significant changes in concentrations of Ca, P, Na, K, Cl, Fe, PCT and AST in German shepherds. Rapid increase in concentration of APP after exercise and subsequent rapid decline during the quiescent phase when the animals were not exposed to additional stimulus, makes it possible to use APP as a biomarker for routine screening of health and condition of these animals. When evaluating the levels of CRP as a non-specific indicator of inflammation, one should consider the fact that intensive exercise can also affect the level of this parameter.

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Received April 27, 2017
Accepted May 9, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0017

FOLIA VETERINARIA, 61, 2: 44—49, 2017

HAEMATOPATHOLOGICAL CHANGES IN DOGS AFFECTED WITH *EHRLICHIA CANIS* IN LESVOS

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ABSTRACT

Canine Ehrlichiosis is an important immunosuppressive tick borne disease in dogs. The geographical distribution and transmission is mostly related with *Rhipicephalus sanguineus* which acts as a vector. There is no predilection of age or sex; all breeds may be infected with Canine Monocytic Ehrlichiosis (CME). The primary targets are monocytic cells. Platelet disorders and serum protein alterations are the principal hematological and biochemical consequences of infections. Clinical signs are almost non-specific. A definitive diagnosis requires: visualization of morulae within monocytes on cytology, detection of serum antibodies with *E. canis,* the IFA test, or the PCR. The objective of this study was to present information about haematological and biochemical tests of *E. canis* infected dogs in Lesvos island in Greece, which is an endemic area.

Key words: dog; *Ehrlichia canis*; hematology; Lesvos island

INTRODUCTION

The species *Ehrlichia canis,* a gram negative obligate intracellular bacterium of the genus *Ehrlichia* (family *Anaplasmataceae),* is the primary cause of Canine Monocytic Ehrlichiosis (CME). It is transmitted by the brown dog tick *Rhipicephalus sanguineus* [1]. The disease was first described in Algeria in 1935 and in southern Africa in 1938. It has a worldwide distribution, apart from Australia and New Zealand, although it is more prevalent in subtropical and tropical areas [15]. A wide variety of clinical and haematological signs and 3 phases have been recognized: acute, subclinical and chronic. The non-specific clinical signs of the acute disease are: high fever, depression, lethargy, and anorexia. The physical examination reveals: lymphadenomegaly, splenomegaly and haemorrhagic tendency with dermal petechiae, ecchymoses, and epistaxis. Thrombocytopenia is the most common hematological finding. Non-regenerative anaemia and a decline in leukocyte count may also occur during this phase. During the subclinical phase, which is characterised by persistent rickettsemia, there are no overt clinical signs and the haematological parameters

usually fall into the normal range, although the platelet count may be in the lower normal range. The chronic form is characterised by pancytopenia due to suppression or destruction of the bone marrow. In this stage, dogs may succumb to fatal secondary bacterial infection and/or bleeding [4, 8, 10].

This research was focused on Ehrlichiosis in dogs in the area of Lesvos, a Greek island in North East Aegean Sea. The vegetation and the climate appears to be favourable for ticks and other ectoparasites. The results coming from dogs in different local areas of this island will be presented, since in endemic areas like Greece, it is a major cause of persistent and life threatening thrombocytopenia and a large number of stray and domestic dogs get infected by *Ehrlichia canis* every year in Lesvos.

MATERIALS AND METHODS

Area and investigated material

Lesvos is the 3rd biggest Greek island situated in the North East Aegean Sea. It has 1,633 km^2 and a population of 100,000 habitants. The climate consists of dry hot summers and short cool winters. The air temperature has an annual average of 18 °C, 10 °C from January — February, and 36 °C in July-August. Rainfall occur during October — March. The vegetation consists of olive trees, pine forests and low bushes and grass. Agricultural activities with many small goat and sheep farms, but also many stray dogs and cats make *Ehrlichia Canis* a very common disease. The selection of 24 dogs, 12 domestic and 12 stray, was made in equal number, 8 dogs (4 domestic and 4 stray) from 3 different areas of the island, A, B, and C. (Fig. 1). Different breeds, sex and ages were tested at a local private clinic.

Methods

A random selection of 24 dogs was made in Lesvos island and these dogs were brought to a private clinic for examination. History, clinical examination, blood collection and rapid Speed Ehrli tests were made. By this test the positive infected were distinguished from the negative dogs. Blood samples were analysed and the results evaluated, so a more definite haematological and biochemical presentation of *E. canis* in Lesvos was demonstrated.

Blood sampling

Blood samples were collected by aspiration from jugular or cephalic veins using a 24 gauge needle and a 0.2 ml syringe, under aseptic conditions. The blood was transferred into tubes with EDTA to prevent clotting and into two tubes containing no anticoagulants. The unclotted sample was used for the determination of haematological parameters employing an Exigo veterinary haematology analyser.

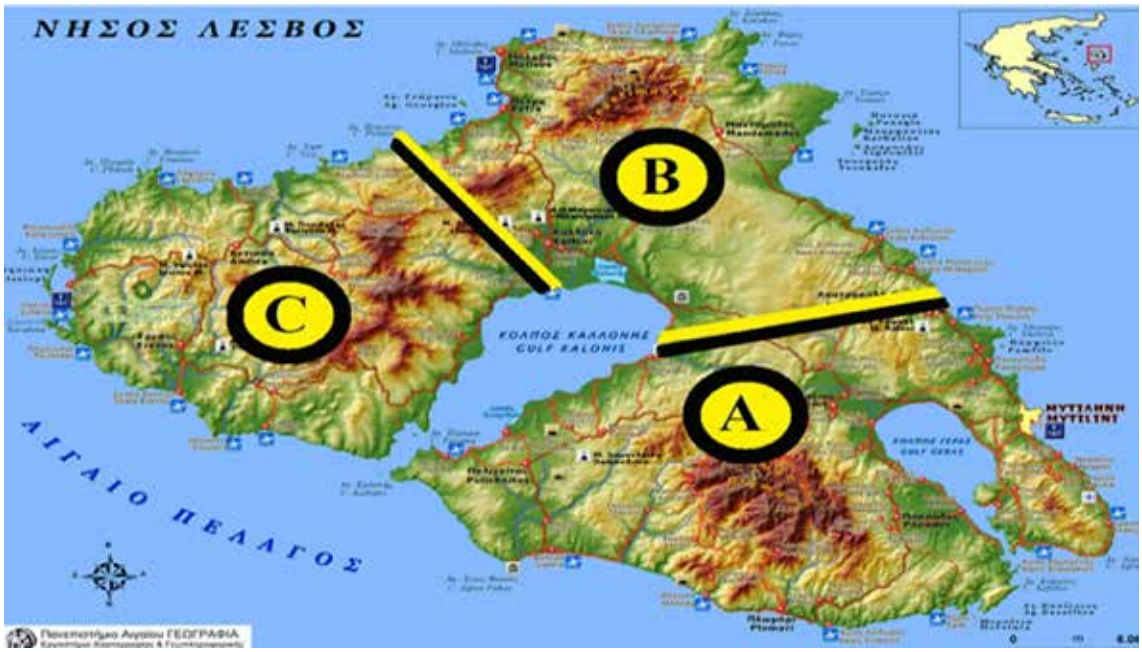

Fig. 1. Map of Lesvos island with areas A, B, C

The two tubes were centrifuged to separate serum from the blood. One tube was used for the determination of biochemical parameters using spot chemII. The other tube was refrigerated for further use.

Speed Ehrli Test

Speed Ehrli is a highly sensitive test which ensures diagnosis before the disease becomes chronic. It is based on the detection of anti *Ehrlichia canis* antibodies and it is a membrane immunochromatography method. Whole blood with or without an anticoagulant, serum or plasma can be used. It has reliability compared with a reference technique, indirect immunofluorescence (IFAT) shows 87 % sensitivity and 95 % specificity. The equipment consists of one test device, one single use pipette and one bottle of reagent. One drop of the sample is pipetted into a well when using an anticoagulant. When using whole blood without anticoagulant, 2 drops of the sample are transferred directly into a well from a syringe. Then 5 drops of the reagent are added and the reading is made after 15 minutes of incubation. The result appears in a result window. The negative test is one single pink band in the right hand side of the window. A positive test is 2 pink bands in the window. After the treatment, the antibodies falls slowly. The result that remains positive may be due to re-infestation. Seroconversion is slow (4 weeks), so if the results are negative but clinical signs show infection, the test should be repeated 15 days later. For this study, blood sample with anticoagulant was used.

RESULTS

From the 24 dogs examined with Speed Ehrli test, 58.33 % were found positive (Patient No. 1 — Patient No. 14), while 41.67 % were negative (Patient No. 15 — Patient No. 24). The hematological parameters of positive dogs are presented in Table 1.

The levels of biochemical parameters in positive dogs are presented in Table 2.

The distribution of positive dogs in areas A, B, C is presented in Table 3. From the 24 examined dogs, 14 were found positive.

The haematological parameters of negative dogs are presented in Table 4.

The biochemical parameters of negative dogs are presented in Table 5.

DISCUSSION

From the 24 randomly chosen dogs examined with Speed Ehrli test in Lesvos from October 2015 until October 2016, 58.33 % were found positive. The remaining dogs (41.67 %) were found negative and their haematological profile showed no signs of anaemia or thrombocytopenia. Patents No. 1 — No. 14 were positive when tested by speed Ehrli test. More precisely, in area A, 50 % of the dogs were positive and from them 25 % were domestic and 75 % were stray dogs. In the area B, 62.5 % of animals were positive and from them 60 % were domestic and 40 % stray dogs. In the area C, 62.5 % of the dogs were positive, of them, 60 % were domestic and 40 % stray dogs. In area A, where the capital town is situated, there was a slightly lower prevalence of infected dogs, while in the areas B and C with low vegetation and many agricultural activities and small farms, the prevalence of infection was higher.

Most of the dogs found infected had clinical signs such as: petechiae, ecchymoses, epistaxis, anorexia, lethargy, weight loss, pale mucous membranes, and oculonasal discharges [2]. Ticks and other ectoparasites were found on many of them, maybe due to insufficient protective measures taken by their owners, such as collar and spot on, or due to a total absence of protection from the stray dogs which moved around quite freely. Even domestic dogs, because of the mild climate, spend most of their time outside households, in gardens, fields and had access to neighbouring small individual farms with sheep and goats,.

In area A there was an increased number of stray dogs, in addition to domestic dogs. Thrombocytopenia is considered to be the most common haematological abnormality in dogs infected with *E. canis* [6, 7, 9, 14]. In our study, platelet (PLT) values below 200 x $10^9.l^{-1}$ were found in 100 % of the dogs which indicated thrombocytopenia. Among them, 35.71 % had PLT ranges lower than $150 \times 10^9.l^{-1}$ and 64.29 % had platelet ranges between $151—199 \times 10^9.l^{-1}$. Many of them had petechiae, ecchymoses, epistaxis and retina haemorrhages as clinical signs (9 of 14 infected dogs). Anaemia is also a common pathological clinical abnormality related to *E. canis* infection [10, 12]. In this study, haemoglobin was found below the normal range in 57.14 % of the infected dogs. A low haemoglobin concentration is a sign of anaemia. The haemoglobin ranges of 28.57 of dogs were above normal and 14.29 % had normal ranges, maybe due to increased haematopoiesis or

Table 1. Percentage of dogs with positive haematological values

Parameter	Reference range	Below [%]	Within [%]	Above [%]
WBC [$10^9.l^{-1}$]	6.0—17.0	–	100	–
LYM [$10^9.l^{-1}$]	0.9—5.0	–	100	–
MID [$10^9.l^{-1}$]	0.3—1.5	–	92.86	7.14
GRAN [$10^9.l^{-1}$]	3.5—2.0	–	92.86	7.14
HGB [$g.dl^{-1}$]	12—18	57.14	14.29	28.57
HCT [%]	37—55	64.29	35.71	–
RBC [$1012.l^{-1}$]	5.5—8.5	64.29	35.71	–
MCV [fl]	60—72	57.14	35.71	7.14
MCH [pg]	19.5—22.5	35.72	7.14	57.14
MCHC [$g.dl^{-1}$)	32—38.5	42.86	50	7.14
RDW [%]	12—17.5	28.58	21.42	50
RDWa [fl]	35—53	21.43	57.14	21.43
PLT [$10^9.l^{-1}$]	200—500	100	–	–
MPV [$10^9.l^{-1}$]	5.5—10.5	–	85.72	14.28
LYM [%]	0.0—99.9	–	100	–
MID [%]	0.0—99.9	–	100	–
GRAN [%]	0.0—99.9	–	100	–

WBC — white blood cells; LYM — lymphocyte; MID — rare cells and white blood cell precursors; GRAN — granulocyte; HGB — haemoglobin; HCT — haematocrit; (packed cell volume); RBC — red blood cell; MCV — mean corpuscular volume; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; RDW — red cell distribution width expressed in %; RDWa — red cell distribution width expressed in fl; PLT — platelet; MPV — mean platelet volume

Table 2. Percentage of dogs with positive biochemical values

Parameter	Reference range	Below [%]	Within [%]	Above [%]
BUN [$mg.dl^{-1}$]	6—33	7.14	78.57	14.29
GLU [$mg.dl^{-1}$]	75—117	14.29	71.42	14.29
ALP [$IU.l^{-1}$]	< 132	–	92.86	7.14
TP [$g.dl^{-1}$]	5.3—7.9	7.14	78.57	14.29
ALT [$IU.l^{-1}$]	< 123	–	92.86	7.14
CRE [$mg.d^{-1}$]	0.6—1.6	7.14	85.72	7.14

BUN — blood urea nitrogen; GLU — glucose; ALP — alkaline phosphatase TP — total protein; ALT — alanine transferase; CR — creatinine

Table 3. Positive dogs found in areas A, B, C

	Area A	Area B	Area C	Total
Stray	3	2	2	7
Domestic	1	3	3	7
Total	**4**	**5**	**5**	**14**

Tab. 4. Percentage of dogs with negative haematological values

Parameter	Reference range	Below [%]	Within [%]	Above [%]
WBC [$10^9.l^{-1}$]	6.0 —17.0	–	100	–
LYM [$10^9.l^{-1}$]	0.9—5.0	–	100	–
MID [$10^9.l^{-1}$]	0.3—1.5	–	100	–
GRAN [$10^9.l^{-1}$]	3.5—12.0	–	100	–
HGB [$g.dl^{-1}$]	12—18	–	100	–
HCT [%]	37—55	10	90	–
RBC [$10^{12}.l^{-1}$]	5.5–8.5	–	100	–
MCV [fl]	60–72	10	90	–
MCH [pg]	19.5—22.5	–	10	90
MCHC [$g.d^{-1}$]	32.3—8.5	–	100	–
RDW [%]	12—17.5	–	40	60
RDWa [fl]	35—53	–	70	30
PLT [$10^9.l^{-1}$]	200–500	–	100	–
MPV [$10^9.l^{-1}$]	5.5—10.5	10	90	–
LYM [%]	0.0—99.9	–	100	–
MID [%]	0.0—99.9	–	100	–
GRAN [%]	0.0—99.9	–	100	–

WBC — white blood cells; LYM — lymphocyte; MID – rare cells and white blood cell precursors; GRAN — granulocyte; HGB — haemoglobin; HCT — haematocrit; (packed cell volume); RBC — red blood cell; MCV — mean corpuscular volume; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; RDW — red cell distribution width expressed in %; RDWa — red cell distribution width expressed in fl; PLT — platelet; MPV — mean platelet volume

Table 5. Percentage of dogs with negative biochemical values

Parameter	Reference range	Below [%]	Within [%]	Above [%]
BUN [$mg.dl^{-1}$]	6—33	10	90	–
GLU [$mg.dl^{-1}$]	75—117	–	100	–
ALP [$IU.l^{-1}$]	< 132	–	100	–
TP [$g.dl^{-1}$]	5.3—7.9	40	60	–
ALT [$IU.l^{-1}$]	< 123	–	100	–
CRE [$mg.d^{-1}$]	0.6—1.6	–	100	–

BUN — blood urea nitrogen; GLU — glucose; ALP — alkaline phosphatase; TP — total protein; ALT — alanine transferase; CRE — creatinine

due to a different stage of the disease. The haematocrit was found below the normal range in 64.29 % of dogs which is also an indication of anaemia, while 35.71 % of the dogs had normal haematocrit maybe because they were at the subclinical phase of infection when no anaemia is usually detected. The red blood cells were below the normal range in 64.28 % of the dogs indicating anaemia. Normal values were determined in 35.72 % of the dogs indicating probably a subclinical phase of the disease.

The mean corpuscular volume (MCV) was below the normal range in 57.14 % of the dogs suggesting microcytic anaemia and 35.72 % showed normal values. The mean corpuscular haemoglobin (MCH) was below the normal range in 35.72 % of the dogs which indicated hypochromic anaemia while in 57.14 % of the dogs its level was above normal indicating macrocytic anaemia. The mean corpuscular haemoglobin concentration (MCHC) was below the normal range in 42.86 % of the dogs indicating anaemia, while in 50 % the MCHC was in normal range. The red blood cells distribution width (RDW %) was below normal in 28.58 % suggesting anisocytosis. This parameter was normal in 21.42 % of the dogs and exceeded the upper limit in 50 % of the animals. The RDWa was below normal limit in 21.43 % of the dogs, within normal range in 57.14 %, and exceeded the upper limit in 21.43 %.

The MPV was increased above normal in 14.28 %, probably indicating subclinical stage of an infection and was in the normal range in 85.72 %. The blood urea nitrogen (BUN) was in the normal range in 78.57 % of the infected dogs and in patient No. 10 (7.14 %) it was below the lower limit. An increased level of the BUN was detected in 14.29 % of the dogs (patients No. 9 and 13), probably because of kidney damage, in conjunction with elevated values of total protein (TP) and creatinine (CRE). The alanine transferase (ALT) was above the normal range in patient No. 12 (7.14 %), in conjunction with decreased TP which is an indication of liver damage [3]. The increased values of ALT indicated liver damage and early detection can be made by this. The total protein values were above normal in patients No. 5 and No. 13 (14.29 %), suggesting subclinical infections. A decreased TP was found in patient No. 12 (7.14 %) which, in conjunction with high ALT, indicated liver damage. Creatinine values were above normal in patient No. 13 (7.14 %) which in conjunction with the BUN and TP values suggested kidney damage. Lower creatinine and BUN was detected also in patient No. 10, indicating kidney failure. Normal values were detected in 85.72 % of the infected dogs. ALP exceeding the upper limit was found in patient No. 14 (7.14 %), suggesting potential liver disease. In patient No. 12, we detected a value of 80 % IU/L which is within the normal range but higher than in the rest of the patients. Normal values were detected in 92.86 % of the dogs. White blood cells and lymphocytes were within the normal range in all dogs. MID and GRAN were above normal in 7.14 % of the infected dogs and normal in 92.86 %.

The haematological results in patients No. 15 — No. 24, negative in the Speed Ehrli test, were within the normal ranges. Platelet values were normal in 100 % of the dogs, while RDW was normal in 70 % and exceeded the upper limit in 30 %. The RBC values were normal in 100 % of dogs, while the haematocrit was normal in 90 % and below the lower limit in 10 % of the normal dogs. All dogs showed normal haemoglobin values. The MCV were normal in 90 % of the dogs and below the normal limit in 10 %. The MCH exceeded the upper limit in 90 % of the dogs and were in the normal range in 10 % of them. The MCHC were normal in all dogs. The MPV were normal in 90 % of dogs and below the lower limit in 10 % of them. The WBC, MID, LYM, GRAN, GLU, ALT, and CRE were in the normal range in 100 % of the dogs. The BUN was within the normal range in 90 % of the dogs, while in 10 % it was below the lower limit. The TP was normal in 60 % and below the normal limit in 40 % of the dogs.

Variations in haematological profiles in *E. canis* infected dogs may be related to: differences in the virulence of the *E. canis* strains, antigen heterogeneity of this bacterial agent, and the clinical form of the disease [4, 5]. Further studies are required to determine the influence of contaminant infections with other bacteria or parasite such as *Leishmania infatum*, *Bartonella* spp., *Babesia* spp., etc., on the observed haematological abnormalities [5, 11, 13, 15]. *Leishmania infatum* has a high prevalence in Lesvos, as well as in the rest of Greece and other Mediterranean countries. According to our study, it seems that the percentage of infection of dogs in Lesvos is high. This can also vary with the season, because the dogs get ticks more often during the summer, from May to October, which was also observed in our study. No official records can be found related to *Ehrlichia canis* infection in Lesvos except for individual private veterinary clinics. Compared with other studies conducted in other parts of Greece or neighbouring countries which have similar weather conditions and harbour ticks

as *R. sanguineus*, our research showed no significant differences [15]. It appears that more preventative measures should be taken by owners of domestic dogs and also by the state for stray dogs which are numerous in Lesvos island.

CONCLUSIONS

Early detection of *E. canis* in dogs is very important, so monitoring and control programs should be established. In Lesvos there was noticed a raised awareness of this disease by local veterinarians and many owners. Serological and preventive measures towards dogs and ticks could help to decrease further the number of infected animals. Our study allowed us to conclude that Speed Ehrli test contributes toward quick diagnosis of symptomatic and asymptomatic patients. From the 24 examined dogs, 14 were found positive. The occurrence of the disease seemed to be more frequent during May-October, which correlates with the timing of ticks being active and also with increased temperatures. The determination of haematological and biochemical parameters detected thrombocytopenia and anaemia in all positive dogs, as well as liver and kidney damage in some of them. A combination of haematological and biochemical values with clinical diagnosis can allow veterinarians to obtain a better clinical picture, administer the best treatment and recommend relevant prophylactic and preventive measures.

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Received May 10, 2017
Accepted May 24, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0018

FOLIA VETERINARIA, 61, 2: 50—55, 2017

IS PORCINE KOBUVIRUS 1 A TYPICAL DIARRHOEIC PATHOGEN OF PIGLETS?

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ABSTRACT

The objective of this study was to show if porcine kobuvirus 1 (PKV-1) participates in the development of diarrhoea in piglets. The experiments were focused on comparing the occurrence of PKV-1 with the occurrence of rotavirus A (RVA) infection in suckling pigs on Slovak pig farms. A total of 91 rectal swabs of piglets (age < 28 days) were collected from 8 pig farms. RT-PCR was employed to detect PKV-1 through amplification of the 495 bp fragment of the 3D gene using primers KoVF/KoVR, and RVA was detected through amplification of the 309 bp fragment of the VP6 gene using primers rot3 and rot5. As expected, the detection of RVA in diarrhoeic piglets was 56.8 % (P < 0.01), while only 14.8 % in healthy animals. These results confirm that RVA is one of the main causes of diarrhoea in young piglets. Comparatively, PKV-1 was detected in approximately equal numbers in the same group of both healthy and diarrhoeic pigs, with 74.1 % in healthy animals and 81.1 % in diarrhoeic animals, which was not statistically significant (P < 0.05). The level of co-infection of both viruses was 11.1 % in healthy animals. A portion of 48.6 % (P < 0.01) of diarrhoeic animals were found with RVA and PKV-1 co-infections. The results of this study indicate that while RVA is an enteric virus, PKV-1 cannot confidently be confirmed as an enteric pathogen.

Key words: diarrhoea; piglet; porcine kobuvirus; rotavirus

INTRODUCTION

One of the major health problems in the swine industry is the high frequency of diarrhoea in piglets, which is one of the main causes of mortality and morbidity in neonatal pigs. The aetiology of enteric disease is diverse, including viral, bacterial and protozoal pathogens, but viruses are the predominant factor. In 2008, a novel virus was detected in faecal samples from clinically healthy pigs in Hungary [21]. This virus belongs to the family *Picornaviridae* and demonstrates similarity to members of the genus *Kobuvirus*; *Aichivirus A* (previously known as human aichi virus) and

Aichivirus B (formerly bovine kobuvirus). The virus was named porcine kobuvirus 1 (PKV-1), which is also referred to as *Aichivirus C*.

The kobuvirus genome ranges from 8.2 to 8.4 kb in size with a viral protein genome (VPg) linked to the 5'-untranslated region (UTR) and a poly (A) tail at the 3'-UTR [12]. The polyprotein precursor is further processed to generate a leader (L) protein, three structural proteins (VP0, VP3 and VP1), and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) [23]. The RNA-dependent RNA polymerase (3D) is the most conserved region among kobuviruses [22].

PKV-1 has been detected in diarrhoeic pigs either in individual infections or in mixed infections with other established diarrhoeic viruses. In more recent years, the virus has been detected worldwide, with studies demonstrating PKV-1 in Asia [10, 14, 16, 17, 28,], Europe [7, 13, 21, 29], North America [25], South America [2] and East Africa [1]. In the scientific literature, PKV-1 is traditionally described as an enteric virus, however, its pathogenesis and contribution to the development of enteric disease remain poorly understood.

Rotaviruses were first described in pigs in 1976, and they are known enteric pathogens which cause gastroenteritis in young children and animals [27]. Classification of members of the Rotavirus genus is based on the serological reactivity and genetic variability of VP6, and at present there are 8 different groups which are differentiated alphabetically from A-H (RVA-RVH) [18]. The RVA group is antigenically the most diverse species amongst the Rotavirus genus, and they are also the most important due to their high prevalence and pathogenicity in both humans and animals.

The goal of this study was to determine the prevalence of porcine kobuvirus 1 in both healthy and diarrhoeic piglets in Slovakia, and to determine the relationship between kobuvirus and rotavirus A in their ability to cause diarrhoea. This information will contribute to the present knowledge available on porcine kobuvirus 1, and ultimately aid the understanding of its clinical impact on the swine industry.

MATERIALS AND METHODS

Samples

Rectal swabs were collected from 8 farms across Eastern and Western Slovakia, using transport swab applicators (Sarstedt AG & Co, Germany). The samples were collected from 91 suckling piglets before they were of weaning age (< 28 days) which were divided into clinically healthy (n = 54) and diarrhoeic (n = 37) groups.

RNA isolation

Elution of the rectal swabs was achieved by adding 1 ml of 0.01 mol.l^{-1} PBS (Merck Millipore Corp., USA) and then allowing the samples to incubate for 30 minutes. The samples were then vortexed for 3 minutes at 2000 rev.min^{-1} and then centrifuged for 5 minutes at 14,000 × g. Then 200 µl of the sample was added to 700 µl of TRIzol Reagent (Life Technologies, USA), followed by gentle hand-mixing and a 5 min incubation. 200 µl of chloroform was added to each sample to better separate the solution into water and organic phases. 500 µl of the top aqueous layer was removed and then 500 µl isopropyl alcohol was added to each test tube. The precipitated nucleic acid was washed with 75 % alcohol.

cDNA synthesis

The synthesis of cDNA was carried out using reverse transcrip2196tase and random hexamers as described by Jackova et 98al. [13].

Detection of rotavirus A and porcine kobuvirus 1 using single RT-PCR

Amplification of a 309 bp fragment of the VP6 gene was performed to detect the RVA genome, by using primers rot3 and rot5 [9]. For the detection of PKV-1 with PCR, the same cDNA, as prepared for RVA, was used. The single RT-PCR for PKV-1 was based on the amplification of a 495 bp fragment of the 3D gene using primers KoVF/KoVR [28]. Details of both PCR methods were described elsewhere [13].

Gel electrophoresis

The size of the PCR products was checked by electrophoresis in 2 % agarose gel. The DNA Ladder 100 bp marker (AppliChem, GmbH, Germany) was used to aid approximation of the size of the DNA fragments.

Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5 for Windows (GraphPad Software, USA). The data was analysed using the chi-square ($\chi 2$) test, with

confidence limits of 95 %, $P < 0.05$ (statistically significant) or 99 %, $P < 0.01$ (highly statistically significant).

RESULTS

Prevalence of rotavirus A in clinically healthy and diarrhoeic pigs

An electrophoresis image for RVA detection is displayed in Fig. 1A, based on RT-PCR amplification of the 309 bp DNA fragment. Results on the detection of RVA in healthy and diarrhoeic piglets are presented in Fig. 2. The total percentage of positive suckling pigs for rotavirus A was 31.9 % (29/91). Only 8 healthy animals were positive, which produces a positivity rate of 14.8 %. 21 of the 37 diarrhoeic suckling pigs tested positive for rotavirus, giving a 56.8 % figure for positive diarrhoeic animals. The chi-square ($\chi 2$) test showed that there was a highly statistically significant correlation (level of 99 %) between RVA and diarrhoea ($\chi 2 = 17.7887$; $P = 0.000025$).

Prevalence of porcine kobuvirus 1 in clinically healthy and diarrhoeic pigs

An electrophoresis image for PKV-1 detection is displayed in Fig. 1B. Clinical samples positive for PKV-1 were represented with a 495 bp electrophoretic band. The summary of results on the detection of PKV-1 RNA in the clinical samples is presented in Fig. 2. Of the 91 suckling pigs, 70 tested positive for PKV-1, which produces a positive percentage of 76.9 %. Forty of the healthy 54 suckling pigs were PKV-1 positive, representing a 74.1 % positivity rate. Diarrhoeic suckling pigs showed a higher positive percentage of 81.1 %, with 30 of the 37 diarrhoeic pigs testing positive. Despite observation of some differences between healthy and diarrhoeic animals, there was no statistically significant correlation at the level of 95 % ($P < 0.05$) between PKV-1 and diarrhoea ($\chi 2 = 0.6072$; $P = 0.4358$).

Levels of co-infection of rotavirus A and porcine kobuvirus 1 in clinically healthy and diarrhoeic pigs

Both PKV-1 and RVA were detected together in 24 pigs, with a positivity rate of 26.4 %. Healthy pigs had a positivity rate of 11.1 % (6 positive; 48 negative), whereas a higher rate of 48.6 % was detected in diarrhoeic pigs (18 positive;

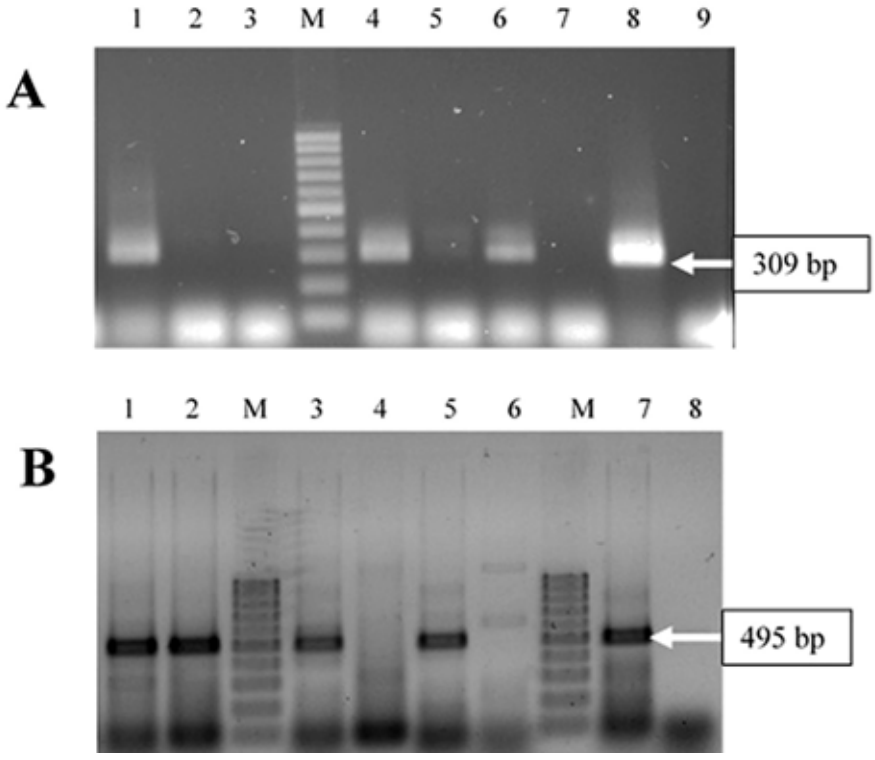

Fig. 1. Gel electrophoresis of PCR products. A — Samples 1, 4, 6 and 8 are positive for rotavirus A B — samples 1, 2, 3, 5 and 7 are positive for porcine kobuvirus 1

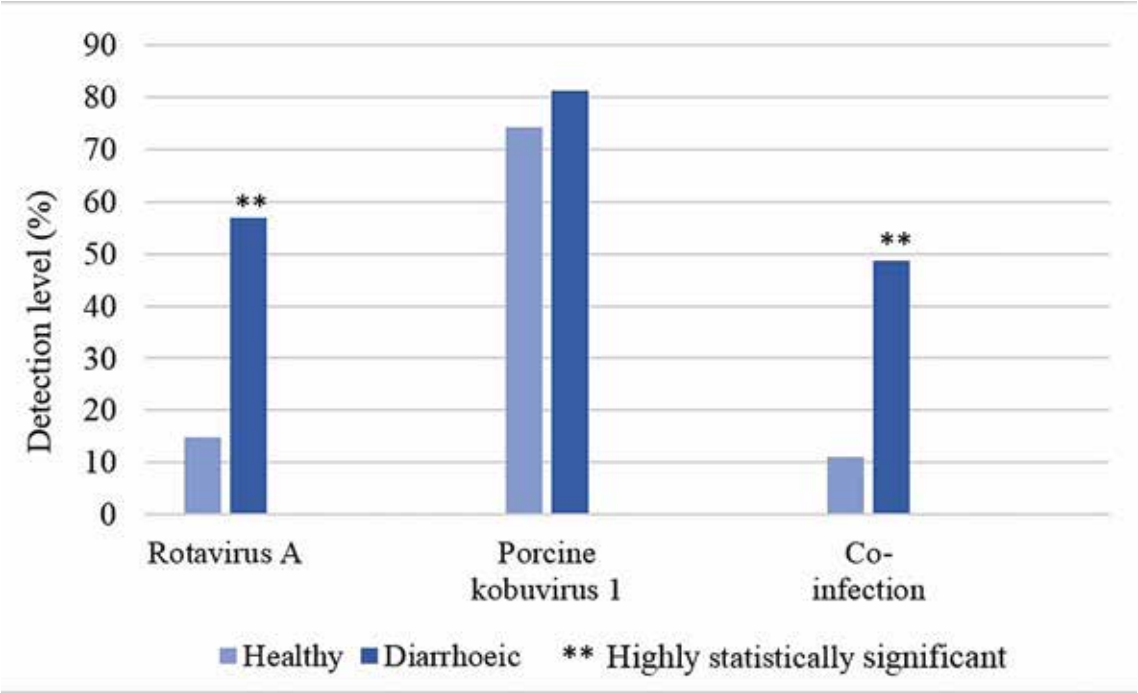

Fig. 2. Comparison within the same group of piglets for the levels of detection of rotavirus A, porcine kobuvirus 1 and co-infection

19 negative). There was a highly statistically significant correlation (level of 99 %, $P < 0.01$) between co-infection and diarrhoea ($\chi 2 = 15.9324$; $P = 0.000066$). All results on co-infection study are summarized in Fig. 2.

DISCUSSION

It is important to determine the prevalence of PKV-1 and RVA in pigs within a region, in order to contribute to the available knowledge on the global prevalence of these viruses and their role in porcine diarrhoea. The results from this study indicated that there was a highly statistically significant correlation between RVA infection and the occurrence of diarrhoea. Comparatively, no statistically significant difference was determined between PKV-1 infection and diarrhoea.

The overall prevalence of RVA in piglets was 31.9 %. Similar levels of incidence were also detected in Slovenia and Spain, with 20.0 % and 16.7 %, respectively [19]. In contrast, other European countries showed a lower incidence of RVA, such as 4.2 % in Hungary, and 10.1 % in Denmark [19].

RVA was detected in 56.8 % of diarrhoeic piglets but only in 14.8 % of clinically healthy piglets. This result was relatively anticipated, as RVA has been proven in scientific literature to be a predominant enteric pathogen and therefore a cause of diarrhoea. Publications on the association between RVA and diarrhoea are long-established, since its discovery in pigs in 1976 [27]. It is believed that group A rotaviruses are responsible for 53 % of pre-weaning and 44 % of post-weaning diarrhoea in pigs [11]. Specifically regarding RVA, it is reported to account for 89 % for all rotavirus diarrhoea in commercial pig populations [26]. The prevalence of diarrhoea in the sampled age group of piglets also corresponds with the scientific literature, as RVA infections tend to have the greatest implications on animals less than 28 days old [15, 24].

In total, a 76.9 % positivity rate was determined for PKV-1 infection in pigs originating from Slovakia. This figure of prevalence within Slovakia concurs with the European levels detected by Zhou et al. [29] in a study across 5 European countries (Austria, Germany, Hungary, Spain and Sweden), with an overall prevalence of 56.7 %. When comparing the levels of PKV-1 in healthy and diarrhoeic pigs, the present study detected nearly similar levels

in both clinical categories, with 74.1 % in healthy animals and 81.1% in diarrhoeic animals. Zhou et al. [29] detected similar levels, with an average finding of 54.5 % in healthy pigs in the 5 European countries, and 58.2 % detected in diarrhoeic pigs.

PKV-1 was incidentally detected at a level of 13.3 % in clinically healthy animals in Hungary when searching for astroviruses [21]. Since then, many studies have aimed to compare the level of detection in healthy and diarrhoeic animals. The successive study was performed by Yu et al. [28], in which they discovered a 30.1 % prevalence rate amongst clinically healthy pigs in China. More recent studies, such as that performed by Di Bartolo et al. [6] on Italian farmed pigs, subsequently confirmed the results of previous studies, with PKV-1 detection levels of 57.5 % in healthy pigs and 49.7 % in diarrhoeic pigs.

However, other studies have demonstrated higher levels of PKV-1 in diarrhoeic animals, which suggests a potential link to causing enteric disease. Khamrin et al. [16] identified a 99 % positivity rate for PKV-1 in diarrhoeic pigs in Thailand, however this study did not provide a comparison with the levels of PKV-1 in asymptomatic pigs. A study in Korea by Park et al. [20] stated a statistically significant correlation between the occurrence of diarrhoea and PKV-1, however, only 3.57 % of the diarrhoeic pigs tested for PKV-1 alone, thereby the possibility of another enteric pathogen causing diarrhoea cannot be excluded.

If kobuvirus infection was responsible for causing diarrhoea, we would expect to see a statistically significant correlation between diarrhoea and PKV-1, though this was not confirmed in the present study. Similar difficulty in establishing significance is also true for other emerging viruses, including porcine sapovirus, porcine enterovirus G and porcine astrovirus, as they are also detected in healthy and diarrhoeic pigs without known clinical significance [3, 4, 5, 8].

The levels of co-infection were also investigated in this study. A total of 24 out of the 91 sampled piglets tested positive for both RVA and PKV-1, giving a percentage of 26.4 %. When comparing the prevalence of co-infection between healthy and diarrhoeic animals, there was a highly statistically significant relationship between co-infection and diarrhoea. Only 11.1 % of healthy animals were positive for both viruses, in comparison to 48.6 % of diarrhoeic animals. Interpreting the individual factors of co-infection is a challenging task, as it is difficult to attribute the occurrence of diarrhoea with a specific aetiological agent.

CONCLUSIONS

The results of this study clearly confirmed that while RVA is a causative agent of diarrhoea in piglets, PKV-1 cannot confidently be confirmed as a typical diarrhoeic pathogen. Although PKV-1 has a high prevalence in piglets in Slovakia, they survive with absence of significant disease. Further investigation is required to determine the role of PKV-1 in the enteric system and such kind of research requires a more complex approach including the application of the next generation of sequencing techniques.

ACKNOWLEDGEMENTS

This study was prepared as a student scientific work which was a part of broader project under the supervision of Stefan Vilček. The study was supported by project INFEKT-ZOON (ITMS 26220120002) co-financed by EU.

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Received May 10, 2017
Accepted May 31, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0019

FOLIA VETERINARIA, 61, 2: 56—60, 2017

MONITORING OF PHYSIOLOGICAL CHANGES OF URIC ACID CONCENTRATION IN THE BLOOD OF SNAKES

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ABSTRACT

The evaluation of uric acid concentrations in the blood of snakes is a crucial tool in the diagnosis of gout and renal disease; both prevalent diseases in captive reptiles. However, without an understanding of the physiological fluctuations in uric acid levels and the absence of distinction that makes pathological changes, biochemical parameters are devalued. This study focuses on investigating the relationship between feeding rate and plasma-uric acid concentrations of snakes. The aim of this investigation is to facilitate a better understanding of the feed-induced changes that occur, and to render the analysis of this biochemical parameter as a more potent diagnostic tool. A total of 10 snakes were used in the study and the basal concentration of uric acid was established prior to feeding via blood biochemical analysis. The snakes were then fed rats and successive postprandial blood samples were taken for the monitoring of uric acid levels. The results demonstrated that feeding led to substantial elevations in the uric acid values, whereby postprandial concentrations were significantly elevated for up to 5 days after feeding. The postprandial elevations in uric acid documented in these snakes were of similar levels reported in snakes afflicted with gout or renal disease. The results demonstrated the significant changes that occur to uric acid levels after feeding, and highlights the resemblance between postprandial increases in uric acid and concentrations reported in snakes suffering from renal disease or gout. To avoid a misdiagnosis and to distinguish transient postprandial hyperuricemia from pathological elevations, collecting sufficient anamnestic data on time since last feeding in performing repeated sampling after one week period of fasting is suggested.

Key words: feed-induced changes; uric acid; snakes

INTRODUCTION

The analysis of biochemistry is a powerful diagnostic tool and an essential part of any diagnostic work-up of patients in the veterinary field. However, unlike in small

animal medicine, there remain significant gaps in our understanding of physiological changes and variables affecting reptilian biochemistry resulting in the devaluation of biochemical analysis as a diagnostic tool at this time. With the exponential rise in the number of reptiles in the UK, it is becoming increasingly crucial that our understanding of these animals develop alongside their increase in numbers.

Metabolic diseases are among some of the most frequently presented conditions in reptiles in captivity. Between 1992 to 1996 McWilliams and Leeson [6] found a prevalence of 84.4 % of metabolic disease in lizard patients were attributed to husbandry malpractice and dietary indiscretions. With the increased awareness of reptile owners and progression in exotics medicine, this statistic may improve but metabolic diseases remains a current issue in reptilian medicine.

Certain species of reptile exhibit elevated uric acid levels during hibernation, thought to be due to the reduced renal tubular blood flow at low temperatures experienced during the hibernation period [8].

Gout may be classified as either primary or secondary. Primary gout is the result of an overproduction of uric acid related to an innate metabolic disorder. Secondary gout is attributed to chronic disease states that offset the normal balance between production and excretion of uric acid (e.g. chronic renal disease, starvation, hypertension, use of nephrotoxic pharmaceuticals, such as gentamycin) [9].

However, gout is a common affliction in reptilian patients, it is not a common problem in general veterinary medicine. Current diagnostics of gout in reptiles combines clinical presentation, assessment of the biochemical panel (namely uric acid), radiography and biopsies, with the latter being definitive. However, in the detection of gout, diagnostic imaging and biopsies are insensitive in the early stages of the disease, leaving the veterinarian only the clinical presentation and biochemical evaluation as diagnostic options [7].

Numerous factors have been reported to influence the biochemistry of reptiles, including the season, hormones and diet (depending on the time of the blood collection) [5]. Understanding variations in serum uric acid concentrations in healthy reptiles is essential for assessing hyperuricemia in patients potentially affected by gout. However, reference ranges for uric acid provided in the current literature are broad and do not account for such physiological fluctuations rendering such information less sensitive and potentially misleading conclusions [1].

With the ability to detect hyperuricemia prior to the extensive deposition of urate crystals in tissues would allow clinicians to identify risk patients and develop appropriate therapeutic strategies.

The aim of this study was to determine feed-induced changes to serum uric acid concentrations in 7 species of snake.

MATERIALS AND METHODS

For this study, ten adult snakes of various species were used: Burmese Python (*Python bivittatus*), Bulgarian Ratsnake (*Elaphe quatuorlineata*), Boa Constrictor (*Boa constrictor*), Carpet Python (*Morelia spilota*), Taiwanese Beauty Snake (*Orthriophis taeniurus freiesi*), Common Kingsnake (*Lampropeltis getula*) and Rainbow Boa (*Epicrates cenchria*).

The snakes were housed individually in terrariums with glass front panels and with the slots in the side panels for ventilation. Each snake was kept in temperature controlled conditions at approximately 25 °C (± 5 °C). Prior to pre-prandial sampling, each snake underwent a minimum fasting period of 14 days to avoid anomalies from previous meals. The snakes were fed rats (*Rattus norvegicus*) and mice (*Mus musculus*) and the body weight was recorded with each meal. Water was given *ad libitum*.

Blood samples were collected and run prior to feeding to establish baseline concentrations of uric acid in each snake. Uric acid concentrations were measured subsequent to feeding and daily thereafter until serum concentrations returned to near basal levels.

The blood was collected via the ventral coccygeal vein with a 23-gauge needle and the samples were collected into heparinized blood collection tubes. If blood collection from the ventral coccygeal vein was not possible, it was obtained via cardiocentesis. The samples were then centrifuged and a volume of 5 µl of serum was obtained with a micropipette and was aliquoted for analysis. For the measurement of uric acid concentrations from the serum, immunoassays were performed with the LifeAssays Canine CRP test kit (Life Assays AB, IDEON Science Park, Sweden), evaluating each sample, the level of CRP in $mg.l^{-1}$.

The one-way analysis of variance (ANOVA) was performed to determine any statistically significant differences

between the means of pre-prandial and post-prandial serum uric acid concentrations; the significance was assumed when $P < 0.05$.

RESULTS

The mean pre-feeding and post-feeding serum uric acid concentrations in the blood of snakes are shown in Table 1. and Fig. 1.

Table 1. Mean pre-feeding and post-feeding serum uric acid concentrations in blood of snakes

Days post feeding	Mean serum uric acid [mg.l^{-1}]
0	253.01
1	784.65
2	1251.88
3	988.56
4	642.80
5	414.33
6	403.52
7	363.13
8	326.34
9	299.08

Table 2. Pre-feeding against post-feeding uric acid concentration results from one-way ANOVA, with significance assumed at P < 0.05

Days post feeding	P-value
1	0.00015
2	0.00013
3	0.00003
4	0.00001
5	0.01729
6	0.07928
77	0.12153
8	0.25678
9	0.45148

The post-prandial levels of uric acid rose substantially in all snakes with a mean peak concentration reaching 1251.88 mg.l^{-1} (Table 1). Serum uric acid reached peak concentrations between 1 and 2 days in the blood of all snakes, with maximum recorded levels reaching 2524.3 mg.l^{-1} in Morelia spilota and minimum 578.4 mg.l^{-1} in Python bivittatus. The majority of the snakes studied showed similar patterns of sharp elevations in uric acid, followed by a more gradual decline and returning to basal concentrations approximately nine days after feeding.

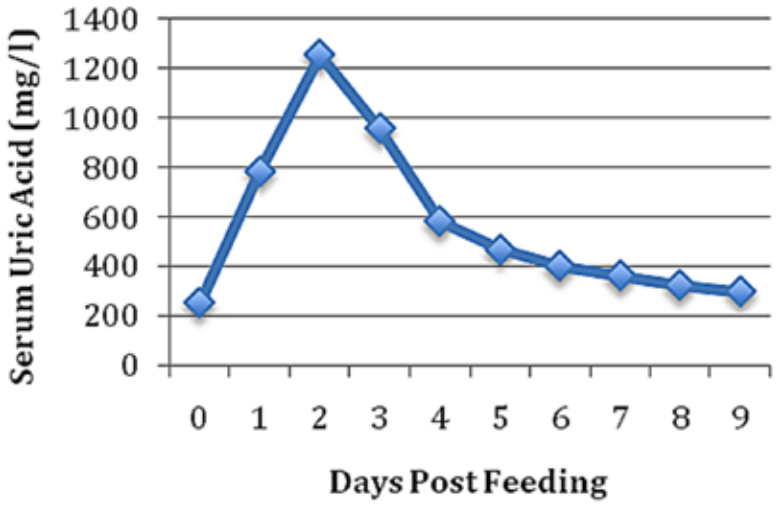

Fig. 1. Changes in mean pre-feeding and post-feeding serum uric acid concentrations in snakes

Results from the one-way ANOVA demonstrated pre-prandial and post-prandial uric acid profiles to be statistically significant ($P < 0.05$) up until the fifth day after feeding. From days 1—4 uric acid values were substantially elevated; $P < 0.001$, indicating marked deviations from the basal concentrations. From day 5, we observed a reduction in the mean uric acid concentration (414.33 $mg.l^{-1}$) with a significance value of $P < 0.05$. From day 6 onwards, there was a steady reduction in the mean concentration and we found no significance in these samples ($P > 0.05$).

DISCUSSION

This study has visibly demonstrated that significant post-prandial increases of serum uric acid concentration occur in snakes. Maixner et al. [4] demonstrated the effect of feeding on uric acid concentrations in other species of captive reptile: savannah monitor lizards (*Varanus exanthematicus*), black rat snakes (*Elaphe obsoleta*) and the Gila monster (*Heloderma suspectum*). Post-prandial serum uric acid levels found in this study revealed levels of hyperuricemia similar to those documented in reptiles suffering from renal disease or gout. Reptiles presented with gout show uric acid levels over 2-fold higher than the recorded baseline levels [2], analogous with post-prandial concentrations documented in this study. Although it is difficult to find a definitive measure of hyperuricemia due to differing physiologies among snake species, some reports define hyperuricemia as serum uric acid concentration greater than 150 $mg.l^{-1}$ [3]. Post-prandial values recorded in our present study were substantially higher than levels with peak concentrations reaching 5 times higher than recorded pre-prandial levels. These results provide compelling evidence that feeding has a quite marked effect on uric acid measurements to the extent at which the reptilian clinician may misinterpret these results as an indication of disease.

An investigation into the effect of temperature on the metabolic rate in the Bolivian Silverback (*Boa constrictor amarali*) found that the ambient temperature had a profound effect on the duration of digestion and also digestive efficiency [10]. With this, we can assume that under conditions with more variable temperatures, in particular lower temperatures (not uncommon in captive reptile husbandry), we may see changes in the relationship between feeding and uric acid concentrations.

For the exotics veterinarian this has quite significant implications on the way biochemistry panels are evaluated. It highlights the importance of collecting sufficient anamnestic data prior to carrying out further diagnostic measures. As highlighted, post-prandial effects on serum uric acid concentrations may last up to 5 days or more. It is therefore rational to suggest that information on the time interval since last feeding should be included in the anamnesis. Clinicians should also note that reptiles presented with hyperuricemia should be sampled and re-assessed no sooner than one week of fasting to allow for more accurate interpretation of uric acid concentrations and to avoid misdiagnosis.

CONCLUSIONS

To conclude, this study highlights the importance of feed-induced changes in serum uric acid concentrations in the blood of snakes. It is evident that post-prandial changes in uric acid may mimic degrees of hyperuricemia as those seen in reptiles suffering from gout or renal disease. We also discovered that with the exclusion of such information in the anamnesis, there is room for error and misinterpretation in the diagnostic work-up. The clinical relevance of these results are found while determining the time interval required for re-sampling for a more accurate representation of true basal levels of uric acid in a patient and shown to be approximately 1 week after feeding. This study also highlights the difficulties encountered when providing generalized reference values for biochemical parameters in reptiles. With substantial differences among snake species and broad reference values, there lay a risk of misinterpretation and potential misdiagnosis if it is not possible to control major variables (i.e. feeding). With the further development of reptilian medicine, we would hope to find more species specific databases, allowing for more accurate reference values available for the clinician, rendering the analysis of biochemistry a more powerful diagnostic tool.

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Received May 15, 2017
Accepted June 5, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0020

FOLIA VETERINARIA, 61, 2: 61—66, 2017

THE EFECT OF PROBIOTIC LACTOBACILLI AND ALGINITE ON THE CELLULAR IMMUNE RESPONSE IN SALMONELLA INFECTED MICE

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ABSTRACT

Alginite is organic matter rich in humic substances and commonly found in nature, but despite that, the knowledge of its biological effects is limited. In our study we focused on monitoring the effects of alginite alone, as well as its effect as a carrier of probiotic lactobacilli on the cellular immune response in SPF mice after infection with *Salmonella* Typhimurium. Sixty six conventional SPF female mice of the Balb/c line were divided into 4 groups: 1. infection free negative control (NK) supplied neither alginite nor probiotic lactobacilli in the feed; 2. infection free alginite control (Alg) supplied feed with 10 % alginite; infected control supplied alginite in the feed but no lactobacilli; 3. infectious control (Alg + Sal) — animals infected with salmonella and supplied 10 % alginite in the feed but no lactobacilli;and 4. probiotic group (Lab + Alg + Sal) — animals infected with salmonella and administered 10 % alginite and *Lactobacillus reuteri* 2/6 in the feed. On day 21 of the experiments, the mice were bled and their mesenteric lymph nodes were taken after their death. The peripheral blood of the mice was analysed for the activity of phagocytes and the percentage of selected lymphocyte subpopulations was determined in the mesenteric lymph nodes and blood. The significantly highest phagocytic activity (FA) was noted in the infected group with alginite (Alg + Sal). The FA was significantly increased in groups Alg and Lab + Alg + Sal in comparison with the NK group. The highest engulfing ability of phagocytes (phagocytic index) was observed in the Lab + Alg + Sal group in comparison with other groups, but also in Alg group in comparison with NK. In the Lab + Alg + Sal group,we observed a significantly higher percentage of B-lymphocytes, $CD4^+CD8^+$ and natural killer T cells (NKT), but more significant impact on the numbers of subpopulations of lymphocytes was observed in the mesenteric lymph nodes, with the significantly highest proportions of $CD4^+CD8^+$lymphocytes and NK and NKT cells. Our results indicated immunomodulatory properties of alginite and *L. reuteri* 2/6 in salmonella infected mice, especially at the level of the innate immune system components. This activation of phagocytosis and NK cells can be used in the treatment of various infections.

Key words: alginite; immune cells; immunomodulation; *L. reuteri*; mice

INTRODUCTION

The optimum composition of intestinal microflora is important for the correct development and function of the immune system and contributes to the sustainment of good health. Proportions of intestinal bacteria are significantly affected by the lifestyle and eating habits of the population and that has changed in many ways in the recent period. Disturbances of the natural microflora have resulted in a number of health disorders. This increased interest in probiotics is due to their capability of restoring physiological microflora, ensuring the proper function of the intestine and strengthening of the immune system. It is highly probable that in the future probiotics could help to decrease the risk of serious diseases and make their prevention and treatment more effective [9].

Alginite is a sedimentary laminated rock occurring in oil shales that originated from biomass of fossil algae during several millions of years in volcanic craters [7]. Abundant deposits of alginite are located in Slovakia, close to the village Pinciná in Lučenec basin, or in Hungary in the village Pula, where it was discovered for the first time [4]. Alginite is rich in amorphous organic matter and well preserved cells of green algae *Botryococcus braunii* [2]. Besides organic substances, alginite contains many macro-elements, e. g. P, K, Ca, Mg, and also a number of trace elements. The content of heavy metals in alginite (As, Cd, Pb, Ag, Cr, Se) does not exceed toxicity limits [8]. Owing to its enormous specific surface (300 to 650 $m^2.g^{-1}$), alginite exhibits high water retention capacity (approximately 110 %). With regard to its pH values, alginite belongs among neutral substances with relatively high proportion of humus. Humins are a class of organic substances insoluble in water, currently used in human and veterinary medicine, particularly due to their antiviral, antibacterial, detoxification and anti-carcinogenic properties.

Owing to the high specific surfac e of alginate, it can be used as a carrier of probiotic lactobacilli and serve, at the same time, as an important source of biogenic substances with immunomodulation properties.

The aim of this study was to investigate the influence of the administration of probiotic strain *Lactobacillus reuteri* and alginite on cellular immune response of SPF mice.

MATERIALS AND METHODS

Experimental design and animals

The experiments were carried out within the study: "The influence of probiotic strain stabilised on fossil carrier on the intestinal micro-environment of conventional SPF mice infected with *Salmonella* Typhimurium", which was approved by the State Veterinary and Food Administration of SR under No. 1177/14-221.

Sixty six conventional female mice of Balb/c line (Velaz s.r.o., Prague, Czechia) were kept in rearing boxes,

Table1. Experimental groups and experimental schedule

Group	Alginite (Day 0—21)	*L. reuteri* 2/6 (Day 0—21)	Infection with *Salmonella* Typhimurium (Day 7)
NK (negative control) (n = 16)	None	None	placebo = administration of uncontaminated BHI broth on day 7 after on set of administration of additives *per os* at a dose of 0.1 ml per mouse
Alg (n = 16)	10 % addition to feed throughout the experiment	None	placebo = administration of uncontaminated BHI broth on day 7 after the on set of administration of additives *per os* at a dose of 0.1 ml per mouse
Alg+Sal (n = 17)	10 % addition to feed throughout the experiment	None	10^8 CFU.ml^{-1} *S.* Typhimurium in BHI broth *per os* at a dose of 0.1 ml per mouse on day 7 after the on set of administration of additives
Lab+Alg+Sal (n = 17)	10 % addition to feed throughout the experiment	10^8 CFU.g^{-1} Stabilised on alginate throughout the experiment	10^8 CFU.ml^{-1} *S.* Typhimurium in BHI broth *per os* at a dose of 0.1 ml per mouse on day 7 after onset of administration of additives

BHI broth — Brain Heart Infusion broth

6—7 mice per box, were fed *ad libitum* a complete mixed feed for mice Altromin 1311 (Altromin International, Germany) and they had unrestricted access to water. The experimental schedule is presented in Table 1.

Characteristics of bacterial strains and their cultivation

In the experiment we tested probiotic, exopolysaccharides-producing porcine strain *Lactobacillus reuteri* 2/6. This strain was isolated from the faeces of a clinically healthy pig at the Institute of microbiology and gnotobiology of the University of Veterinary Medicine and Pharmacy in Košice. For accurate identification of this lactobacillus in the organism of mice, we used spontaneous rifampicin-resistant isolates of this strain obtained by cultivation on MRS (deMan, Rogosa and Sharpe) agar (Becton Dickinson and Company Microbiology systems, USA), containing 30 $\mu g.ml^{-1}$ rifampicin (Sigma Chemical Co., UK). Lactobacilli were cultivated anaerobically on MRS agar for 48 hours or in MRS broth at 37 °C for 24 hours. For the preparation of the inoculum, we used night (18 h) cultures from MRS broth that was inoculated onto alginite previously saturated with 40 % lactose. The solid state fermentation took place at 37 °C for 24 hours. Alginite was then lyophilised and lactobacilli counts were determined, which reached 10^8 $CFU.g^{-1}$.

The experimental mice from groups Alg + Sal and Lab + Alg + Sal were infected with *Salmonella* Typhimurium CCM 7205, obtained from Masarykova University in Brno, Czechia. They were cultivated at 37 °C for 24 hours on Brilliant Green agar (BGA, Laboratories Conda, Spain). Inoculum for infecting the mice was prepared in BHI broth (BHI, Merck, Germany) at 37 °C from the night culture.

Collection of samples and preparation of lymphocytes

On day 14 post infection (day 21 of the experiment), samples of the peripheral blood were collected from the sinus orbitalis of mice into heparin containing tubes. Eight mice from each group were used for immunological analysis. Mesenteric lymphatic nodes (MLN) were collected from these mice immediately after death (86 $mg.kg^{-1}$ body weight, sodium pentobarbital, cervical dislocation). They were placed into an ice-cold Hank's solution (137 mM NaCl; 5 mM KCl; 1.1 mM $Na_2HPO_4 . 2 H_2O$; 0.4 mM KH_2PO_4; 5 mM D-glucose; 4 mM $NaHCO_3$; 10 mM HEPES; pH 7.1—7.3). Lymphocytes were isolated from MLN by compressing the MLN between 2 sterile slides. The obtained suspension was washed in cold PBS (MP Biomedicals, France) and centrifuged for 5 min at 600 g. The number of lymphocytes was adjusted to $10^6.50\ \mu l^{-1}$.

Phenotyping and phagocytic activity of immune cells

The cells were identified by means of direct labelling with monoclonal antibodies conjugated with fluorochromes (eBioscience, USA) in the following combinations: CD4/CD8a/CD49b and CD3/B220. Specification of the antibodies used is presented in Table 2.

The cells were labelled as follows: 50 µl of blood or cell suspension from MLN were incubated with monoclonal antibodies in tubes for 15 min in the dark at laboratory temperature. Then 500 µl of lysing solution (BD FACS Lysing Solution, BD Biosciences, USA) was added to the tubes with the blood, the tubes were again incubated for 15 min in the dark at laboratory temperature and were washed twice with 1 ml PBS (centrifugation for 5 min at 600 g). Finally, 100 µl PBS was added to each tube and cytometric analysis was performed immediately employing a flow cytometer BD FACSCantoTM (Becton Dickinson Biosciences, USA). The position of lymphocytes was displayed on a basic dot plot FSC (forward-scattered light) against SSC (side-scattered light). Proportions of individual subpopula-

Table 2. Specification of anti-murine monoclonal antibodies used in the experiments

Type	Fluorochrome	Clone	Isotype	Concentration	Volume
anti-CD4	FITC	GK 1.5	IgG2b, κ	0.5 $mg.ml^{-1}$	4 µl
anti-CD8a	R-PE	53-6.7	IgG2a, κ	0.2 $mg.ml^{-1}$	2 µl
anti-CD49b	APC	DX5	IgM, κ	0.2 $mg.ml^{-1}$	1 µl
anti-CD3	PerCP-eFluor710	17A2	IgG2b, κ	0.2 $mg.ml^{-1}$	2 µl
anti-B220	PE-Cyanine7	RA3-6B2	IgG2a, κ	0.2 $mg.ml^{-1}$	2 µl

tions were determined by means of dot plots for relevant fluorescences (CD4 against CD8; CD8 against CD49b; CD3 against B220) and expressed as per cent proportions of all lymphocytes. The obtained data were analyzed using BD FACS DivaTM software.

The phagocytic activity was det-6B2ermined using the above mentioned flow cytometer and commercial kit Phagotest® (Orpegen Pharma, Germany).

Statistical analysis

Statistical analysis was performed by the one-way analysis of variance (one-way ANOVA) and Tukey test, using software GraphPad Prism. The level of significance was set to $P < 0.05$.

RESULTS AND DISCUSSION

In the experimental groups Alg, Alg + Sal, and Lab + Alg + Sal, we observed a significant increase in phagocytic activity and engulfing ability of phagocytes (phagocytic index). The highest phagocytic activity was recorded in group Alg + Sal and lower in the group with simultaneous administration of lactobacilli (Lab + Alg + Sal), which may be related to better clinical status of mice in this group (Fig. 1a). The highest engulfing ability of phagocytes was observed in group Lab + Alg + Sal (Fig. 1b). The induction effect of lactobacilli on phagocytic activity was reported in the study by Perdigón et al. [5] in which *per os* administration of *L. casei* significantly increased phagocytic activity of macrophages in mice. The immunostimulating effect of humic acids was also confirmed by Habibian et al. [3], who observed dose-dependent potentiation of phagocytic activity of mononuclear cells in rats.

The significantly highest percentage proportion of CD4+ lymphocytes in the peripheral blood was observed in group Alg + Sal, and alginate itself significantly decreased this proportion in healthy animals in comparison with group NK (Fig. 2a). In group Lab + Alg + Sal we recorded the highest number of both $CD4^+CD8^+$ lymphocytes (Fig. 2b) and NKT ($CD49b^+CD8^+$) cells (Fig. 2c), which contradicts the results of Gill et al. [1] who failed to observe any noticeable effect of the administration of probiotics on subpopulations of lymphocytes in mice. However, subpopulation of NK cells ($CD49b^+CD8^-$) in lactobacilli and alginite infected groups in our study was significantly lower in comparison with alginite control (Fig. 2d).

In mesenteric lymph nodes we observed a significant stimulation of cellular response in group Lab + Alg + Sal, which showed the significantly highest proportion of double-positive $CD4^+CD8^+$ lymphocytes (Fig. 3a), NK cells (Fig. 3b) and NKT cells (Fig. 3c). The proportion of $CD4^+$ lymphocytes was significantly increased in both infected groups (Fig. 3d). The positive influence of lactobacilli on local immune response was observed also in the study by PerdIgón et al. [6], who also recorded an increase in $CD4^+$ cells in the small intestine of mice after the administration of *L. casei* and *L. plantarum*.

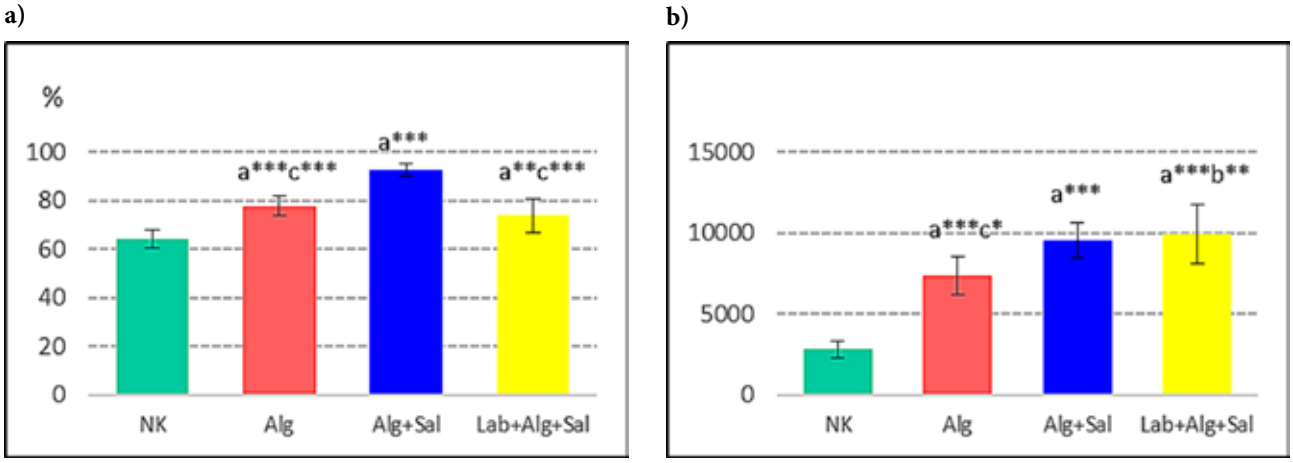

Fig. 1. The influence of the administration of probiotic strain *L. reuteri* and alginite on: a) Phagocytic activity and b) Phagocytic index in mice infected with *Salmonella* Typhimurium
a — significant difference compared to NK; b — significant difference compared to Alg;
c — significant difference compared to Alg+Sal; * — $P < 0.05$; ** — $P < 0.01$; *** — $P < 0.001$

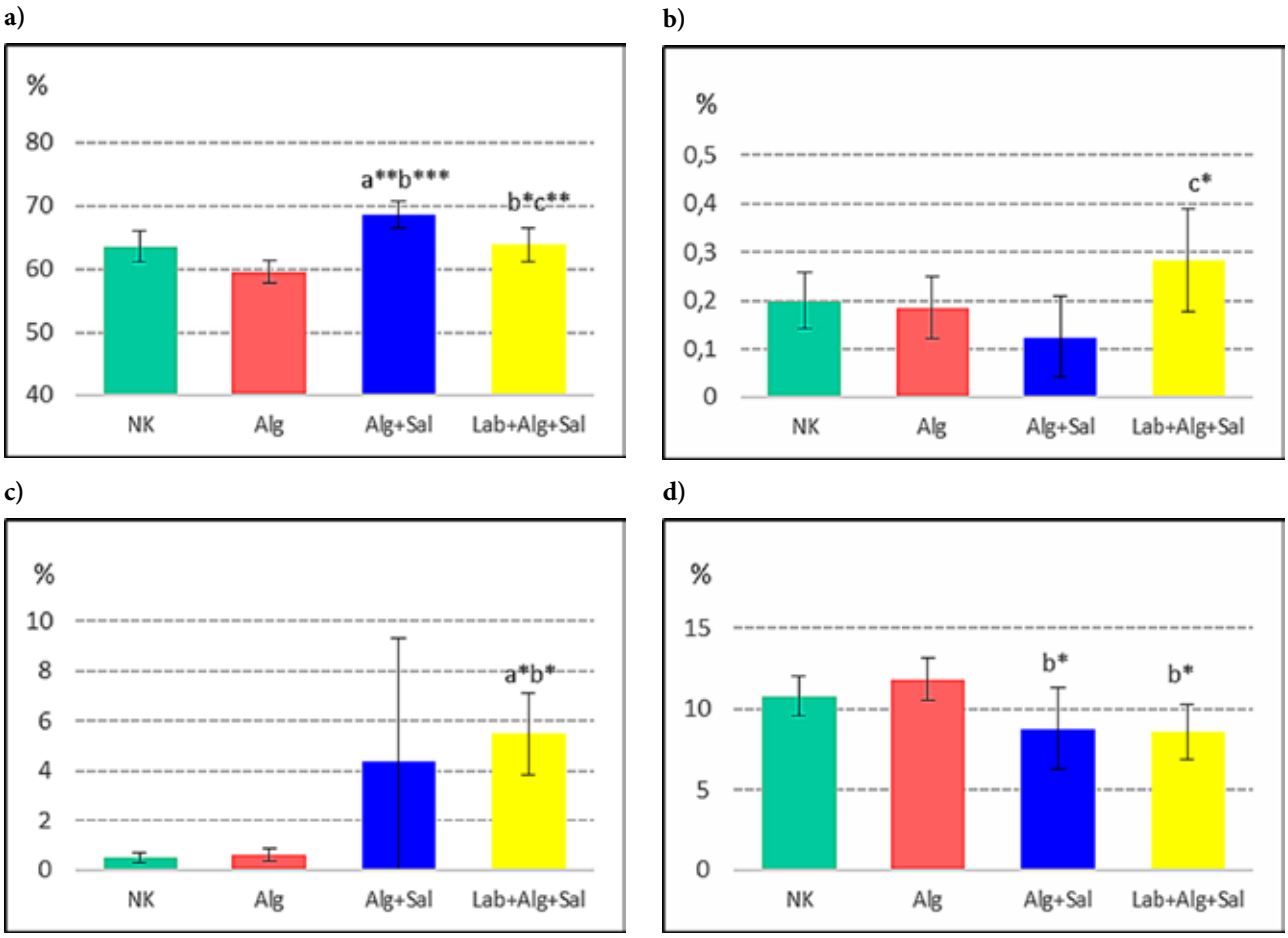

Fig. 2. The influence of the administration of probiotic strain *L. reuteri* and alginite on proportions of the following lymphocytes in peripheral blood of mice infected with *S.* Typhimurium: a) $CD4^+$; b) $CD4^+CD8^+$; c) $CD49b^+CD8^+$; d) $CD49b^+CD8^-$
a — significant difference compared to NK; b — significant difference compared to Alg; c — significant difference compared to Alg+Sal; *— $P < 0.05$; ** — $P < 0.01$; *** — $P < 0.001$

CONCLUSIONS

Our experiment indicated an immunomodulation potential of lactobacilli and humic substances found in alginite. In the group infected with *S.* Typhimurium, the cellular immune response was significantly stimulated by the administration of *L. reuteri* and alginate, particularly at the local level, in mesenteric lymph nodes of mice by activation of $CD4^+CD8^+$ lymphocytes, NK and NKT cells, and at the level of the innate immune system component by activation of phagocytosis.

ACKNOWLEDGEMENT

This study was supported by the Slovak Research and Development Agency on the basis of the Agreement No. APVV-0199-11 and by the structural project EU INFEKTZOON (ITMS 26220120002).

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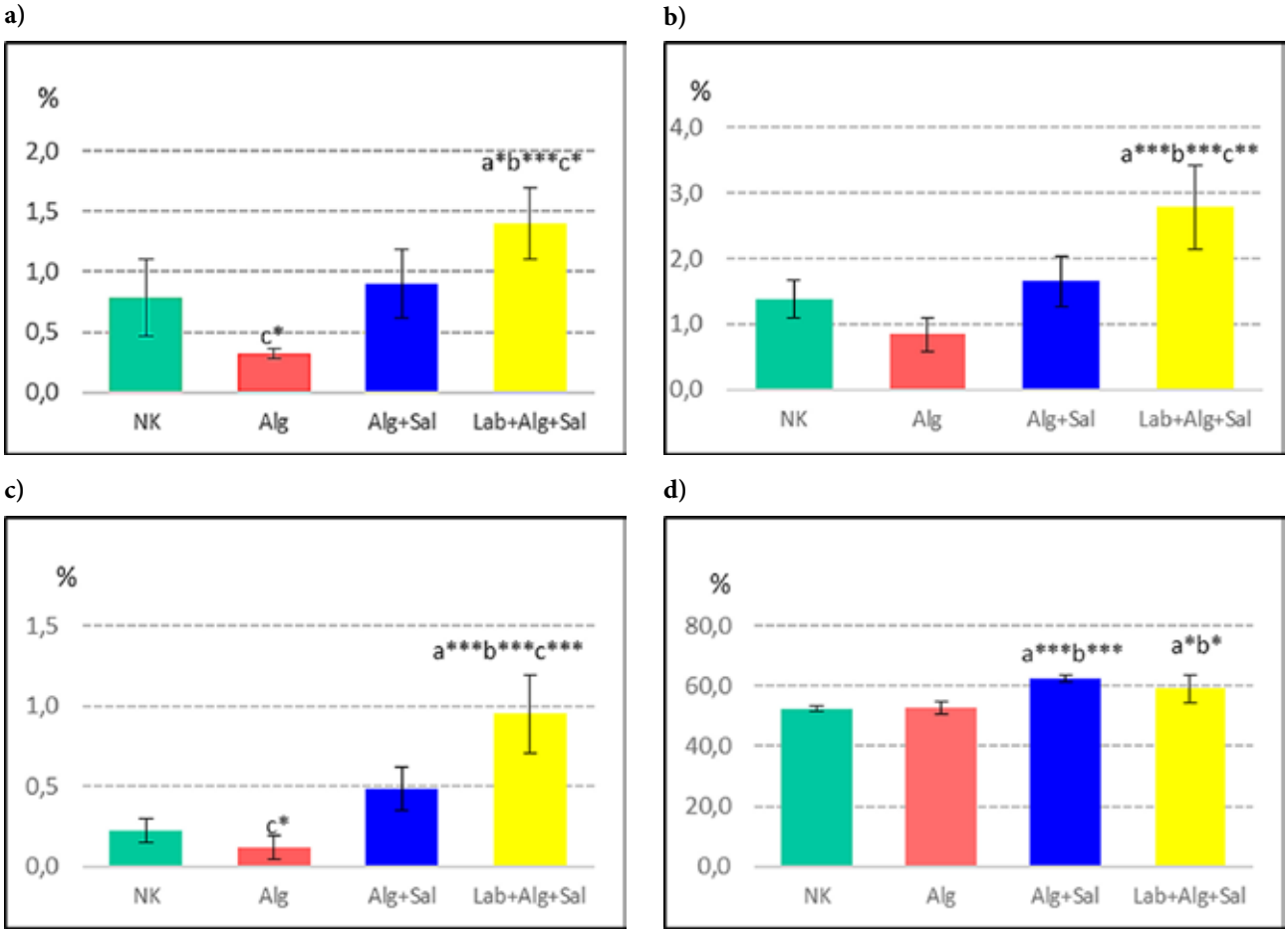

Fig. 3. The influence of the administration of probiotic strain *L. reuteri* and alginite on proportion of the following lymphocytes in mesenteric lymph nodes of mice infected with *S.* Typhimurium: a) $CD4^+CD8^+$; b) $CD49b^+CD8^-$; c) $CD49b^+CD8^+$; d) $CD4^+$
a — significant difference compared to NK; b — significant difference compared to Alg; c — significant difference compared to Alg + Sal; * — $P < 0.05$; ** — $P < 0.01$; *** — $P < 0.001$

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Received May 17, 2017
Accepted June 6, 2017