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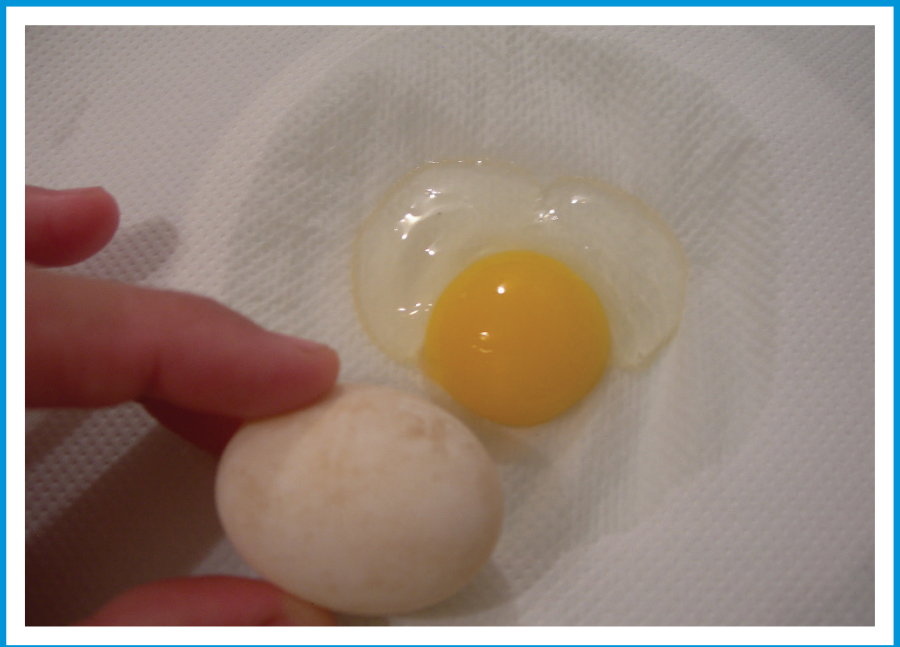

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Original Research Article

SEROPREVALENCE OF *Neospora caninum* IN CATTLE OF NEISHABOUR, NORTHEAST IRAN

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Abstract: *Neospora caninum* is a worldwide distributed pathogen which causes abortion in cattle and leading to economic loss in the cattle industry. The aim of this study was to determine the seroprevalence of *N. caninum* antibodies in cattle in Neishabour (Northeast of Iran). From September 2012 to October 2013 a total of 100 serum samples were analyzed for antibodies against *N. caninum* antigen using a commercial *N. caninum* ISCOM ELISA kit. Antibodies to *N. caninum* were found in 26 of the 100 (26%) sera based on ELISA test results. With regard to seropositivity, no significant difference was observed regarding origin, sex and age (P>0.05).The results showed that *N. caninum* is relatively prevalent in cattle in the Northeast part of Iran and the evaluation of potential of infection can be useful when considering control programs.

Key words: seroprevalence; *Neospora caninum*; Neishabour; cattle; Iran

Introduction

Neospora caninum is a heteroxenous cyst-forming apicomplexan protozoan which is considered as a major causative of infectious bovine abortion worldwide and has been associated with sporadic, endemic and epidemic abortions (1, 2).The infection causes important economic loss to the cattle industry due to reproductive failure associated with abortion and mortality in congenitally infected calves. *N. caninum* infection has been reported in dairy cattle herds on all continents (3, 4, 5, 6).

Dogs and coyotes are the definitive hosts in *N. caninum* life cycle (7, 8, 9) whereas cattle and other mammals act as natural intermediate hosts (10, 11, 12). In cattle, *N. caninum* infection may occur by horizontal transmission due to ingestion of sporulated oocysts shed by the definitive host (13, 14). However, vertical transmission is the predominant route of infection (1, 14). Vertical transmission occurs when tachyzoites cross the placenta of a persistently infected dam and infect the fetus (15).

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Transplacental transmission can occur in consecutive pregnancies in the same cattle and so the infection can persist in cattle herds through many generations.The infection usually has a chronic course and persists throughout the life of an infected animal (16, 17). *N. caninum* DNA has been reported in fresh and frozen semen of naturally infected bulls and the possibility of venereal transmission in bovine neosporosis has been suggested (16, 18).

The economic impact of *Neospora*-induced abortions depends on direct costs and the value of fetuses lost. Indirect costs include those associated with establishing the diagnosis, rebreeding cows that aborted and possible loss of milk yield (19). As clinical diagnosis is difficult, serological tests are necessary for an exact diagnosis. Several serological tests, including the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody technique (IFAT), the direct agglutination test (DAT), and immunoblots (IB) can be used to detect anti *N. caninum* antibodies (13).

Iran is a very diverse country in terms of climate. To evaluate the impact of climate on the prevalence of *N.caninum*, the incidence of this parasite should be measured in different climatic area. Since recognition of *N. caninum* in 1980, there are only few studies about the seroprevalence of bovine neosporosis in Iran, which are mostly carried out in Central and South-East Iran (20, 3). This study was performed to determine the prevalence of antibodies to *N. caninum* in cattle in Neishabour city, Northeast of Iran.

Materials and methods

Serum samples

During September 2012 and October 2013, One hundred serum samples were collected from cattle of Neishabour, the animals being randomly selected. Blood samples were taken using disposable needles. The owners were questioned about animal management, age and the information obtained was recorded. This study was performed between September 2012 and October 2013. All samples were immediately transported to the diagnostic laboratory. Serum was removed after centrifugation at 1000×*g* for 10 min. All sera were divided equally into two microtubes and stored at -20 °C until laboratory testing.

ELISA test

Serum samples were stored at -20°C until tested. They were analyzed for antibodies to *N. caninum* using ELISA. Anti-Neospora antibodies were detected using a commercially available *N. caninum* iscom ELISA kit (Svanova Biotech AB, Sweden). The kit was used according to the manufacturer's instructions. Briefly, 100 microlitres of pre-diluted serum sample added as first antibody and the plate incubated at 37 °C on shaker for 1 hour. The wells were washed three times with PBS Tween Buffer and 100 microlitres of HRP conjugate added to each well and incubated for one hour at 37 °C. The plate was washed again and 100 microlitres of substrate solution added and incubated at room temperature for 10 minutes. Then 50 microlitres of stop solution were added to stop the reaction and the plates were read in an ELISA microplate reader (Anthos 2020, Austria) at a wavelength of 450 nm. The optical density (OD) of the ELISA was read on an automatic plate reader and the Percent Positivity values (PP) of the test samples were calculated by the following formula:

PP = Mean OD value (sample or Negative Control) × 100) / Mean OD value Positive Control

The results were expressed as the percent positivity (PP) of the high positive control sera. The manufacturer's current recommendations for the interpretation of the test are that a test result of below 20 PP indicates a negative result, and a test result of above or equal to 20 PP indicates a positive result.

Statistical analysis

Descriptive statistics with 95% confidence interval (CI) was used to estimate the prevalence of infection and a chi-square test was used to analyze associations between infection by *N. caninum* and other factors studied in the present study. For statistical analysis, the SPSS 12 computer program was used and $P<0.05$ was considered to be significant.

Table 1: Seroprevalence of *Neospora caninum* in relation to age (95% CI)

Age	The number of animals tested	No. of positives	Seroprevalence (%)
<18 months	29	6	20.6%
≥18 months	71	20	28.1%

Table 2: Comparison of *Neospora caninum* antibodies in relation to sex (95% CI)

Sex	The number of animals tested	No. of positives	Seroprevalence (%)
Bull	9	2	22.2%
Cow	91	24	26.3%

Results

Results obtained from the sera using ELISA are given in Tables 1 and 2. The results were expressed as the percent positivity (PP) of the high positive control sera. Antibodies to *N. caninum* were found in 26 of the 100 (26.0%; 95% CI: 17.8-35.7) sera based on ELISA results. Among the 29 sera in the cattle <18 month age group, 6 (20.7%; 95% CI: 8.0-39.7) were seropositive, whereas among the 71 sera above 18 months old, 20 (28.2%; 95% CI: 18.1-40.0) were seropositive (Table 1). Among the 9 bulls, 2 (22.2%; 2.8-60.0) were seropositive whereas of the 91 cows, 24 (26.4%; 95% CI: 17.7-36.7) were seropositive (Table 2). There was no statistically significant relationship between seroprevalence of sex and age groups (P>0.05)

Discussion

Our study showed more than one fourth of samples were positive for *N. caninum*, which can cause abortion in cattle leading to economic loss in the cattle industry in this region. In this study there was no significant difference in seroprevalence between the different age groups.

Akca et al. (2005) reported that 8.2% of Simmental tested cows were positive in Kars province, Turkey (21). Sevgili et al. (2005) found antibodies to *N. caninum* in 23 of the 305 (7.5%) cow sera based on ELISA test results in the province of Sanliurfa, Turkey (22). With regard to seropositivity, no significant difference was observed in origin, animal breed, and age (P >0.05). The presence of antibodies against *N. caninum* in cows only indicates exposure to the parasite. Wouda et al. (1998) and Sadrebazzaz et al. (20) reported for most herds that the seroprevalence levels were equal across all age groups (23). The relationship between age and seroprevalence in bovine neosporosis is speculative. Jensen et al. (1999) suggested that seroprevalence increases with age (24). In contrast, Sanderson et al. (2000) reported that cows below 3 years of age had higher CI-ELISA inhibition percentage values than cows above 6 years of age (25). They also suggested that infected cows can infect fetuses, and if these calves have not been reinfected, antibody titers decline over time, resulting in an apparent decrease in seroprevalence with cow age. In our previous study (3) in Kerman province, antibodies to *N. caninum* were found in 36 of the 285 (12.6%) sera based on ELISA test results. Data showed that no significant difference regarding origin, sex and age (P>0.05).

Heidari et al (2012) reported that the seroprevalence of *N. caninum* infection in the native cattle of Kurdistan province of Iran is 7.80% (26). Their results showed that there was no significant difference in the prevalence rate of abortion between seropositive and non-seropositive cattle (p = 0.588). These findings may be due to that native breeds are genetically resistant to neosporosis. In investigation in Brazil on the infection of *N. caninum* in different cattle breeds (Zebu, Holstein, crossbreed Zebu/Holstein) there was close association between cattle breeds and the frequency of infection by *N. caninum* (10). The result of previous study

indicated that breed, which is inherent factor to host, could be considered of high relevance for the distribution and frequency of infection by *N. caninum*. The authors of that study suggested that genetic distance between Holstein and Zebu breeds possibly leads to the significant difference. These results were similar to finding of a study performed in Ontario which showed that there was a genetic susceptibility to infection by *N. caninum* in Holstein cattle (27).

Due to the lack of information about the prevalence of infection in the definitive host, the dog, in Iran, it is not possible to know which method of transmission (horizontal or vertical) is the main route of infection. However, further studies on the epidemiological evidence for a relationship between *N. caninum* infection in dogs and cattle and the relationship between abortion in cows and infection with *N. caninum* in Iran are required.

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SEROPREVALENCA *Neospora caninum* PRI GOVEDU V SEVERNEM IRANU

S. R. Nourollahi-Fard, M. Khalili, O. Fazli, H. Sharifi, M. H. Radfar

Povzetek: *Neospora caninum* je po vsem svetu razširjen patogen, ki povzroča zvrge pri govedu, kar ima za posledico v velike gospodarske izgube v govedoreji. Cilj naše raziskave je bil ugotoviti prekuženost s protitelesi proti *N. caninum* pri govedu v mestu Neishabour (severovzhodni Iran). Od septembra 2012 do oktobra 2013 je bilo analiziranih skupno 100 vzorcev seruma za protitelesa proti antigenu *N. caninum* z uporabo komercialno dostopnega kita *N. caninum* ISCOM ELISA. Rezultati testa ELISA so pokazali protitelesa proti *N. caninum* v 26 od 100 vzorcev (26 %) serumov. V zvezi s seropozitivnostjo niso opazili pomembnih razlik glede pripadnosti, spola in starosti ($p > 0,05$). Rezultati so pokazali, da je *N. caninum* dokaj razširjena pri govedu v severovzhodnem delu Irana in da je ovrednotenje možnosti okužbe lahko koristno pri pripravi kontrolnih programov.

Ključne besede: seroprevalenca; *Neospora caninum*; Neishabour; govedo; Iran

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Original Research Article

EVALUATION OF DIFFERENT CHEMICAL COMPOSITIONS IN EGGS OF THE HERMANN'S TORTOISE (*Testudo hermanni*)

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Abstract: In this study, Hermann's tortoise (*Testudo hermanni* boettgeri) eggs were studied. The aim was to evaluate the basic composition, amino and fatty acid profiles, as well as the presence of certain trace elements in the eggs. The average size of THB eggs was 29.9 × 39.5 mm and weight 20.7 g. The shell accounted for 12.5 %, albumen 46.9 % and yolk 40.6 % of the entire THB egg. The refractive index was 1.3341 in the albumen and 1.5120 in the yolk. The albumen contained 98.2 % water, 0.9 % proteins, 0.7 % ash and traces of fat. The yolk contained 60.6 % water, 21.0 % protein, 14.2 % fat, and 4.0 % ash. The pH of albumen was 8.8.
The fatty acid composition was measured in egg yolk; oleic acid, palmitic acid, palmitoleic acid and, vaccenic acid were the most abundant. The amino acid composition was measured in egg albumen, and 18 amino acids were detected. The major amino acids present in albumen were glutamic acid, aspartic acid, leucine, phenylalanine, lysine, glycine, serine, threonine, and alanine. Concentrations of the trace elements, in descending order were in the shell: iron (Fe), nickel (Ni), copper (Cu), manganese (Mn), cobalt (Co), zinc (Zn), chromium (Cr), arsenic (As) and selenium (Se); in the albumen: Fe, Cu, Cr, Se; and in the yolk: Fe, Zn, Cu, Mn, Se, Cr, Ni, Pb, Co.
The purpose was to determine the chemical composition the of eggs, which can be used as a basic study for further comparison with free-living tortoises, and also as a comparison for the possible impact of environmental pollution.

Key words: *Testudo hermanni*; eggs; chemical composition; fatty acids; amino acids; trace elements

Introduction

Hermann's Tortoise (*Testudo hermanni*) (TH) is a medium-sized terrestrial species that can be found in nature in the European Mediterranean area. Currently, two subspecies are distinguished: *Testudo hermanni* hermanni (THH) in Western Europe and *Testudo hermanni* boettgeri (THB) in Eastern Europe (1). Scientists disagree about the existence of a third subspecies *Testudo hermanni* hercegovinensis (2).

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Egg reproduction is common for all tortoises. The size and shape of turtle eggs can vary within species and even for individual clutches. The clutch size ranges from only one or two eggs in the Asian black marsh turtle (*Siebenrockiella crassicollis*) or the pancake tortoise (*Malacochersus tornieri*) to over hundred eggs in sea turtles (3). THH lay a maximum of seven eggs per clutch and THB nine eggs; a mean clutch size is 3.3 and 4.3 eggs, respectively. Most frequently, TH lay one to two clutches per year, rarely three (1). Among turtles, the weights of their eggs range from 2.2 g in the Common musk turtle (*Sternotherus odoratus*) to

110 g in the Galapagos giant tortoise (*Geochelone elephantopus*) (3). In comparison to turtle eggs, the average weight of chicken eggs is 58 g (4). Typical reptile eggs are in most cases symmetrical, i.e. spherical or elliptical and are not tapered (5). TH eggs are white, hard-shelled, and almost elliptical in shape (1). Typical avian eggs are asymmetrical and tapered at one end (5). Reptiles exhibit a greater range in eggshell type then avian eggs (5). Irregularly shaped eggs occur commonly in turtles from the Flat-headed turtle (*Platemys*), the Neotropical wood turtles (*Rhinoclemmys*), the Freshwater terrapins from genus *Heosemys* and the genus *Melanochelys*. Pillow-shaped (i.e. elliptical in cross-section) eggs are the rule in the Hinged tortoises (*Kinixys* sp.). Most Testudinidae produce elliptical eggs, but some larger species, e.g. the African spurred tortoise (*Geochelone sulcata*), produce spherical eggs. Regardless of the actual size or shape of the egg, or the type of eggshell, the proportions of egg components are relatively constant among the species (5).

All reptile eggs have an outer shell that is either flexible (soft-shelled) or rigid (hard-shelled). The eggshell consists of a fibrous inner layer (egg membrane) that contains proteins and an outer layer that is primarily composed of calcium carbonate. The main functions of the eggshell are to protect the contents from mechanical and microbial influences and to serve as a mineral reservoir for the growing embryo. The eggshell contains pores through which, especially in hard-shelled eggs, moisture and gases are exchanged (3). The structure of the egg shell ranges from pliable to brittle in the former and entirely brittle in the latter (6). The reptile eggs have a thick fibrous membrane between the inner surface of the shell and albumen. Shortly after laying, contraction of the yolk and other contents causes an air cell to appear, usually at one end. This effect can be seen quite clearly if the egg is examined against a bright light source (7). The same effect is seen in the chicken egg (8).

A tortoise egg's albumen accounts for about 58 % of the entire egg weight and has a protein content of 10-12 %, comprising mainly ovalbumin, ovotransferrin, ovomucoid, globulins and lysozyme (9). The albumen fraction is an important reservoir of water (6). Chicken albumen consists primarily of about 90 % water into which 10% proteins are dissolved (including albumins, mucoproteins, and globulins). Unlike the yolk, which is high in fats, albumen contains almost no fat, and the carbohydrate content is less than 1 % (10). Chalazae are absent in reptile eggs and present in bird eggs (11; 12).

Reptile eggs contain large amounts of yolk, ranging 32-55 % for turtles and 72-99 % for lizards and snakes. The eggs contain a considerable 50-70 % more yolk than what the embryo requires for its development. The surplus yolk serves as an energy reserve and an important reservoir for vitamins, minerals, and trace elements for the newly hatched animal during the first few weeks of life (3). The yolk membrane, also called the vitelline membrane, surrounds the white and yellow yolk material on which the embryonic disc is situated. Morphologically, several structures are seen in the yolks of reptiles, birds, and five mammal species of monotremes (amniotic eggs) (5). The latebra, the neck of the latebra, the nucleus of the pander, and the embryonic disc originate from the white yolk, which represents only 2 % of the whole yolk (11; 12).

The chemical compositions of turtle eggs have not yet been investigated in detail, especially because the eggs are not commercially used for human consumption, as chicken eggs are. Only a few articles are found on this topic (13; 14). The aim of our study was to evaluate the chemical composition, amino and fatty acid profiles, and certain trace elements of the Hermann's tortoise eggs. The purpose was to determine the chemical composition of eggs, which can be used as a basic study for further comparison with free-living tortoises, and also as a comparison for the possible impact of environmental pollution.

Materials and methods

All tortoise species of the family Testudinidae have been included in Appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1977. The species Hermann's tortoise (*Testudo hermanni*) is covered by Regulation (EC) No. 338/97 and listed in Annexes A. It is also strictly protected by Council Directive 92/43/EEC, the "Habitats Directive". According to the national legislation, the breeder has obtained a permit for the breeding of Hermann's tortoises issued by the Ministry for the Environment and Spatial Planning.

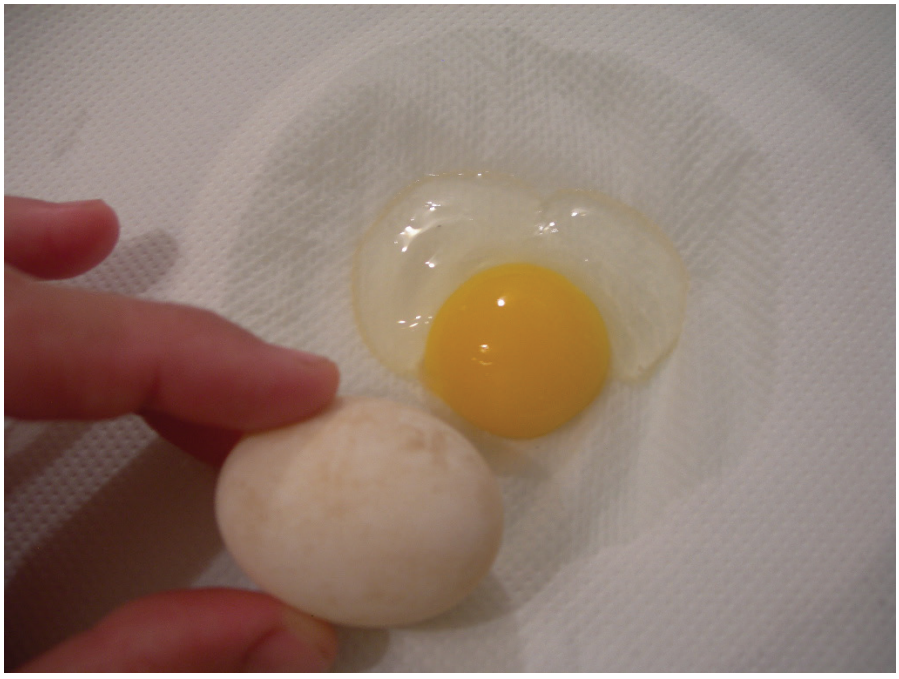

Figure1: Hermann's eggs

All the Hermann's tortoises in this study are captive bred in Slovenia, and they are born and bred in captivity in accordance with the conditions laid down in Article 54 of the Commission Regulation (EC) No 865/2006 and in CITES Res. Conf. 10.16 (Rev.). To use the eggs of protected species for scientific purposes, according to the national legislation (nature conservation or veterinary), no special permit is required. The farm of Hermann's tortoises was designated for the trade of tortoises as pet animals. The tortoises live throughout the year in outdoor enclosures. The average age of females was 30 years, and their average weight was over 2000 g. The diet of our breeding group was based on grass with additional seasonal vegetables and fruits.

In June 2014, eggs from three clutches of THB (N=15) were collected (Fig. 1). Eggs were laid within 24 hours. The next day, each egg was weighed at a precision of 0.01 g and measured with a vernier calliper with the precision of 0.05 mm. The yolk was separated from the albumen, and yolk, albumen, and shell from each egg were weighed individually. After that, the pooled samples of the shell, albumen and yolk were prepared for further analyses by homogenizing fractions from fifteen eggs with a laboratory blender. The pooled samples were prepared because the contents of one egg would not be enough for all the intended analyses. The refractive index value of albumen and yolk was measured with the Abbe refractometer, and the pH of albumen was determined using PHM210 standard pH meter (Meter Lab) immediately after the preparation of pooled samples. For further analyses samples were stored in a freezer (-20 °C).

For the determination of chemical and fatty acid composition, the contents of amino acids and trace element validated methods were used. Analyses were performed in duplicates. Moisture was determined by drying to a constant weight at 102 °C. Total ash was determined by incineration in a muffle furnace at 550 °C. Proteins were estimated by measuring the total nitrogen via the Kjeldahl method and multiplying by 6.25. Total fat was determined via the Weibull-Stoldt method. For the determination of fatty acids in the yolk, gas chromatography with a flame ionisation detector (GC-FID) was used. Tryptophan in albumen was determined via high-performance liquid chromatography (HPLC) and the concentration of other amino acids in albumen via GC-FID. The concentrations of trace elements in the shell, albumen and yolk, were determined via inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion of the samples.

Data were analysed using Microsoft Excel software.

Results

The results of the arithmetic mean (am), standard deviation (SD), the minimum value (min), and the maximum value (max) for eggs length, width, shell thickness and weight of shell, albumen and yolk are presented in Table 1. The moisture, ash, protein and fat contents in albumen and yolk are presented in Table 2. In the albumen, the pH value was 8.8 and the refractive index value was 1.3341. In the yolk, the refractive index value was 1.5120.

The quantity of each fatty acid was expressed as the percentage of the total fatty acid content (Table 3). Oleic acid was the major acyl component of the yolk, forming almost half of the total fatty acid mass (53.26%). The yolk profile was dominated by monounsaturated fatty acids (76.96%); saturated fatty acids contributed 18.60%. The fatty acid profile of the yolk lipid differed slightly from the results of the THB egg yolk obtained from Speake et al. (13), as seen in Table 3.

The results of amino acid composition in albumen are presented in Table 4. Of the non-essential amino acids, glutamic acid, and aspartic acid dominated. The largest part of the essential amino acids was represented by leucine, phenylalanine, and lysine.

The concentrations of trace elements Pb, Cd, As, Se, Zn, Ni, Cu, Cr, Mn, Fe, and Co in the shell, albumen and yolk are shown in Table 5. The concentrations of trace element in the yolk were far higher than those from the albumen, except for Cr for which the concentration did not differ from those in the yolk. The results showed a considerable difference between shell trace element concentrations of As, Ni, Cr, Mn, Fe and Co versus yolk or albumen concentrations. Interestingly, the contents of Se and Zn were the highest in the yolk. Regarding toxic elements, Pb was found only in the yolk, while As was found only in the shell. Cd was not found in any fraction of the egg.

Table 1: Hermann's tortoise eggs characteristics and compositions (N=15)

Egg's characteristics	am (mm)	SD (mm)	min – max (mm)
Egg length	39.5	±1.3	37.3 – 42.7
Egg width	29.9	±1.0	26.8 – 30.9
Shell thickness	0.5	±0.07	0.4 – 0.6
Egg's compositions	am (g)	SD (g)	min – max (g)
Shell	2.6	±0.15	2.3 – 2.9
Albumen	9.7	±1.4	7.1 – 11.0
Yolk	8.4	±0.6	7.7 – 9.1
Whole egg	20.7	±1.4	17.2 – 22.4

am – arithmetic mean, SD – standard deviation, min – minimum value, max – maximum value

Table 2: Composition of the albumen and yolk of Hermann's tortoise eggs

Constituent	Albumen (%)	Yolk (%)	Albumen and Yolk together (%)*
Moisture	98.2	60.6	80.7
Ash	0.7	4.0	2.3
Protein	0.9	21.0	10.2
Fat	<0.5	14.2	<6.8

* the calculated value based on the data: the shell 12.5 %, the albumen 46.9 % and the yolk 40.6 % of the total content of eggs.

Table 3: Fatty acid composition of Hermann's tortoise egg yolk

Fatty acids	Percentage our results (MU)	Percentage Speake et al. (13) ± SD
Caprinic acid (C 10:0)	< 0.05	/
Lauric acid (C 12:0)	0.09 (±0.04)	/
Myristic acid (C 14:0)	0.92 (±0.17)	0.8 ± 0.1
Myristoleic acid (C 14:1)	0.13 (±0.04)	/
Pentadecylic acid (C15:0)	0.06 (±0.03)	/
Palmitic acid (C 16:0)	13.84 (±2.04)	14.1 ± 0.3
Palmitoleic acid (C 16:1)	12.58 (±1.97)	8.9 ± 1.2
Heptadecanoic acid (C 17:0)	< 0.05	/
Heptadecenoic acid (C 17:1)	0.20 (±0.05)	/
Stearic acid (C 18:0)	3.63 (±0.67)	5.3 ± 0.9
Oleic acid (C 18:1 n-9)	53.26 (±5.14)	45.6 ± 1.5
Vaccenic acid (C 18:1 n-7)	9.33 (±1.72)	9.4 ± 0.3
Linoleic acid (C 18:2 n-6)	1.78 (±0.33)	6.6 ± 1.5
α-linolenic acid (C 18:3 n-3)	0.67 (±0.13)	3.8 ± 0.6
γ-linolenic acid (C 18:3 n-6)	< 0.05	/
Octadecatetraenoic acid (C 18:4 n-3)	< 0.05	/
Arachidic acid (C 20:0)	0.06 (±0.04)	/
Eicosenoic acid (C 20:1 n-9)	1.40 (±0.26)	1.3 ± 0.1
Eicosadienoic acid (C 20:2 n-6)	0.06 (±0.04)	/
Eicosatrienoic acid (C 20:3 n-6)	0.13 (±0.04)	/
Arachidonic acid (C 20:4 n-6)	0.84 (±0.16)	1.5 ± 0.3
Eicosatetraenoic acid (C 20:4 n-3)	< 0.05	/
Eicosapentaenoic acid (C 20:5 n3)	0.18 (±0.05)	0.4 ± 0.0
Erucic acid (C 22:1 n-9)	0.06 (±0.04)	/
Docosapentaenoic acid (C 22:5 n3)	0.29 (±0.06)	0.6 ± 0.1
Docosahexaenoic acid (C 22:6 n3)	< 0.01	0.0
Lignoceric acid (C 24:0)	< 0.01	0.0
Tetracosenoic acid (C 24:1)	< 0.05	/
Conjugated linoleic acid	0.33 (±0.07)	/
Saturated fatty acids	18.60 (±2.16)	20.2
Mono-unsaturated fatty acids	76.96 (±5.76)	65.2
Poly-unsaturated fatty acids	4.28 (±0.41)	/
Total omega 3	1.14 (±0.15)	/
Total omega 6	2.81 (±0.37)	/
Total omega 3/ Total omega 6	0.41 (±0.08)	/

MU – measurement uncertainty, SD – standard deviation, / – not done

Table 4: Amino acid composition of Hermann's tortoise egg albumen

Amino acid	g/100 g (MU)
Tryptophan	< 0.01
Aspartic acid	0.101 (±0.014)
Threonine	0.052 (±0.009)
Serine	0.064 (±0.011)
Glutamic acid	0.115 (±0.015)
Proline	< 0.01
Glycine	0.072 (±0.011)
Alanine	0.042 (±0.008)
Valine	< 0.01
Isoleucine	< 0.01
Leucine	0.095 (±0.013)
Tyrosine	< 0.01
Phenylalanine	0.083 (±0.012)
Lysine	0.081 (±0.012)
Histidine	< 0.01
Arginine	< 0.01
Cysteine	0.0607 (±0.0074)
Methionine	< 0.001

MU – measurement uncertainty

Table 5: Eleven trace element concentration (mg/kg wet weight) in the shell, albumen, and yolk of Hermann's tortoise eggs

Element	Shell (mg/kg)	Albumen (mg/kg)	Yolk (mg/kg)
Pb	< 0.01	< 0.01	0.03
Cd	<0.005	< 0.005	< 0.005
As	0.068	< 0.01	< 0.01
Se	0.017	0.005	0.188
Zn	0.5	< 0.4	26
Ni	9.7	< 0.005	0.031
Cu	1.0	0.23	1.0
Cr	0.251	0.076	0.077
Mn	0.922	< 0.020	0.369
Fe	> 40	4.1	31
Co	0.873	< 0.002	0.005

Discussion

Relationships between egg size, egg components, and neonate size have been investigated across a wide range of oviparous taxa (15). The average size of THB eggs examined was 29.9 × 39.5 mm and weight 20.7 g (Table 1). Bertolero et al. (1) obtained a slightly lower average value: THB eggs measured 27.9 × 37.4 mm and weighed 17.1 g. Values for THB eggs, which were measured by Highfield, were bigger but lighter: 40 × 29 mm and weighing 12 to 14 g (7). In Greece, the measured average weight for THB was 17.8 g, ranging from 10.5 to 23.5 g (16). According to available literature, no data was found for shell thickness, but we think it is an important indicator of the relationship between the size of eggs and their weight. The average shell thickness for THB eggs in our study was 0.5 mm (Table 1).

In terms of overall turtle egg weight, the shell contributes about 11 to 12 % (range: 4.3-24.7 %), the albumen contributes about 46 to 47 % (range: 34.4-61.9 %), and the yolk contributes about 41 to 42 % (range: 32.1-55.0 %) (5). Wallace et al. (15) examined relationships between the egg weight, the egg composition and the hatchling size in the Leatherback sea turtles (*Dermochelys coriacea*). Albumen comprised 63 % of egg weight and explained most of the variation in egg mass, whereas yolk comprised only 33 %. However, Hewavisenthi and Parmenter (17) reported that the Flatback sea turtle (*Natator depressus*) eggs contained roughly equal proportions of yolk and albumen. The shell contributed approximately 5 %, the albumin 45 %, and the yolk 50 %. In our study, we found out that shell represented 12.5 %, albumen 46.9 % and yolk 40.6 %. The weight of albumen varied the most. Speake et al. (13) found 42.5 % yolk in the same species. Proportions of the Spur-thighed tortoise (*Testudo graeca*) eggs are the most similar to our results. Specifically, the eggs of the Spur-thighed tortoise consist of 16 % shell, 44 % albumen and 40 % yolk (7). Unlike reptilian eggs, chicken eggs consist of about 9.5 % shell, 63 % albumen, and 27.5 % yolk (18). From the results seen in tortoises, a greatly lower yolk content is seen in chicken eggs.

Turtle females supply eggs with more or less equal proportions of solid nutrients, but there may be considerable variations in the amount of water in the eggs. Among clutches of the Diamondback terrapin (*Malaclemys terrapin*) and the Common snapping turtle (*Chelydra serpentina*), small eggs tend to have proportionally less water than large eggs do (3). From the results of our study we calculated the composition of THB egg contains (the albumen and the yolk, without shell), moisture 80.7 %, ash 2.3 %, protein 10.2 %, and fat 6.8 %. According to available literature data, the composition of THB eggs has not yet been published. In comparison to the snail-eating turtle (also known as 'Rice Field Terrapin') (*Malayemys macrocephala*) eggs, less moisture (72.9 %) and ash (1.5 %) but more protein (12.4 %) and fat (8.6 %) were found (14). The composition of the Olive Ridley sea turtle (*Lepidochelys olivacea*) eggs was: moisture 43.4 %, ash 3.5 %, protein 49.2 %, and fat 3.9 % (19). The results of our study show a great difference especially in moisture and protein content in comparison to the sea turtle eggs. The main components of chicken eggs are moisture 74 %, proteins 12 % and fats 11 % (4). THB eggs contain less protein and fat, and they are more watery than chicken eggs.

Fatty and amino acid compositions of different species of turtle eggs were previously examined. Comparing our results detected in THB (Table 3) with results for the snail-eating turtle (14), the contents of fatty acids in THB eggs were lower for all fatty acids except for oleic acid, vaccenic acid, and eicosenoic acid. The ratio of omega-3/omega-6 fatty acid found in our study (0.41 %) was almost two times higher than in the snail-eating turtle (0.23 %). Thompson and Speake (20) interpreted that the fatty acid composition of yolk lipids is partly determined by maternal diet and partly by the expression of maternal biochemical pathways for the interconversion of fatty acids that have evolved to adjust the dietary supply to the embryonic requirements. Our results regarding fatty acid composition in the yolk in comparison to those of Speake et al. (13) differ slightly in palmitoleic acid, stearic acid, oleic acid, and arachidonic acid. The obvious difference is seen in linoleic acid and α-linolenic acid. The reason was probably due to differences in diet and environmental conditions. The diet of our breeding group is based on grass complemented with additional seasonal vegetables and fruits. It should be noted that *Testudo hermanni* has a postnuptial reproduction cycle which means after the completion of the reproductive phase an intense foraging period to permit nutritional

accumulations for full gonadal growth in the late fall prior to the next hibernation occurs (21). For a more detailed examination among feeding of parents, environmental conditions and contents of female eggs would be required to make more detailed investigations. Linolenic acid formed 1.78 %, and α-linolenic acid formed 0.67 % of the total fatty acid composition of lipid in the yolk. Speake et al. (13) fed tortoises only with green plants, which contribute α-linolenic acid as the main polyunsaturated acid. The linolenic acid formed 6.6 %, and α-linolenic acid formed 3.8 %. The same as Speake et al. (13), we did not detect docosahexaenoic acid (DHA) in yolk which is essential for embryonic development.

There is very little detailed information available on the amino acid composition of the turtle eggs. Results from the present study are shown in Table 4. The essential amino acids profiles of egg albumen found in chicken and quail values were higher than in Hermann's tortoise egg. By comparing our results with the snail-eating turtle (14), lower values for arginine, prolyne, tyrosine, isoleucine, methionine, tryptophan and valine were found. Other amino acids were present in higher values: cysteine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, leucine, phenylalanine, lysine, and histidine. Further investigations considering the importance of amino acids for single turtle's species should be examined.

Very few published studies exist regarding tortoise eggs; it is thus difficult to compare the composition of relative trace elements. In our study, concentrations of the trace elements were shown in descending order in the shell: iron (Fe), nickel (Ni), copper (Cu), manganese (Mn), cobalt (Co), zinc (Zn), chromium (Cr), arsenic (As), selenium (Se); in the albumen: Fe, Cu, Cr, Se; and in yolk: Fe, Zn, Cu, Mn, Se, Cr, Ni, lead (Pb), Co (Table 5). Pb and cadmium (Cd) were not detected in the shell; Pb, Cd, As, Zn, Ni, Mn, Co were not detected in the albumen; Cd and As were not detected in the yolk. Lam et al. (22) also measured the presence of trace elements for the Green sea turtle (*Chelonia mydas*) in the shell and both composites of eggs. Their results were all higher except for Mn in the shell, Cu in albumen and Cu and Mn in the yolk, which were lower than our concentrations. The main reason to study elements in turtle eggs is pollution and its potential threat to sea turtles. The trace element concentrations found worldwide could also be a consequence of both diet and environmental pollution, as described Lam et al. (22) for the Green sea turtle (*Chelonia mydas*).

Concentrations of different amino acids, fatty acids and elements are found in different proportions in different turtle species. Farms that breed turtles are a unique living system, and further investigation needs to be done in this field, especially concerning the nutrition of breeding parents and its impact on the composition of eggs.

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DOLOČANJE RAZLIČNIH KEMIČNIH SESTAVIN V JAJCIH GRŠKE KORNJAČE (*Testudo hermanni*)

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Povzetek: Namen raziskave je bil proučiti sestavo jajc grške kornjače (Testudo hermanni boettgeri), vsebnost aminokislin in maščobnih kisli, kot tudi vsebnost nekaterih elementov v sledovih. Povprečna velikost jajc THB je bila 29,9 × 39,5 mm, masa pa 20,7 g. Celotno jajce je bilo sestavljeno iz 12,5 % lupine, 46,9 % beljaka in 40,6 % rumenjaka. Indeks refrakcije beljaka je znašal 1,3341 in rumenjaka 1,5120. Beljak je vseboval 98,2 % vode, 0,9 % beljakovin, 0,7 % pepela in sledove maščobe. Rumenjak je vseboval 60,6 % vode, 21,0 % beljakovin, 14,2 % maščob in 4,0 % pepela. Vrednost pH beljaka je bila 8,8.
Najvišji delež maščobnih kislin v rumenjaku so predstavljale oleinska kislina, palmitinska kislina, palmitooleinska kislina in vakcenska kislina. V beljaku smo potrdili 18 različnih aminokislin. Najvišji delež le-teh so predstavljale glutaminska kislina, asparginska kislina, levcin, fenilalanin, lizin, glicin, serin, treonin in alanin.
Koncentracije elementov v sledovih v lupini so bile v naslednjem padajočem zaporedju: železo (Fe), nikelj (Ni), baker (Cu), mangan (Mn), kobalt (Co), cink (Zn), krom (Cr), arzen (As), selen (Se); v beljaku: Fe, Cu, Cr, Se, v rumenjaku pa: Fe, Zn, Cu, Mn, Se, Cr, Ni, Pb, Co.

Ključne besede: *Testudo hermanni*; jajca; kemična sestava; maščobne kisline; aminokisline; elementi v sledovih

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Original Research Article

THE EFFECT OF CRUDE *Nigella sativa* OIL AGAINST THE ACUTE TOXICITY OF DICLOFENAC SODIUM AND IBUPROFEN ON THE LIVER OF ALBINO MICE

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Abstract: The present research work is an investigation of the effect of *Nigella sativa* oil against the acute toxicity induced by the drugs diclofenac and ibuprofen on the liver enzymes, body weight and Hepatosomatic index of Swiss albino mice. Thirty-six healthy, adult Swiss albino mice were used to assess diclofenac- and ibuprofen-induced hepatotoxicity and the hepatoprotective effect of *N. sativa* oil. The animals were divided into the control group and five experimental groups. The animals in the control group were given saline (0.9 percent of NaCl) only, whereas the animals in experimental groups were given single sub-lethal doses of diclofenac, ibuprofen and *N. sativa* oil alone and together. A significant ($p<0.05$) reduction in the body weight of the diclofenac- and ibuprofen-treated groups was recorded. The hepatosomatic index showed significant ($p<0.05$) changes in the combined treated groups. Hepatotoxicity can be confirmed by comparing the significant ($p<0.05$) and highly significant ($p<0.01$) increase in liver enzymes in all the treated animals. The hepatoprotective effect of *N. sativa* oil was confirmed.

Key words: diclofenac; ibuprofen; hepatotoxicity; hepatoprotective; *N. sativa oil*

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are responsible for roughly 10 percent of the total of cases of drug-induced hepatotoxicity (1). NSAIDs induce anti-inflammatory activity. The mechanism of action of NSAIDs occurs via the inhibition of the cyclooxygenase enzyme (COX), thus inhibiting prostaglandins synthesis and inducing its anti-inflammatory action (2). NSAIDs induce liver damage due to the formation of reactive oxygen species (ROS), such as HO, H_2O_2, NO, and O_2. Recent studies have revealed that the administration of NSAIDs significantly increases the lipid peroxidation (LPO) by decreasing the glutathione level, which induces hepatotoxicity due to the generation of free radicals (3). A group of enzymes found in cytosol is released into the blood due to the disturbance of hepatocytes transport functions, and it results in increased enzyme levels in the blood serum, which indicates hepatocellular damage (4).

Nigella sativa (*N. sativa*), popularly known as 'black seed', is used as a spice and food preservative, and its oil extract has been reported

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to possess a plethora of activities that include anti-inflammatory, anti-cancer, anti-diabetic, anti-hyperlipidemic, anti-oxidant, nephroprotective and hepatoprotective actions (5). Thymoquinone, an active constituent of *N. sativa*, acting as an antioxidant, provides significant protection against free radical-induced lipid per oxidation (LPO) and DNA damage (6). A broad range of studies proving the hepatoprotective effects of *N. sativa* exist. Recently, Ait Mbarek *et al.* (2007) demonstrated *N. sativa* decreases hepatic metastasis from tumours, such as mastocytomas (7). Thymoquinone exhibits a hepatoprotective effect against liver damage induced by carbon tetrachloride (8) and prohibits tertbutyl hydroperoxide (TBHP)-induced depletion of glutathione (GSH). GSH is an antioxidant that depletes free radicals; however, thymoquinone increases the activities of antioxidant enzymes and protects against various forms of cancer (9).

N. sativa, through thymoquinone, increases the ratio of helper to suppressor T cells, has a stimulatory effect on macrophages, and enhances the natural killer cell activity and production of interleukin 3 (IL-3) (10). Mohamed and co-workers reported the harmful effect of dimethylaminoazobenzene (DAB) on the livers of Swiss albino mice and the protective effect of *N. sativa* oil treatment on these animals (11). *N. sativa* oil administration showed improvement in the elevation of liver enzymes induced by malathion in albino rats (12). The current study was designed to investigate the hepatoprotective effects of *N. sativa* oil against the hepatotoxicity induced by the oral administration of diclofenac and ibuprofen.

Material and methods

Housing and feeding conditions

Thirty-six healthy, adult, (12 weeks old) female Swiss albino mice, weighing 23-41 grams (g), were purchased from a local market in Lahore, Pakistan. They were kept in clean iron cages in the animal house of the Department of Zoology, Lahore College for Women University, Lahore. The study duration was 24 hours. All mice were maintained on a 12-h light/dark cycle with the temperature maintained at 22 °C (±3 °C). Mice were fed with commercial rodent chow in pellet form; drinking water was provided *ad libitum* throughout the experiment. The experiments were approved by the Research Ethical Review Committee of Lahore College for Woman University, Lahore on 4th of June, 2015 (Memo number RERC - ZOO - 099).

Chemical used

- Diclofenac: Dicloran (Diclofenac Sodium) (50 mg) SAMI Pharmaceuticals Pakistan Ltd.
- Ibuprofen 200 mg was purchased from a pharmacy. (Abbott Laboratories Pakistan Ltd).
- Crude *N. sativa* oil (TOP TREATMENTS, Pakistan Ltd).

Experiment protocol/ Dosage

The LD_{50} of diclofenac sodium in albino mice is 95mg/kg B.W, as reported by Basavraj *et al.* (2012) (13). In the current study, 20 mg/kg B.W (~1/5th of LD_{50}) of diclofenac sodium was administered to the animals. According to Zayed and Hassan (2014), the LD_{50} of ibuprofen is 740 mg/kg B.W (14). In the current study, 74 mg/kg B.W (~1/10th of LD_{50}) of ibuprofen was used. *N. sativa* oil was administered as 2.5 mL/kg B.W (15). Both drugs were first converted to powdered form, dissolved in distilled water for the preparation of the stock solution, and were finally diluted in distilled water in order to make the final volume 0.3 ml for each animal. The dose was administered using gavage after two hours of feeding.

Experiment Design

After the five days of acclimatization, the thirty-six mice were randomly divided into six groups; each group consisted of six mice.

Group 1 (Control): All the animals received 0.3 ml (0.9 percent w/v) normal saline.

Group 2 (Diclofenac treated): Mice were orally administered with sub-lethal dose i.e. 20 mg/kg B.W.

Group 3 (Ibuprofen treated): Mice received a single dose of 74 mg/kg B.W of ibuprofen orally.

Group 4 (*N. sativa* oil treated): Mice received 2.5 ml/ kg B.W of crude oil of *N. sativa.*

Group 5 (Diclofenac and *N. sativa* oil): Single oral dose of 20 mg /kg body weight of diclofenac along with 2.5 ml/kg B.W of crude oil of *N. sativa* oil was administered.

Group 6 (Ibuprofen and *N. sativa* oil): Single oral dose of 74 mg /kg B.W of ibuprofen along with 2.5 ml/kg of crude oil of *N. sativa* oil was administered.

Blood Sampling

Twenty-four hours after treatment, the mice were anesthetized, and their blood samples were collected through cardiac puncture; finally, the mice were dissected. Blood samples were centrifuged at 3000 rpm for 10 min to separate the serum, which was stored at -40 °C. The liver was removed and weighed using an electronic balance; this weight was then used to calculate the hepatosomatic index (HSI) using the formula given below.

$$\text{HSI: } \frac{\text{liver weight}}{\text{Mice weight}} \text{ x } 100$$

Biochemical parameters (ALT, AST, and ALP)

Serum alanine aminotransferases (ALT), serum aspartate aminotransferases (AST) and serum alkaline phosphatases (ALP) were analysed using standard protocols. Analysis was carried out using a semi-automated chemistry analyser (URIT 800 chemistry analyser URIT Medical Co., Ltd Guangxi, China). AST and ALT estimation was carried out using the IFCC (International Federation of Clinical Chemistry) method using AST kit by Crescent Diagnostics, Jeddah Industrial City, Phase III, Jeddah Kingdom of Saudi Arabia. According to DGKC, optimized standard method ALP was measured using Fluitest ALP DGKC ALP diagnostic kits, Analytical Biotechnologies AG 35104 Lichtenfels, Germany. Then, assay values were compared with biochemical control (ELI Tech Clinical Systems). After every ten samples, quality control was run for the calibration of equipment.

Statistical Analysis

Data were analysed using (SPSS version 19) one-way ANOVA of variance followed by the Tukey *post hoc* test for establishing a significant difference between treated groups. $p<0.05$ was considered significant, and $p<0.01$ was considered highly significant. Data were presented as mean $\pm$ S.E.M.

Results

The current study was undertaken to evaluate the hepatoprotective effects of *N. sativa* by observing changes in body weight, the hepatosomatic index, and biochemical parameters. The data in Table 1 demonstrate a significant decrease in the mean body weight of animals treated with diclofenac, ibuprofen, and diclofenac plus *N. sativa*, while the effect of *N. sativa* alone or in combination with ibuprofen was not significant (Table 1). The percentage changes in the mean body weight of respective groups are shown in Table 1.

The changes in the hepatosomatic index showed a non-significant ($p>0.05$) reduction in the groups treated with the diclofenac (D), ibuprofen (B), *N. sativa* oil (N) as compared to the control group. In contrast, the combined treatment of both diclofenac and ibuprofen with *N. sativa* (DN, BN) showed a significant change ($p < 0.05$) (Table 1).

Serum biochemical analysis

The effects of *N. sativa* on diclofenac- and ibuprofen-induced hepatotoxicity were evaluated by recording changes in serum AST, ALT, and ALP levels. There was an increase in the serum AST levels in diclofenac- and ibuprofen-treated animals in comparison to the control group. This effect was reversed in one group that received *N. sativa* along with diclofenac (Table 2).

The data in Table 2 demonstrate a trend of increased levels of serum ALT in the diclofenac- and ibuprofen-treated animals in comparison to the control. This effect was reversed in the group that received *N. sativa* along with ibuprofen (Table 2).

There is an increase in serum ALP levels in diclofenac- and ibuprofen-treated animals in comparison to their control group. This effect was reversed in both groups of *concomitant treatment of N. sativa* with diclofenac and *N. sativa* with ibuprofen. In contrast, mice treated with *N. sativa* alone remained within normal levels in comparison to the control group (Table 2).

Table 1: Comparison of the percentage increase or decrease in mean Body Weight (B.W) before and after the treatment (Mean ± SEM) and Hepatosomatic Index in the control and experimental group (D, B, N, DN and BN) after 24 hours of treatment (n=6 each group)

Treatment	Mean Body Weight			
	Before treatment(g)	After Treatment(g)	Percentage Increase or Decrease	Hepatosomatic Index (HSI)
Control (C)	23.67 ± 0.67	24.33 ± 0.67	2.78	10.06± 0.952
Diclofenac (D)	29.83 ± 0.87	27.17 ± 0.95*	8.91	9.11 ± 0.57
Ibuprofen (B)	26.50 ± 0.81	24.33 ± 0.715*	8.19	9.35 ± 0.337
N. sativa oil (N)	36.17 ± 0.703	35.33 ± 0.558	2.32	7.98 ± 0.378
Diclofenac + *N. sativa* oil (DN)	34.17 ± 0.70	32.33 ± 0.67*	5.38	7.22 ± 0.19*
Ibuprofen + *N. sativa* oil (BN)	35.83 ± 0.946	34.83 ± 1.014	2.79	7.07 ± 0.213*

*$P<0.05$

Table 2: Serum level of AST, ALT, and ALP (IU/L) enzymes following 24 h after' different treatments (n=6 each group)

Treatment	AST(IU/L)	ALT (IU/L)	ALP (IU/L)
Control (C)	91.8 ± 3.20	50.13 ± 1.67	47.64 ± 1.40
Diclofenac (D)	163.4 ± 4.22**@@	90.37±4.25**	143.20 ± 2.86**@@
Ibuprofen (B)	109.68± 3.93*##	78.24± 3.61**	106.08 ± 3.79**##
N. sativa oil (N)	96.7 ± 2.67##	61.88 ± 3.05##@	56.62 ± 2.43##@@
Diclofenac +*N. sativa* oil (DN)	143.4 ± 4.50**#@@	82.60 ± 3.70**	121.38 ± 3.80**##@
Ibuprofen +*N. sativa* oil (BN)	102.19 ± 4.32##	69.27± 3.92##@	81.74 ± 3.72**##@@

*= in comparison to C, # in comparison to D, @ in comparison to B, *, #, @ p< 0.05, **, ##, @@ p<0.01

Discussion

The current study has shown a decrease in mean body weight, which might be due to tissue damage, because it was previously reported that significant decreases in the body weights of the broiler chicks and pigeons were observed in all diclofenac-treated animals, indicating its dose-dependent toxicity (16). Recently, Mohamed et al., 2010 observed the significant decrease in body weight in DAB and DAB plus *N. sativa* treated groups compared to the untreated control group (11). Al-Khafaji (2013) also reported significant decreases in the body weight of mice that received paracetamol and crude oil of *N. sativa* together (17). In our study, the decrease in body weight might be due to the acute dose of *N. sativa oil*, as the metabolism of fats commences with the administration of this oil, and the digestion of fats (oil) requires more energy. In this case, it first reduced the body weight and then its protective effect began. *N. sativa* also produced a significant reduction in the blood level of cholesterol, triglycerides, high-density lipoproteins (HDL) and low-density lipoproteins (LDL) in albino rats after 4 days of treatment (18). The rats treated with *N. sativa* oil showed decreased body weight which might be due to reduced food intake, thus diminishing serum lipids and glucose levels (19, 20). In another study, *N. sativa* oil treatment

showed a slight decrease in the mean body weight of Sprague-Dawley rats (21). According to Al-Khafaji (2013), liver weight is not affected by non-steroidal anti-inflammatory drugs (NSAIDs) and *N. sativa* oil in all treatments (17). In the current study, a comparison of hepatosomatic indexes among all groups has shown a non-significant decrease in liver weight, except the treated group of concurrent administration. The liver was already undergoing oxidative stress, so it could not metabolize oil as it should have if it were functioning normally. Liver weight in the combined treatment group significantly decreased, which may be due to the plethora of activities associated with *N. sativa*. Thymoquinone (an active ingredient of *N. sativa*) can increase the expression of antioxidant enzymes (e.g. GSH peroxidase and superoxide dismutase), thus reducing the NADH/NAD+ ratio leading to an inhibition in lipogenesis in the hepatocytes (22). Guiloski and co-workers (2015) reported that the diclofenac caused a non-significant reduction in the liver size (HSI) of fish (23).The current study has shown an increase in serum enzyme levels in the treated groups (i.e., diclofenac and ibuprofen), which might be due to hepatotoxicity caused by diclofenac and ibuprofen (24, 25). These enzyme levels are generally used in toxicological studies to assess hepatic function (26). It has been suggested that the raised AST levels occur due to extensive tissue necrosis, in the case of liver disease (27). Increased levels of these enzymes might be due to cellular leakage or the loss of functional integrity of cell membrane in liver (28). It was previously reported that diclofenac sodium-treated animals showed increased LPO (29, 30). Previous studies showed that the elevation of serum enzymes after ibuprofen is indicative of cellular injury to the liver (31). The current study has shown that the *N. sativa* treatment alone showed normal liver enzyme levels as that of the control group. Moreover, the biochemical results of the current study demonstrate that the combination of diclofenac and *N. sativa* attenuates the toxic effect of diclofenac as indicated by their serum AST and ALP levels. It also prevented hepatotoxicity induced by ibuprofen, as shown by their serum ALT and ALP levels. Previously, it was reported by Al-Khafaji (2013) that combined treatment of *N. sativa* with paracetamol prevents a rise in serum AST, ALT, and ALP levels because of its antioxidant properties (17). In another study, *N. sativa* oil maintained the serum levels of AST and ALT close to normal, and it showed a hepatoprotective effect against D-Galactosamine (D-GalN)/Lipopolysaccharide-induced hepatotoxicity and oxidative stress in rats (32). The severity of diclofenac is greater than that of ibuprofen because of its high dose in comparison to ibuprofen. *N. sativa* oil offers greater protection against diclofenac in comparison to ibuprofen. Previous studies showed that higher doses and longer durations of ibuprofen exposure increased hepatic toxicity (31). It was found that *N. sativa* treatment prevented CCL4-induced hepatotoxicity in rats by decreasing the lipid peroxidation and increasing the antioxidant defence system activity (8). In a study carried out for 24 hours on mice, similar findings were reported as that of the current study, which showed that the concurrent administration of *N. sativa* oil along with diclofenac and ibuprofen moderately affects serum enzyme levels (17).

Conclusion

From the results of the current study, it is concluded that when treated groups were compared with the control group, all enzymes (AST, ALT, and ALP) increased in both drug-treated groups. This elevation showed moderate decrement toward control values when herbs were combined with drugs (diclofenac and ibuprofen).

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VPLIV OLJA ČRNE KUMINE (*Nigella sativa*) NA AKUTNO ZASTRUPITEV JETER, POVZROČENO Z NATRIJEVIM DIKLOFENAKOM IN IBUPROFENOM, PRI ALBINO MIŠIH

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Povzetek: Predstavljena raziskava opisuje učinke olja črne kumine (*Nigella sativa*) na aktivnost jetrnih encimov, telesno težo in hepatosomatski indeks pri švicarskih albino miših pri akutni zastrupitvi, povzročeni z natrijevim diklofenakom in ibuprofenom. Za oceno škodljivega učinka diklofenaka in ibuprofena na jetra ter zaščitnega učinka olja *N. sativa* na jetra je bilo testiranih šestintrideset zdravih odraslih miši. Živali so bile razdeljene v kontrolno skupino in v pet poskusnih skupin. Živali v kontrolni skupini so prejele fiziološko raztopino (0,9 % NaCl), medtem ko so dobile živali v poskusnih skupinah različne subletalne odmerke diklofenaka in ibuprofena samostojno ali pa v kombinaciji z oljem črne kumine. Rezultati so pokazali statistično značilno ($p < 0,05$) znižanje telesne mase pri skupinah mišk, ki so dobivale diklofenak in ibuprofen. Pri vseh skupinah, tretiranih z diklofenakom in iboprufenom, so se pokazale statistično značilne razlike med kontrolno in tretiranimi skupinami, kar potrjuje hepatotoksični vpliv tretiranja. Hepatosomatski indeks je pokazal statistično značilne ($p < 0,05$) razlike med skupinami, ki so bile tretirane s kombinacijo učinkovin, in skupinami, ki so prejemale tudi olje črne kumine, kar kaže na hepatoprotektivni učinek olja črne kumine.

Ključne besede: diklofenak; ibuprofen; hepatotoksičnost; hepatoprotektivnost; olje *N. sativa*

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Original Research Article

MODULATING EFFECT OF MYCOAD® ON PERFORMANCE, MUCOSAL AND SYSTEMIC IMMUNITY IN CHICKEN AFLATOXICOSIS

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Abstract: To determine the aflatoxin effect on performance, and humoral and mucosal immunity, 240 one-day old chicks were divided into 4 equal groups. Treatment groups include Group 1: chickens that received a standard diet based on corn and soy as negative control, Group 2: chickens fed with a basal diet containing 3 ppm aflatoxin as positive control, Group 3: chickens fed with 0.25% Mycoad® in basal diet, and Group 4: chickens fed with diet containing 0.25% Mycoad® plus 3 ppm aflatoxin. All chickens continuously received diets from hatching until 28 days old. Growth indices, such as weight gain, feed consumption and food conversion rate, were determined weekly. At 28 days, all chickens were sacrificed. After blood sampling, serum was prepared to measure serum IgG titer against Newcastle disease vaccine using the HI method. Moreover, the heads were collected for nasal-tracheal lavage for assaying IgA against infectious bronchitis vaccine in the mucosa of the respiratory tract. The measurement of mucosal IgA was carried out using the ELISA method with specific goat anti-chicken IgA. That results indicated that chickens that received aflatoxin demonstrated lower growth indices, and fewer serum IgG and mucosal IgA titers than others did, while performance and immune responses in chickens that received Mycoad® plus aflatoxin were significantly higher than chickens fed with aflatoxin alone. Overall, it seems that aflatoxin can affect mucosal immunity in the upper respiratory tract as well as performance and humoral immune responses. Supplementation of Mycoad® to diet contaminated with aflatoxin can reduce the adverse effects of aflatoxin on performance, as well as mucosal and systemic immune responses.

Key words: aflatoxin; chicken; immunity; Mycoad®

Introduction

Aflatoxins (AFs) are secondary metabolites of various *Aspergillus* species, e.g. *Aspergillus falvus* and *Aspergillus parasiticus*. Chemically, aflatoxins are furanocoumarin compounds, and the most important ones are B_1, B_2, G_1, and G_2 (1). Among the various types of aflatoxins, aflatoxin B_1 (AFB_1) is most commonly encountered, and it is also considered to have higher toxicity than other aflatoxins (2). Aflatoxins commonly contaminate a wide variety of tropical and subtropical food/feedstuffs (3). Poultry can be exposed to high concentrations of aflatoxin via feedstuffs which lead to large economic losses (4). The toxicity of aflatoxins (aflatoxicosis) in poultry is characterized by mortality, listlessness, anorexia, reduced growth rates, negative feed conversions, fatty liver, reduced egg production, poor pigmentation, and increased susceptibility to other diseases (5, 6, 7, 8). Aflatoxin is known to have strong hepatotoxic and carcinogenic effects (9). A practical approach to detoxification is the utilization of sorbents in the

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diet that adsorb aflatoxin in the gastrointestinal tract of poultry, thereby reducing bioavailability and toxicity (7, 8).

Since the discovery of aflatoxins, the negative effects of them on animal health have been an active area of research. Based on this, research during the last five decades has well elucidated the negative effects of aflatoxins on animal performance and immunity (2). Aflatoxin can be a primary immunosuppressive agent (4) that can influence the efficacy of immune response in poultry. Most studies in this field focused on the effect of this mycotoxin on the humoral and cellular immune system. To date, there has been no scientific research on the effect of aflatoxin on mucosal immunity in chickens. In this research, the mucosal respiratory immune response against infectious bronchitis (IB) vaccine in chickens affected by experimental aflatoxicosis was analyzed. Moreover, the level of mucosal Immunoglobulin A (IgA) against IB vaccine was determined in chickens fed with aflatoxin plus one standard mycotoxin adsorbent (Mycoad®) for decreasing the reverse effects of aflatoxins on mucosal immunity.

Materials and methods

Production and assaying of aflatoxin

Aflatoxin provided by *Aspergillous parasiticus* (PTCC: 1850) belongs to Iranian Scientific and Industrial Research. Aflatoxin was produced according to the Shotwell method (10) on maize with a few modifications. The aflatoxin was assessed with the HPLC method utilizing reverse-phase C18 column (250×4.6 mm, 5 μm), equipped with fluorescence detector set at 370 (excitation) and 440 (emission). The mobile phase was deionized water/methanol/acetonitrile (60:20:20) with a flow rate of 0.8 ml/min and an injection volume of 20 μL.

Experimental design

A total of 240 one-day-old broiler chicks (Ross strain) were randomly divided into four groups with three replicates of 20 chicks in each separated pen during the 28-day experiment. A basal diet based on corn-soybean was balanced in accordance with the recommendation by NRC (11).

Treatment groups include Group A: chickens fed a basal diet, Group B: chickens fed 3 ppm aflatoxin in a basal diet, Group C: chickens fed a basal diet containing 0.25% Mycoad® and Group D: chickens received a basal diet containing 3 ppm aflatoxin and 0.25% Mycoad®.

In this study, the basal diets were tested for contamination by aflatoxins, fumonisins, and zearalenone. The diets were not contaminated by any mycotoxins. The maize containing aflatoxin was added to the basal diet to increase the concentration of aflatoxin in experimental diet to 3 ppm. In groups that did not receive aflatoxin, the same amount of uncontaminated maize (without aflatoxin) was added to the basal diet. All treatment groups received experimental diets throughout the growing period from hatching until 28 days old. The diet formula is presented in Table 1. Feed and water were supplied *ad libitum* to all groups, and 24-hour light was utilized throughout the experiment. All chickens were vaccinated against Newcastle disease (ND) (at 8 and 18 days old), IB (at 1 and 10 days old), and infectious bursal disease (IBD) (at 14 days old) with B1, LaSota, H120, and D78 vaccines. Data on body weight, body weight gain, feed intake, and feed conversion ratio were recorded at weekly intervals. Moreover, cumulative data were assessed.

Mycoad®

Mycoad® is a commercial broad spectrum mycotoxin adsorbent for all types of feed formulated as hydrated sodium and calcium aluminosilicate (HSCAS) by Special Nutrients Inc., USA.

Mucosal and systemic immunoglobulin assay:

Blood samples from all chickens at 28 days of age were collected for measurement of serum immunoglobulin G (IgG) titer against ND vaccine by conventional haemagglutinin inhibition (HI) test (12). Thereafter, all the chickens were slaughtered, and samples of the trachea and nasal mucosa were separated immediately. The mucosal surface of the trachea and nasal mucosa were washed with 1 ml of phosphate buffer saline (PBS) (pH = 7.4) containing 0.1% of bovine serum albumin (BSA) three times. Immediately after washing, the liquid extract was centrifuged,

Table 1: Composition of the experimental diets for broiler chicks

Ingredients %	0-18 (days old)	19-28 (days old)
Corn grain	53.00	58.50
Soybean meal	39.00	33.65
Vegetable Oil	4.00	4.00
DCP	1.35	1.18
Oyster shells	1.45	1.50
Methionine D-L	0.25	0.23
Vitamin E	0.10	0.10
Edible Nacl	0.25	0.24
Vitamin Premix*	0.30	0.30
Mineral Premix*	0.30	0.30
Calculated nutrient content		
ME (Kcal/Kg)	3005	3070
CP (%)	22.82	19.90
Ca (%)	0.95	0.92
Available Phosphorus (%)	0.45	0.41
Lysine (%)	1.24	1.15
Na (%)	0.16	0.15
Methionine+Cystine (%)	0.95	0.88

*Supplied Per Kilogram of Feed: 8.000 IU of Vitamin A, 2000 IU Vitamin D3, 50 mg Vitamin E, 1.5 µg Vitamin B1, 2.2 mg B, 6.5 mg Vitamin K, 7 mg Vitamin B2, 2 mg Vitamin B1, 40 mg nicotinic acid, 160 µg vitamin Biothine, 12 mg Calcium pantothenate, 1 mg Folic acid 30 mg Fe, 70 mg Mn, 100 µg Se, 40 mg Zn, 6 mg Cu, 1.14 mg I.

Table 2: The growth indices in chickens at end of fourth week

Indices	Negative control	Chickens that received aflatoxin	Chickens that received Mycoad®	Chickens that received aflatoxin and Mycoad®
Feed Intake	1568±20^{bc}	1321±15^{a}	1635±30^{b}	1508±25^{c}
Weight Gain	1131±35^{b}	782±18^{a}	1230±43^{b}	1093±25^{b}
Feed Conversion Ratio	1.38±0.04^{b}	1.68±0.03^{a}	1.33±0.06^{b}	1.37±0.02^{b}

*Data presented as Mean±SD.
^{a,b}Different letters in each row represent the existence of significant differences between groups ($P < 0.05$).

Table 3: The systemic and mucosal immune responses in different groups

Indices	Negative control	Chickens that received aflatoxin	Chickens that received Mycoad®	Chickens that received aflatoxin and Mycoad®
Serum IgG titer against ND vaccine	4.85±0.39^{a}	2.95±0.21^{b}	5.22±0.32^{a}	4.45±0.35^{a}
Mucosal IgA titer against IB vaccine	4.92±1.65^{a}	3.15±1.68^{b}	5.57±1.30^{a}	4.61±1.24^{a}

* Data presented as Mean±SD.
^{a,b}Different letters in each row represent significant differences between groups ($P < 0.05$).

and the supernatant was collected (13, 14, 15). To measure the level of specific IgA against IB vaccine in trachea and nasal mucosa, the lavage samples were tested with a commercial ELISA kit (Synbiotic co.). Based on the lack of commercial kits for measuring the IgA, commercial IBV ELISA kit was utilized, but the conjugated HRP goat anti-chicken IgG was replaced with conjugated HRP goat anti-chicken IgA. The goat anti-chicken IgA was conjugated to horseradish peroxidase purchased separately (Bethyl Laboratories, Cat. no. A 30-103P).

For the determination of IgA titer in mucosal respiratory lavage, the mean optical density (OD) of the negative control was calculated, and three times the standard deviations were added to the OD of the negative control. Thereafter, the OD of the negative control and positive-negative threshold was utilized for the calculation of titers (16, 17). In this study, the IgA titer in each sample was calculated utilizing the KPL software program according to negative control and positive-negative threshold. The IgA titers were expressed based on log 2.

Statistical Analysis

All data were analyzed using the one-way ANOVA method by SAS software (18). Significant differences among treatment groups were recognized at $P < 0.05$ by post-hoc Tukey test.

Results

Evaluation of Growth Parameters

The statistical comparison of weight gain, feed intake, and feed conversion ratio at the end of the fourth week showed that chickens that received aflatoxin had the lowest weight gain and feed intake and the highest feed conversion ratio ($P< 0.05$). The weight gain and feed conversion ratio in the other groups did not show significant differences. In terms of feed intake, chickens that received Mycoad® had a higher feed intake than the chickens that received aflatoxin, and aflatoxin plus Mycoad®. There was a significant difference in feed intake between chickens fed with Mycoad® and chickens that received aflatoxin, or aflatoxin plus Mycoad® ($P< 0.05$) but with the negative control, no significant difference was observed (Table 2).

Evaluation of the humoral immune response against ND vaccine

The results of the HI test demonstrated that the IgG titer against ND vaccine in chickens that received aflatoxin was significantly less than other treatment groups ($P <0.05$). There were no significant differences between other groups for serum IgG titer against ND vaccine (Table 3).

Evaluation of the mucosal immune response against IB vaccine

There were significant differences in the mucosal respiratory IgA titer against IB vaccine in the group fed with aflatoxin alone in comparison with other groups ($P<0.05$), while there was no significant difference between other groups (Table 3).

Discussion

Growth parameters, such as weight gain, feed intake and feed conversion ratio in the chickens that received 3 ppm aflatoxin, were significantly less than other with experimental groups. Review of previous studies indicates that 2.5 ppm and higher amount of aflatoxin can significantly decrease growth and food conversion efficiency. For instance, Huff et al. (19) observed that the addition of 2.5 ppm aflatoxin reduced weight gain. Moreover, Harvey et al. (20) indicated that the addition of 3.5 ppm of aflatoxin in food can decrease feed intake and weight gain. The results of the current study indicate that the addition of Mycoad® to the contaminated feed at 3 ppm level of aflatoxin can completely inhibit the reduction of weight gain, feed intake and feed conversion efficiency. The comparison of growth parameters in control chickens and chickens that received aflatoxin plus Mycoad® revealed that, although Mycoad® could not increase growth parameters in chickens that received aflatoxin, it could improve growth indices and prevent the loss of growth parameters caused by aflatoxin. Previous studies have indicated that the presence of silicate in the composition of Mycoad® as an aluminosilicate compound can tightly bind with available aflatoxin in the gastrointestinal tract and markedly decrease the bioavailability and toxicity of aflatoxin (21, 8).

In this study, the reduction in mucosal IgA titers against infectious bronchitis vaccine and serum

IgG against ND vaccine in chickens receiving aflatoxin may be due to the effects of aflatoxin on the immune system. However, suppression of immune system caused by aflatoxin in poultry has already been reported (4), but there is no report on the effects of aflatoxin on mucosal immunity.

Infectious bronchitis virus is a major poultry pathogen that is endemic worldwide and leads to serious economic losses in the poultry industry. The mucosal immunity in respiratory tract acts as a first line of defense against IB virus infection. Some studies have indicated the significant relationship between the level of mucosal respiratory IgA and resistance against IB virus infection (22, 15). Therefore, it seems that measuring the level of mucosal respiratory IgA can be a suitable alternative for monitoring the protection level against IBV. Some reports have indicated that aflatoxin can increase the sensitivity of birds to viruses (4) and cause adverse effects on the production of serum antibodies against ND virus, pasteurellosis, and IBD (23). Moreover, there is some evidence of aflatoxin's effect on the histopathologic features of the thymus, spleen and bursa of Fabricious in chickens. Thymic aplasia, splenic atrophy, and lymphoid depletion in bursa can show the effect of aflatoxin on the cellular immune response in chickens (24). Ibrahim et al. (25) showed that both percentages and means of phagocytic activities were significantly reduced in chicks fed with 2·5 ppm aflatoxin. Moreover, the leucopenia in chickens following aflatoxin toxicity at a level of 3 ppm can demonstrate the cellular immunosupression of aflatoxin (26). The effect of aflatoxin on interferon, complement and serum proteins (27), subsequent liver damage, and inhibition of protein synthesis (8) are the possible causes of immunosuppression. It seems the effect of aflatoxin on the immune system is influenced by the dose and duration of utilizing contaminated diet with aflatoxin. For instance, Pasha et al. (28) revealed that a low level of aflatoxin (100 ppb) has no effect on the size of the bursa of Fabricious. Moreover, Kouwenhoven (29) investigated the systemic antibody production following vaccination against Newcastle disease in chickens fed with aflatoxin. In this study, 0.2-0.5 ppm of aflatoxin B1 could not change the systemic immune response to the ND, *Salmonella pullorum* and *Pasturella multocida* but higher dose (0.6-10ppm) could suppress systemic antibody response to *Salmonella* and sheep RBCs. However, Tessari et al. (30) reported that systemic antibody production following immunization with ND vaccine was affected by 0.2 ppm aflatoxin in the diet. The result of the current study is in line with the results of Ibrahim et al. (25) who clarify that 2.5 ppm aflatoxin has negative effect on the formation of ND antibodies. Nevertheless, the effects observed in the present study may be due to high dietary aflatoxin contamination. Considering the significant difference between the groups receiving the Mycoad® in addition to aflatoxin with aflatoxin alone, it seems that Mycoad® with its binding effect reduces the gastrointestinal absorption of aflatoxin and prevents the side effects of aflatoxin on the upper respiratory mucosal immune system.

The overall results of this study showed that 3 ppm aflatoxin can decrease growth parameters, humoral and mucosal immune responses. However, Mycoad® based on the dose and duration of use as examined in recent studies could prevent inhibitory effects of aflatoxin on the growth parameters, mucosal and humoral immunity system in broiler chickens. Therefore, the control of mycotoxins as immunosuppressive agents by using a commercial mycotoxin binder is valuable for providing better records in performance and immune responses.

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VPLIV DODATKA MYCOAD® NA PRIRAST IN IMUNSKO ODPORNOST PIŠČANCEV Z AFLATOKSIKOZO

M. Amiri, M. Gholami-Ahangaran, M. Jafarian-Dehkordi

Povzetek: Da bi preučili vpliv aflatoksina na prirast in imunski odziv (sistemski in v sluznicah) piščancev, smo 240 enodnevnih piščancev razdelili na 4 enake skupine in jih od izvalitve do 28. dneva starosti hranili s 4 različnimi krmami. Skupina 1 je predstavljala negativno kontrolo (piščanci, krmljeni s standardno krmo na osnovi koruze in soje), skupina 2 je bila pozitivna kontrola (piščanci, krmljeni s standardno krmo, ki je vsebovala 3 ppm aflatoksina), v skupini 3 so bili piščanci, krmljeni s standardno krmo z dodatkom 0,25 % Mycoad®-a, v skupini 4 pa piščanci, krmljeni s krmo, ki je vsebovala 3 ppm aflatoksina in 0,25 % Mycoad®-a plus. Tedensko smo določali različne pokazatelje rasti, kot so povečanje telesne mase, poraba krme in njen izkoristek. Po 28 dneh smo vse živali žrtvovali. Z uporabo metode HI smo v serumu določili titer protiteles IgG proti virusu bolezni Newcastle ter v izpirku nosu in sapnika določili titer protiteles IgG proti cepivu kužnega bronhitisa. Izmerili smo tudi raven sluzničnih protiteles IgA z metodo ELISA s specifičnimi kozjimi protitelesi proti piščančjim protitelesom IgA. Rezultati so pokazali, da imajo piščanci, ki so prejeli aflatoksin, nižje indekse rasti in nižje titre tako serumskih protiteles IgG kot tudi sluzničnih protiteles IgA. Medtem pa sta bila prirast in imunski odziv pri piščancih, ki so prejeli poleg aflatoksina tudi Mycoad® plus, bistveno višja kot pri piščancih, krmljenih samo z aflatoksinom. Naši rezultati kažejo, da vsebnost aflatoksina v krmi piščancev vpliva na lokalni imunski odziv sluznic zgornjih dihalnih poti in na učinkovitost sistemskega imunskega odziva. Dodatek Mycoad® h krmi, onesnaženi z aflatoksini, lahko zmanjša neželene učinke aflatoksina na uspešnost cepljenj pri piščancih.

Ključne besede: aflatoksin; piščanci; imunost; Mycoad®

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Original Research Article

QUANTITATIVE ANALYSIS IN CANINE CUTANEOUS SOFT TISSUE SARCOMAS AND REACTIVE SPINDLE CELL PROLIFERATIONS ON CYTOLOGICAL SMEARS

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Abstract: Stained cytological specimens from twenty-four canine cutaneous soft tissue sarcomas and twenty-four canine spindle cell reactive proliferations were analyzed by computer-assisted nuclear morphometry. In each case, the nuclei of at least 100 neoplastic and reactive spindle cells were measured. The studied morphometric parameter was nuclear roundness (NR). The aim of the present study was to evaluate the possibility of quantitative differentiation between canine cutaneous soft tissue sarcomas and reactive spindle cell proliferations. The mean values of NR were statistically significantly higher in soft tissue sarcomas than in non-neoplastic lesions. Statistical differences in NR were found between liposarcoma and haemangiopericytoma. Cytomorphometric differences between soft tissue sarcomas divided by histologic grade were not revealed. Differences, related to age, sex, breed or location of the lesions between non-neoplastic and neoplastic lesions were also not observed. The results indicated that the morphometric parameter NR could be used as an additional tool for differentiation between canine cutaneous soft tissue sarcomas and reactive spindle cell proliferations.

Key words: computer-assisted morphometry; nuclear roundness; soft tissue sarcomas; spindle cell proliferations; dog

Introduction

Soft tissue sarcomas are a population of mesenchymal neoplasms that account for 15-20% of all cutaneous and subcutaneous tumours in dogs (1, 2). Most of the neoplasms are solitary and affect predominantly middle-aged and senior dogs. There is no sex and breed predilection with the exception of synovial cell sarcomas in dogs (2). Despite their heterogeneity, soft tissue sarcomas have a similar biological behavior. They are locally invasive and post-operative recurrences are reported in 7-30 % of cases (1, 2). Metastases are observed in about 20 % of the patients (3-5). The most important outcome predictor of this type of neoplasms is their grade determined histopathologically (1). Cytological diagnosis of soft tissue sarcomas is a challenge to pathologists. Pathological samples usually contain few cells as neoplastic cells are hardly exfoliated. In addition, the differentiation of sarcoma cells from reactive connective tissue processes by cytology is difficult to reliably differentiate (3-5). Computer-assisted nuclear morphometry is an objective image analysis for estimation of chosen parameters in individual cell (6, 7). The resulting data are objective and the technique is quickly performed using conventional microscopic analysis. The last investigations in

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this area showed that morphometry could be used as an additional tool for diagnosis and prognosis in some neoplastic diseases in small animals (8-10). Moreover, some researchers have used this non-traditional diagnostic method for detailed grading of malignant tumours (11, 12).

The aim of the present study was to evaluate the applicability of quantitative differentiation between canine cutaneous soft tissue sarcomas and reactive spindle cell proliferations.

Materials and methods

Animals

The survey was performed in 24 dogs with spontaneous soft tissue sarcomas -fibrosarcoma, (n=8), liposarcoma, (n=8), haemangiopericytoma, (n=8) and 24 dogs with reactive spindle cell proliferations: granulation tissue, (n=12) and dermal fibrosis, (n=12). All investigated formations were localized on skin epidermis and dermis. Patients' records were obtained from the database of the Department of General and Clinical Pathology, Faculty of Veterinary Medicine, Trakia University, Bulgaria (Table 1). Age, sex, breed of the dogs, location of the lesion, tumour grade and histologic diagnoses were recorded. In difficult cases an immunohistochemically investigation of neoplastic tissues was also performed (Fig. 1).

Cytological and histopathological processing

The material was obtained preoperatively by fine-needle aspiration biopsy, fixed immediately with Merckofix spray (Merck®, Darmstadt, Germany) and stained with Hemacolor (Merck®, Darmstadt, Germany). The cells were taken from four different areas of the formations. Later, material for histopathological examination was obtained from all patients. Samples were fixed in 10% neutral formalin and routinely processed. The final diagnoses were confirmed histopathologically (13). Histologic sections from all tumours were subsequently graded according to a system adopted from human medicine (14). The system was first applied for canine soft tissue sarcomas in 1980 year (14-16). It is based on tissue type and differentiation, mitotic rate, and percent necrosis - Table 1. (5,14).

Computer-assisted morphometry

The material for cytology was morphometrically analysed via trinocular digital microscope [Motic Professional B3 digital microscope (Motic, China Group Co Ltd, Hong Kong, China], and microscope image analysis software [Image Pro Plus® analysis system (Media Cybernetics, Silver Spring, MD, USA, version 4.5.0.29 for Windows 98/NT/2000)], Fig. 2. The measurements were calibrated with the aid of a micrometer ruler. Microscopic fields were randomly selected in the areas of highest cellularity, using x 40 objective lens. Images created by the computer system were stored in the system's digital memory, formatted as .jpeg files and displayed on the monitor screen (Fig. 1). At least 100 nuclei were analyzed in each case. Precautions were taken to include only intact cells. After selection of the proper portion of the cytological specimens and taking the digital photos, the nuclei borders were outlined using the "Draw/Merge object" function with the aid of a computer mouse. The morphometric parameter evaluated in this study was nuclear nuclear roundness (NR). A perfectly circular structure has a roundness value of 1.0 and values > 1 indicate irregular shapes.

Statistical analysis

Data from the morphometric analysis were statistically analyzed by the Mann-Whitney U test (Statistica 6.0, StatSoft, Tulsa, OK, USA) at a level of significance $P < 0.05$.

Results

The mean values of the studied parameter were statistically significantly higher in soft tissue sarcomas than in reactive spindle cell proliferations. The statistical analysis revealed significant difference between liposarcoma and haemangiopericytoma (Table 2). Cytomorphometric differences between soft tissue sarcomas divided by histologic grade were not revealed. Differences, related to age, sex, breed or location of the lesions between non-neoplastic and neoplastic lesions were also not observed.

Table 1: Grading system for soft tissue sarcomas in the dog (Kuntz et al., 1997)

Degree of differentiation	
Score 1	Resembles normal adult mesenchymal tissue
Score 2	Specific histologic type
Score 3	Undifferentiated
Mitotic index (no. of figures per 10 high power field)	
Score 1	0-9
Score 2	10-19
Score 3	> 20
Tumour necrosis	
Score 1	None
Score 2	< 50 % of tissue is necrotic
Score 3	> 50 % of tissue is necrotic
Histologic grade (cumulative score)	
Grade I	≤ 4
Grade II	5-6
Grade III	≥ 7

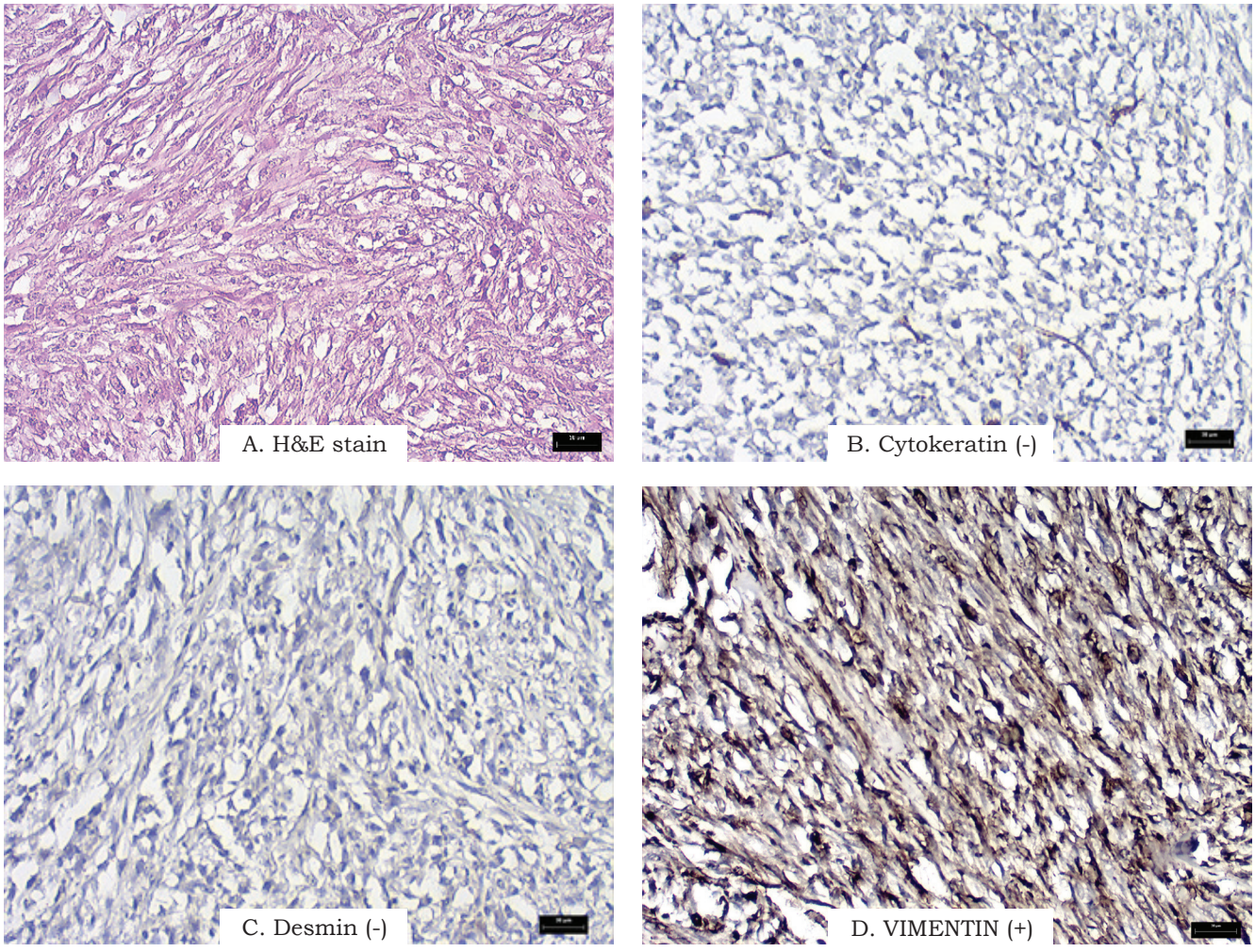

Figure 1: Fibrosarcoma. Immunohistochemistry. Neoplastic cells are Cytokeratin (-), Desmin (-) and Vimentin (+)

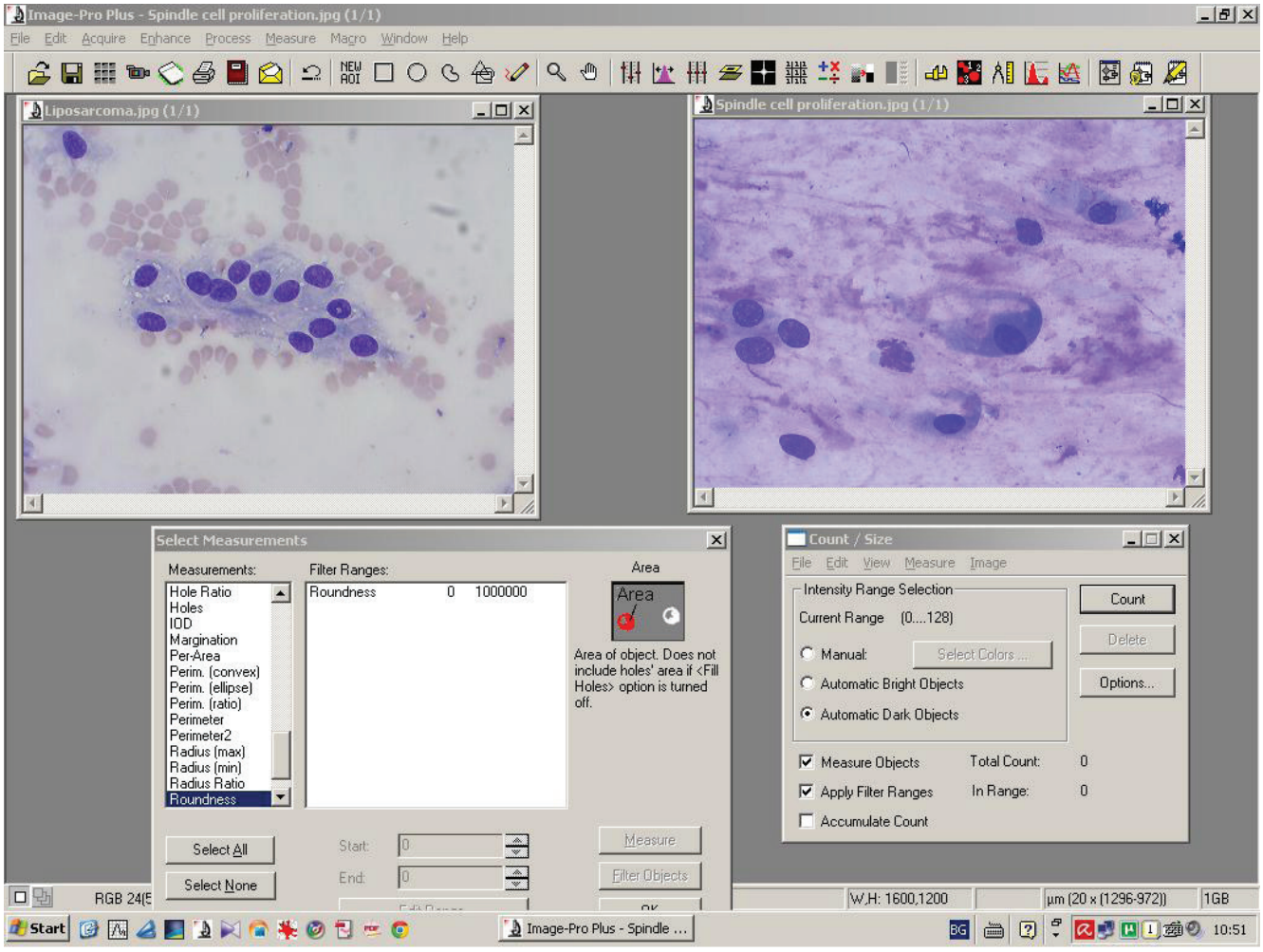

Figure 2: Main window of used software (Image Pro Plus®). Image of cytologic specimens from liposarcoma (left) and spindle cell proliferation (right)

Discussion

Bittinger et al. (17) investigated the diagnostic significance of different morphometric parameters in the differential diagnostics of human soft tissue tumours. The authors concluded that assessment of nuclear parameters may be helpful in the correct diagnosis and differential diagnosis of soft tissue tumours and tumour-like lesions of fibrous origin. Although the image analysis technique is relatively well known in veterinary pathology, there are only few reports of computer-assisted morphometry of NR in neoplastic and non-neoplastic formations. Results from one study (18) showed that NR could be used in the preoperative differentiation of benign from malignant canine mammary gland tumours. In this investigation, the malignant cells had more irregular nuclear shapes than cells in benign tumours. The values of NR were the lowest in adenomas and fibroadenomas and the highest in anaplastic carcinomas. The mean values of NR were as followed in ascending order: adenomas, fibroadenomas, tubulopapillary, solid and anaplastic carcinomas. This indicated that computerized morphometric analysis could be helpful for automated grading of canine mammary gland carcinomas on cytologic specimens. Meachem et al.[5] investigated quantitatively forty-four spontaneous canine soft tissue sarcomas and five reactive connective tissue processes. They found out that in reactive processes the nuclei were bigger and of considerably variable shape and size compared to sarcoma cells. Statistically significant differences ($P<0.05$) between reactive connective tissue processes and neoplasms were established for studied morphometric

Table 2: Clinico-morphological details of the dogs, included in this study

Case number	Breeds	Age (years)	Gender	Localization of lesions	Histologic	Tumour grade
Soft tissue sarcomas						
1	Mixed	11	M	Limbs	Fibrosarcoma	Grade II
2	Mixed	10	M	Head	Fibrosarcoma	Grade I
3	Mixed	9	M	Neck	Fibrosarcoma	Grade II
4	German shepherd	12	F	Limbs	Fibrosarcoma	Grade I
5	Rottweiler	13	F	Trunk	Fibrosarcoma	Grade II
6	Mixed	8	M	Trunk	Fibrosarcoma	Grade I
7	Mixed	7	F	Limbs	Fibrosarcoma	Grade I
8	Mixed	9	F	Limbs	Fibrosarcoma	Grade I
9	Labrador retriever	10	M	Trunk	Liposarcoma	Grade I
10	Doberman pincher	12	F	Trunk	Liposarcoma	Grade II
11	Boxer	11	M	Trunk	Liposarcoma	Grade I
12	Rottweiler	8	M	Neck	Liposarcoma	Grade I
13	Mixed	12	F	Limbs	Liposarcoma	Grade II
14	Mixed	11	F	Trunk	Liposarcoma	Grade I
15	Labrador retriever	10	M	Trunk	Liposarcoma	Grade I
16	Collie	10	M	Neck	Liposarcoma	Grade I
17	Mixed	7	F	Limbs	Haemangiopericytoma	Grade I
18	Boxer	12	F	Limbs	Haemangiopericytoma	Grade II
19	German shepherd	13	M	Trunk	Haemangiopericytoma	Grade I
20	German shepherd	11	F	Trunk	Haemangiopericytoma	Grade I
21	Mixed	9	M	Trunk	Haemangiopericytoma	Grade I
22	Poodle	8	F	Trunk	Haemangiopericytoma	Grade I
23	Mixed	9	M	Trunk	Haemangiopericytoma	Grade I
24	Mixed	9	M	Limbs	Haemangiopericytoma	Grade I
Reactive spindle cell proliferations						
1	Rottweiler	5	M	Trunk		
2	Mixed	4	M	Trunk		
3	Mixed	9	F	Trunk		
4	Collie	10	F	Trunk		
5	Poodle	8	M	Limbs		
6	Poodle	9	F	Limbs		
7	Boxer	8	M	Limbs		
8	Rottweiler	7	F	Limbs		
9	German shepherd	5	M	Trunk		
10	German shepherd	3	F	Trunk		
11	English cocker spaniel	8	M	Trunk		
12	English cocker spaniel	7	M	Neck		
13	Mixed	5	F	Neck		
14	Mixed	8	F	Limbs		
15	Rottweiler	7	F	Limbs		
16	Labrador retriever	9	M	Trunk		
17	Mixed	7	M	Trunk		
18	Mixed	8	F	Trunk		
19	Bulldog	9	F	Neck		
20	Poodle	5	M	Limbs		
21	Collie	5	M	Limbs		
22	Mixed	6	F	Trunk		
23	German shepherd	7	F	Trunk		
24	Mixed	8	M	Trunk		

Table 3. Nuclear roundness in canine cutaneous soft tissue sarcomas and reactive spindle cell proliferations.

Nuclear parameter	Canine soft tissue sarcomas (n = 24)	Reactive spindle cell proliferations (n = 24)	P value
Nuclear roundness	1.38 ± 0.37 [1.05 ± 2.09] Fibrosarcoma (n=8) 1.27 ± 0.31 [1.05 – 1.96] Liposarcoma (n=8) 1.25 ± 0.31* [1.07 – 2.00] Haemangiopericytoma (n=8) 1.63 ± 0.38* [1.07 – 2.09]	1.19 ± 0.09 [1.09 ± 1.44]	P = 0.01

* Means differ significantly, $P < 0.05$.

[ ] – minimum and maximum values

parameters. At the same time, however, they did not find cytomorphometric differences between different type of soft tissue sarcomas divided by histologic grade, mitotic index, or tumour necrosis score. The authors concluded that computer cytomorphometry could distinguish soft tissue sarcomas from reactive connective tissue processes in the dog. The last investigation in this area (19), demonstrated that the quantitative differentiation of reactive connective tissue processes from soft tissue sarcomas in dog is possible, but the same is not true for the different canine soft tissue sarcomas.

In this study for each tumour we used grading system which is relatively simple, reproducible and easy to perform. A total of 24 tumours were graded. Of these 18 (75 %) were classified as grade I, and 6 as grade II (25 %). The sarcoma cells of grade I neoplasms most closely resembled normal adult mesenchymal cells by type. The histologic type of sarcomas grade II tumours could also be determined, although the differentiation was not so clear. In these cases, we used immunohistochemistry markers for detail morphological identification. In our study we did not find any significant differences in NR between grade I and grade II canine soft tissue sarcomas.

The grading system of soft tissue sarcoma is not accepted by some veterinary pathologists mainly to the fact that they applied it for the group rather than to each neoplastic type (20). Moreover, before grading a soft tissue tumour, it is important to confirm the neoplastic disease and this way to eliminate reactive spindle cell proliferations and amelanotic melanomas (21).

Computer-assisted morphometric analysis could be applied both in cytology and histology, but according to us and other investigators (22) the cytological application is more convenient for practical purposes. Moreover, the utilization of kits for rapid fixation and staining of cytological smears allows for standardization of cytomorphometric procedures. Apart the rapid staining, they provide sufficient information for cells and cellular structures (23).

In summary, the results from our study demonstrated that morphometric parameter nuclear roundness could be used as an effective auxiliary tool for differentiation between canine soft tissue sarcomas and reactive spindle cell proliferations. Due to the relative small number of the studied formations, it is obviously necessary to perform further studies in the field of canine soft tissue sarcoma quantitative analysis. This would be of practical value for both pathologists and clinicians.

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KVANTITATIVNE ANALIZE KOŽNIH MEHKOTKIVNIH SARKOMOV PRI PSIH IN PROLIFERACIJE REAKTIVNIH VRETENASTIH CELIC V CITOLOŠKIH BRISIH

R. Simeonov

Povzetek: Namen predstavljene raziskave je bil kvantitativno ovrednotiti razliko med kožnimi mehkotkivnimi sarkomi in proliferacijo reaktivnih vretenastih celic pri psih. Z računalniško podprto analizo smo analizirali morfološke značilnosti jeder v 24 obarvanih prepratih kožnih mehkotkivnih sarkomov in 24 proliferacij reaktivnih vretenastih celic. Pri vsakem primeru smo analizirali vsaj 100 jeder in morfometrijsko določili okroglost jedra (OJ). Povprečna vrednost OJ je bila statistično značilno višja pri mehkotkivnih sarkomih kot pri neoplastičnih lezijah. Ugotovili smo tudi statistično značilne razlike v okroglosti jeder med liposarkomi in haemangiopericitomi. Med različnimi histološkimi tipi mehkotkivnih sarkomov, starostjo, spolom, pasmo in lokacijo poškodbe pa ni bilo citomorfometričnih razlik. Rezultati nakazujejo, da je morfometrični parameter OJ dodaten pokazatelj za razlikovanje med pasjimi mehkotkivnimi sarkomi in proliferacijami reaktivnih vretenastih celic.

Ključne beside: računalniško podprta morfometrija; okroglost jedra; mehkotkivni sarkomi; proliferacija vretenastih celic; pes

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