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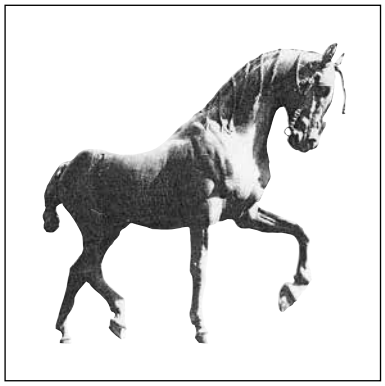

58th STUDENT SCIENTIFIC CONFERENCE

April 9th, 2015

The aim of the 58th Student Scientific Conference (ŠVOČ), organised in the academic year 2014/2015, was to present results of scientific investigations carried out by students of the University of Veterinary Medicine and Pharmacy in Košice. The selected papers were presented in the following five sections:

I. Pre-clinical section. II. Clinical section.
III. Food and environmental section. IV. Pharmaceutical section.
V. Section of bachelor's study.

UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE

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HYPERPLASTIC AND NEOPLASTIC CANINE HEPATOID GLANDS

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ABSTRACT

Perianal or hepatoid glands are modified sebaceous glands in canines located mainly in perianal skin, but they also occur in the skin lateral to the prepuce, in the dorsal lumbosacral region, along the ventral midline area and circumferentially around the proximal third of the tail. Neoplasms of the perianal glands are common in the dog, particularly in the male, and the occurrence of these tumours appears to be related to androgenic hormones. A retrospective study, of 80 histological samples of canine hepatoid gland changes over a 5-year period from 2007—2011, was performed. Canine biopsy specimens were received from veterinary practitioners and were diagnosed at the Department of Pathological Anatomy. This study aimed; to determine the prevalence of the histological types of hepatoid gland changes, their predilection sites, gender, breed and age. Out of the 80 cases, benign adenomas (34; 42.5 %) predominated over malignant adenocarcinomas (27; 33.75 %), mixed types (13; 16.25 %), and hyperplasias (6; 7.5 %). Perianal location (38.75 %) dominated over other common sites occurrence (25 %) such as ventrum of the tail and the prepuce. The ratio of male to female dogs was 6.4/1. Mixed breed dogs (26.25 %) and cocker spaniels (21.25 %) were the highest prevalent breeds in both benign and malignant tumours. The age of the affected dogs showed a range from 7 to 14 years, and exhibited an average of 9.56 years in the benign and 11.5 years in the malignant tumours. Photodocumentation of the typical hyperplastic and neoplastic histological changes were performed and compared.

Key words: adenocarcinoma; adenoma; dog; hepatoid glands; hyperplasia

INTRODUCTION

Hepatoid glands, unique to dogs and marsupials, are non-secretory abortive sebaceous glands situated around the anus in a uniform circle up to 2 cm from the anal orifice, and found also in scattered areas on the; prepuce, tail, hind legs, and trunk [6]. These glands contain two groups

of cells — mature hepatoid cells and peripheral basal reserve cells [2]. Their function is unknown, but referred to as odour producing glands. Perianal gland tumours appear to be androgen dependent. Male dogs show a 5.6-fold-increased risk compared with females. Adrenocortical hormones may play a role in the development of the tumours in female dogs [3].

The proliferative and neoplastic lesions of the hepatoid gland include; hyperplasia, adenoma, epithelioma, well differentiated and poorly differentiated carcinoma [4].

Hyperplasia occurs when the glandular tissue becomes enlarged following androgen overstimulation or an infection. This change is benign and the tissue resembles normal hepatoid glands, so diagnosis is challenging. Histologically, nodular growths are seen containing many lobes of uniform hepatoid cells surrounded by basaloid cells. The size varies from 0.5 to 5 cm so, if large enough, they can be palpated to aid the diagnosis. Benign tumorous growths are divided into adenomas and epitheliomas.

Adenomas are documented as the most common change of hepatoid glands and are reported to be the third most common canine tumour [7], [8]. Together with hyperplasia, they are androgen sensitive, and therefore can be secondary to overstimulation. Histologically, distinct lobular structures are present and the polygonal and round cells are arranged in cords or nests, distinguishing them from other benign growths. Commonly, ulceration and necrosis is seen on clinical examination.

An epithelioma is a benign growth located on the epithelium of organs or other bodily structures. They are uncommonly found in dogs and are distinguished from other benign growths by numerous, large basaloid epithelial reserve cells [1].

Adenocarcinomas are malignant growths, less commonly seen and account for roughly 10 % of all canine hepatoid gland tumours [11]. The tumours are larger and become ulcerated. Histologically, there is a mixture of basaloid cells with irregular and often rudimentary maturation towards typical polyhedral hepatoid cells. Cells of intermediate maturation may predominate. The invasion of these cells into the surrounding normal tissue is the only unequivocal criterion for malignancy [12]. The diagnosis of hepatoid gland changes is only possibly by histological examination by a veterinary pathologist.

The goal of this study was to determine the statistical analyses of data obtained from histological protocols on hyperplastic and tumorous growths. Biopsy and excision samples were provided by veterinary surgeons in Slovakia and they were histologically processed at the Department of Pathological Anatomy.

MATERIALS AND METHODS

Animal characterization: Eighty cases of tumorous hepatoid gland changes were detected in histological samples collected during a 5-year period from 2007—2011. The analysis of; histological types of changes, age, sex, breed predisposition, and anatomical location was done.

Histopathology: Samples were collected by biopsy, surgery or necropsy and send to UVMP for histological examination. Specimens were fixed in 10 % neutral formalin then embedded in wax. Five µm sections were stained with haematoxylin and eosin (HE), and mounted in Entelan onto slides.

Sample analysis and photodocumentation: Light microscopy determined the specific changes in the tissue. The percentage of different changes was recorded and photographs were taken to demonstrate these changes.

RESULTS

During the five year period, a total of 80 cases of hepatoid glands changes were found. Within these cases, we determined the presence of 6 (7.5 %) hyperplasia, 34 (42.5 %) adenomas, 27 (33.75 %) adenocarcinomas, and 13 (16.25 %) mixed tumours. These results demonstrated that adenomas were the most frequent and hyperplasias the least common.

Breed predisposition analysis revealed the dominancy of hepatoid gland changes in cross breed dogs (26.25 %) and Cocker Spaniels (21.25 %). The next most prevalent breeds were poodles and schnauzers. The results demonstrated a higher tendency of occurrence of adenomas (benign tumours) in medium to large breeds and adenocarcinomas (malignant tumours) in medium to small breeds. Age predisposition to the occurrence of tumorous changes was expressed predominantly in the older dogs, ranging from 7—14 years of age. Ten-year old dogs showed the highest incidence (22.5 %). Adenomas were observed in younger dogs (7—10 years) while adenocarcinomas in old-

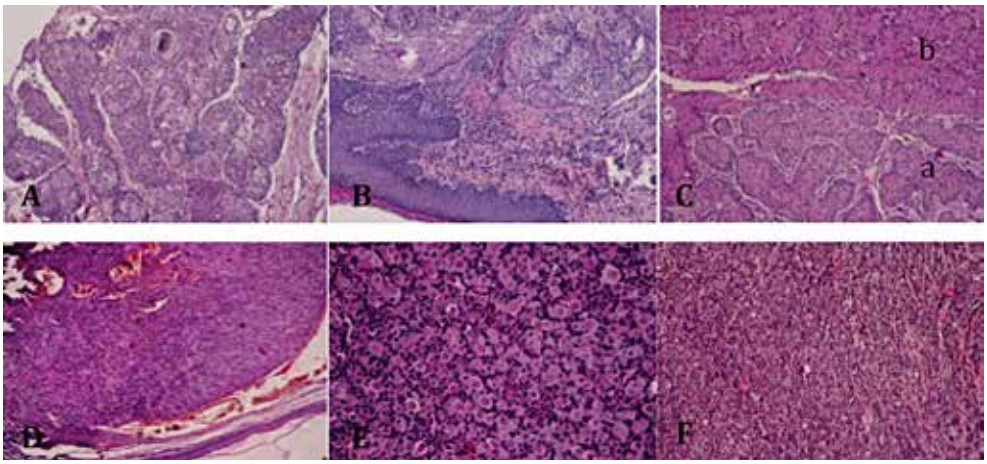

Fig. 1. A, B — Hepatoid glands hyperplasia, 10-year old male cross breed dog; A — Degenerative-necrotic changes of glandular cells (40×); B — Lymphocytic infiltrate in the dermis and interstitium (10×); C — Hyperplasia and adenoma of perianal glands, 12-year old male Dalmatian (HE, 20×), transformation from hyperplasia (a) to adenoma (b); D, E — Adenoma, 10-year old female Bernese mountain dog (HE); D — subepidermal localisation with haemorrhages (10×); E — degenerative-necrotic changes of glandular cells (40×); F — Carcinoma of hepatoid gland, 12-year old female cocker spaniel (HE), diffuse proliferation of basaloid cells with extensive vascularisation (40×)

er animals (10—12 years). Sexual predisposition showed a strong correlation in male dogs — 72.5 % of all cases; female dogs only accounted for 11.25 %. The localisation of changes varied greatly. The skin of perianal area was the most frequent place (38.75 %), however, proliferation of hepatoid gland within skin at the different part of the body was also observed (25 %).

The histological types of hepatoid gland changes (hematoxylin-eosin staining) are documented and closely described at Figure 1.

DISCUSSION

This investigation focused on the prevalence of hepatoid gland changes in correlation to age, breed, sex and anatomical region. Our results determined that benign adenomas were the most common finding (42.5 % of cases), while malignant growths presented 33.75 %. Morrison [9] reported that hepatoid gland tumours are the third most common neoplasias in dogs. This investigator's study involved the examination of 2845 hepatoid gland tumours, and documented 91 % with the benign characteristics of hepatoid gland tumours. Hayes and Wilson [3] performed a study on 472 dogs and found 81.77 % of benign and 18.2 % malignant cases. Breed predilection in our study revealed the highest risk in cross breed dogs for hepatoid gland changes, followed by cockers spaniels and poodles. Morrison [9] reported that benign adenomas are most commonly seen in cocker spaniel, and also Meuten [8] published a high incidence of malignant tumours in cocker spaniels. The age dependency of hepatoid gland changes in our study showed that most of the occurrences were in the older dogs with an average age of 11.01 years. The benign tumours had a higher frequency in younger dogs (average 9.56 years), when compared to malignant tumours observed in older dogs (average 11.5 years). Javanbakht et al. [5] and Morrison [9] found in their studies, that the average age ranged from 10.5—11.3 years and changes were most often seen in dogs over 8 years of age. The sex ratio between males and females in our analysis showed 72.5 % to 11.25 % of the cases respectively. Similar data were published by Pakhrin et al. [10], with 70.37 % in males to 11.11 % in female cases. A survey by Morrison [9] on 145 dogs with hepatoid gland adenocarcinomas found that 93 % were male and 73 % of them were intact. Several published studies have mentioned that intact male dogs showed the highest risk of any hepatoid gland changes. The regional perianal localization was the most affected in our study and consisted of 38.75 % of all cases. Pakhrin et al. [10] found in their study, that 26 (96.30 %) of the cases occurred in the perianal region.

In conclusion, our study demonstrated that benign adenomas of the hepatoid glands were the most common finding in dogs. Predominantly, the dogs were male, aged 10 years, mixed breed or cocker spaniels with a perianal location.

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PREVALENCE OF OSTRICH (*STRUTHIO CAMELUS*) AND RHEA (*RHEA AMERICANA*) PARASITES IN SELECTED HOLDINGS IN ENGLAND

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ABSTRACT

Over a 13 month period, faecal and feather samples were collected from 85 ostriches and rheas from various geographical locations in England. Faecal samples were analysed using sedimentation and floatation methods for the presence of endoparasites. Faecal results showed eggs/oocysts from four genera of parasites in the screened ostrich samples. There was a high prevalence of *Ascaridia* spp. and *Trichostrongylus* spp., followed by *Eimeria* spp. and *Heterakis* spp. which are unusual and have only been documented in a small number of studies. However, the infection may support the theory of cross species contamination. Greater rheas were infected with 5 genera of parasites with an abundance of *Capillaria* spp. Other species included *Eimeria* spp., *Ascaridia* spp., *Trichostrongylus* spp., and *Deletrocephalus* spp. Feather sample results recorded a 100 % infection rate on the ostrich samples identifying just one louse species, i.e. *Struthiolipeurus struthionis*.

Key words: ostrich; parasite; rheas

INTRODUCTION

Ostriches and rheas are large flightless birds from the families *Struthionidae* and *Rheidae*, respectively. Both species are kept for the production of meat, feathers, hides and eggs. Within the United Kingdom (UK) both species are frequently found in Zoological gardens, private collections and specialized farms. The addition of these birds to the list of farmed species has created a new challenge to the veterinary surgeon called upon to provide health care and advice.

Parasites can have a negative influence on overall health status, as well as lowering immunity making birds succumb to disease. Productivity can also be reduced by a heavy parasite burden lowering the number of eggs produced, reducing weight gain and causing loss of body condition. In addition, ostrich and rheas can host zoonotic parasites such as *Toxoplasma gondii* and *Giardia* spp., which can pose a risk to human health. The majority of private 'back yard' owners do not routinely administer anthelminthic drugs to their birds in the belief that ratites are resistant to most worm species that infect other farm birds. Unfortunately, this is

not the case and ratites are susceptible to nematode, cestode, trematode and protozoa infections with at least fifty different species of endoparasites being recoded.

MATERIALS AND METHODS

The samples for this study were collected from various locations in England, United Kingdom, with laboratory analysis occurring in both the UK and Slovakia.

A total of 85 faecal samples (57 greater rheas and 28 ostriches) and 42 feather samples (34 greater rheas and 8 ostriches) were collected over a 13 month period, from November 2013 to December 2014 and examined for the presence of parasites. Only samples from ostriches and greater rheas were looked at in this study, with all ages and sexes being accommodated.

Eleven ratite holdings participated in the current study, including ZOOs, private breeders and collectors, and a commercial ostrich farm. Samples (57 greater rheas and 28 ostriches) were collected through the "free catch" method after individual birds were observed defecating. Samples were collected from the ground by a gloved hand and transferred into coprological sample pots which were placed in a cooler bag with an icepack. Each sample pot was labelled with the date, the owners name, address of sampling area, species of bird and its sex. Once transported back to the laboratory, they were stored for a maximum of five days at 5 °C. In two holdings, repeated sampling was planned at different times of the year, to establish whether seasonal conditions influenced the parasite genus found. A summer collection occurring in June 2014 and winter collection occurring in December 2014 were organised with the relevant holdings.

Feather samples of 34 greater rheas and 8 ostriches were obtained by plucking several feathers from under the bird's wing. Plucked feathers were transferred to a sealable plastic transport bag and taken back to the laboratory for examination under a microscope. Some feathers had noticeable ectoparasites visible to the naked eye, allowing parasites to be removed from the feather using a small paint brush and transferred into a small vial containing 10 % formalin solution. These were transferred back to the laboratory for identification.

In order to view parasitic eggs/oocysts of nematodes, cestodes and protozoa, a simple floatation method can yield satisfactory results, where as sedimentation techniques are useful for identifying the presence of trematode eggs which are too heavy to concentrate by the floatation method.

RESULTS

A total of 85 ratites were examined in the current study for the presence of endoparasites. Twenty-eight birds (32.94 %) were positive for the presence of nematode eggs and 5 (5.88 %) were positive for protozoa oocysts. No ratites were positive for cestode or trematode eggs. Rheas and ostriches were compared as different sample sizes existed with an over representation of greater rheas than ostriches. From the 57 greater rheas, nematode eggs were found in 24 individuals (42.10 %). Two greater rheas (3.50 %) were also positive for protozoa oocysts. In the ostriches, 28 faecal samples were examined; 4 (14.28 %) birds were positive for nematode eggs and 3 (10.71 %) for protozoa oocysts. Rheas in the present study demonstrated a higher infection rate for nematode eggs.

Ostriches were kept at 4 out of the 11 holdings visited during this study. Fifty percent of the ostrich holdings were positive for parasites. Nine out of the 11 holdings housed greater rheas and 6 of these were positive for the presence of parasites (66.66 %).

The current study found that rheas and ostriches could be infected with a single genus parasite infection or have mixed infections where multiple parasite genera occurred. *Eimeria* spp. was the only detected protozoa found in the rhea and ostrich samples. *Trichostrongylus* spp. and *Ascaridia* spp. were the most common nematodes observed in the ostrich population, followed by *Heterakis* spp. (7.7 %). *Capillaria* spp. (31.6 %) was the most abundant nematode found in greater rheas. Other nematode species detected in greater rheas were *Trichostrongylus* spp. (22.8 %), *Deletrocephalus* spp. (8.8 %) and *Ascaridia* spp. (5.3 %). In total, 20 % of the ratite faecal samples tested in this study were infected with *Trichostrongylus* spp. At two separate holdings, repeated faecal samples were performed. Winter and summer samplings concluded no difference in the endoparasite fauna.

Only 42 ratites were examined for the presence of ectoparasites. All 34 greater rheas were negative for ectoparasites. The 8 ostriches which were examined in the study, all had the presence of lice, resulting in the infection rate of

100 %. No other species of ectoparasites were detected on the 8 examined birds. Through microscopy, the lice were identified as S*truthiolipeurus struthionis.*

DISCUSSION

The results for the number of positive samples in greater rheas were similar to those reported in lesser rheas (*Rhea pennata*) by Reissig et al. [3], in which a total of 130 (42 %) of 310 faecal samples examined were positive. This study also had a positive infection rate of 42 %. It seems that lesser rheas and greater rheas share the same susceptibility to *Eimeria* spp., *Trichostrongylus* spp. and *Capillaria* spp.

The infection rate of ostriches was 14.28 % with only 4 birds testing positive from the 28 sampled. Endoparasite infection in the ostriches was limited to two farms. The nematodes detected in this present study were similar to those that occurred in continental Europe. PonceGordo et al. [2], described how *Libyostrongylus* spp. was the most common nematode occurring in Spain, Belgium, Netherlands and Portugal with the prevalence being estimated at 20 %. In addition, *Codiostomum struthonis, Ascaridia* spp. and *Capillaria* spp. had also been documented, although their prevalence was under 1 %.

Libyostrongylus spp. is the most commonly reported nematode to affect ostriches globally. *Libyostrongylus* spp. has adapted to the UK atmospheric climate, hence the parasites exhibits a high prevalence throughout Europe. With this knowledge, the strongyle type nematode eggs detected in this study are presumed to be that of *Libyostrongylus* spp. (Superfamily: *Trichostrongyloidea*) as opposed to species such as *Codiostomum* spp. or *Trichostrongylus* spp. which have minimal detection rates in Europe. However, as eggs are visibly indistinguishable from *Trichostrongylus* spp. or *Codiostomum* spp. there remains a small level of uncertainty as to the exact species found. Differentiation of these three parasites must be made through larvoscopy.

Interestingly, in the present study, two parasites from the family *Ascaridae* were noted; *Heterakis* spp. and *Ascaridia* spp. There are rare accounts of *Heterakis* spp. being documented in ostriches or rheas. Its occurrence in this study could be the result of a spurious parasitosis however, the enclosure was co-inhabited with several guineafowl (*Numidameleagris*) which could result in cross species transmission. No data exists on the egg morphology of *Ascaridia struthionis*, however, due to the *Ascaridia* spp. egg closely resembling *A. gallinae,* the egg observed could be either species. Reissig et al. [3], reported that *Capillaria* spp. were found in 2.8 % of the lesser rheas in Argentina, with a preference for infecting juvenile/adult birds as opposed to chicks. A similar finding was determined during this study. *Deletrocephalus dimidiatus* was first recognized in British rheas in 1994. Taylor et al. [5], reported *Deletrocephalus dimidiatus* in a group of mixed aged rheas in Wales, however, no infection rates were noted. The present study showed infections of 5 birds from the 57 sampled, however, one third of the examined holdings were positive for infected birds. *Deletrocephalus* spp. seems to have a strong residence in England's rheas.

Eimeria spp. has been described in Emu, Ostrich and Rhea [6]. There is little data which supports the exact *Eimeria* species which affects ratites. A study by Sotiraki et al. [4], found the prevalence of coccidian oocysts in ostriches farmed in Greece was relatively low (4.2 %) from 336 faecal samples and was limited to one of twenty farms. In this study, a low infection rate (10.7 %) in ostriches was also present. Mushi et al. [1] found coccidia oocysts in the faeces of 53 (34 %) of 156 apparently healthy ostrich chicks. Birds older than 9 weeks did not have any infection. In contrast, this study found adult ostriches were positive for coccidia oocysts.

CONCLUSIONS

Farming exotic species can be very profitable in today's market, however, an in-depth knowledge on the health and welfare of individual species is paramount in order to make a successful business. Parasites can jeopardise animal health, leading to ill-thrift and occasionally death, and thus, reducing a farmer's income considerably. There is a diverse source of scientific data detailing parasitic fauna and therapy in other farm animal species, however, the same cannot be said in relation to ostriches and rheas. The most common parasite species found in ostriches were *Trichostrongylus* spp. and *Ascaridia* spp. and in rheas *Capillaria* spp. It is highly likely that birds kept in close proximity to domestic fowl, or have enclosures that allow the entry of wild birds, could risk cross species transmission as the majority of parasite genera detected in this study have a wide host range. Even though no ectoparasites were detected on

greater rheas during this study, it is highly likely that birds can suffer from ectoparasites and more research should be conducted on this topic. Recommendations concluded from this study are, to further investigate the parasitic fauna of rheas and ostriches in England, as well as other ratite species such as emus. Faecal egg counts should be established specifically for ratites and ideally be followed up with larvoscopy as many ostrich parasite eggs look similar, particularly those of *Trichostrongylus* spp.

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THE INFLUENCE OF MIGRATION BARRIERS ON ICHTHYOCENOSIS STRUCTURE, PARASITOFAUNA AND CONDITION OF FISH

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ABSTRACT

The aim of this study was to compare the diversity of the ichthyocenosis and parasitofauna of host species of fish in individual fragmented sections of the river Hornád and assess their potential influence on the condition of fish. The fish were caught by means of an electric aggregate in 5 locations separated partially or fully by migration barriers (small water power stations, SWPS). In the entire investigated sector of the river we found 11 species of fish. The greatest differences in species distribution were observed for the riffle minnow (*Alburnoides bipunctatus*) and the European bitterling (*Rhodeus amarus*). On the contrary, the species such as the European chub (*Squalius cephalus*) and the European perch (*Perca fluviatilis*) were observed throughout the river sector. The most prevalent parasites in the observed sector were thorny-headed worms (Acanthocephala: namely the species; *Pomphorhynchus slaevis*, *Pomphorhynchus tereticollis*, *Acanthocephalus anguillae* and *Acanthocephalus lucii*), causing high intensity infections (1-300) with intra- and extra-intestinal localisations and serious damage to the intestinal mucosa. There was a significant relationship between fish species and Clark's coefficient of condition (GLM; d. f. = 2, Chi-square = 10.53, P < 0.05) and between locality and fish condition (GLM; d. f. = 1, Chi-square = 14.081, P < 0.001).

Key words: condition; fish migration; ichthyocenosis; parasitofauna

INTRODUCTION

Migration is an important part of the biology and the life cycle of fish, and the other organisms living in water [8]. Migration barriers result in the fragmentation of the populations of water biota and through changes in ecological factors and parameters of water, affect the structure and species diversity of fish and water invertebrates [1], [2], [9], [15]. Anthropogenic elements are frequently responsible for the development of new adaptations or increased pathogenicity of parasites and also for the development of new host-parasite interactions [14].

The aim of this study was to observe and compare species structure and distribution of ichthyofauna and parasitic endo-helminths in a fragmented sector of a model river, the Hornád, and their influence on the condition and health of the host species of fish.

MATERIALS AND METHODS

In April and August of 2014 we carried out, in a model sector of the river Hornád, an ichthyo-parasitologic monitoring of the fish populations separated by individual migration barriers (small water power stations, SWPS). An electric aggregate was used for catching the fish in 5 locations: at water reservoir (WR) Ružín, characterised by a lower temperature of water, from the hipolimnetic discharge from WR, and thus, from a secondary trout zone extending down to the section after Košice, by the village Ždaňa, where the river changes its character to a lowland river. The downstream locations were: Location 1 – Malá Lodina (48° 52' 53.1" N; 21° 07' 04.6" E) 500 m away from WR Ružín dam; location 2 – Veľká Lodina (48° 51' 36.4" N; 21° 09' 30.7" E); location 3 – Družstevná pri Hornáde (48° 48' 05.4" N; 21° 14' 39.1" E); location 4 – Vyšné Opátske (48° 41' 13.0" N; 21° 17' 36.0" E); and location 5 – Ždaňa (48° 36' 07.4" N 21° 20' 15.5" E).

The fish were examined by an incomplete parasitological dissection using conventional parasitological methods [5], [13]. We determined the basic epizootological parameters of the individual helminths present.

The faunistic similarity of individual locations was calculated according to a formula: $(2c/a+b)\times 100\,\%$, where a = the number of species of fish in location A; b = the number of species in location B; and c = the number of fish species common to both locations.

The condition of the fish was determined by calculating a Clark's coefficient (CC) of condition: $CC = m(g)/l(mm)^3 \times 100\,000$, which uses the body weight of the fish determined after removal of all organs from the body cavity [7]. All statistical analyses were performed using R statistical software (R i386 3.1.1). The aim of the analysis was to identify relationships between condition of fishes and number of parasites at the interaction with locality and fish species. A generalized linear model (GLM) was fitted, with logarithmically transformed values of condition factor as the response variable, and number of parasites, effect of locality and fish species at the two way interaction as explanatory variables, according to Crawley [3]. The insignificant terms of model were gradually removed and refitted. After excluding the insignificant interactions, significances were tested with ANOVA type II, to increase the power of statistical test [6]. Parsimony of refitted models was tested under the decrease of Akaike information criterion (AIC).

RESULTS AND DISCUSSION

Species spectrum of fish

A sample consisting of 222 fish was used to determine the species spectrum of the fish which belonged to 3 families (*Cyprinidae, Percidae, Gadidae*), 9 genera and 11 species. The location with the highest species abundance was location 3, where 6 species of fish were observed. The highest number of fish (37) was caught below SWPS in location 4.

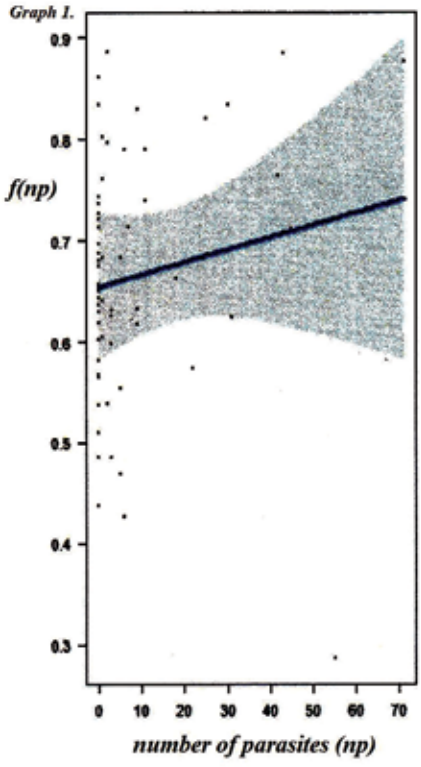
The proportion of individual species and of the gender of fish differed significantly. The fish more frequently caught in the sector were: the European perch (*Perca fluviatilis*), *Cyprinidae species* of the European chub (*Squalius cephalus*), the Carpathian barbel (*Barbus carpathicus*) and the riffle minnow (*Alburnoides bipunctatus*). The mean values of the coefficient of condition are shown in Graph 2. The greatest differences in the distribution of the species were exhibited by the riffle minnow and the European bitterling (*Rhodeus amarus*), but the species, such as *S. cephalusa* and *P. fluviatilis*, were observed throughout the investigated river sector. Barbel (*Barbus barbus*) was caught only once in location 3, where we found also one stone loach (*Barbatula barbatula*) and 3 gudgeons (*Gobio gobio*). The only representative of *Gadidae* in Slovakia, the burbot (*Lota lota*), was caught (11 burbots) only in location 4. The leak (*Alburnus alburnus*) was caught in locations 4 and 5 and the critically threatened species of Slovak ichthyofauna, Danubian longbarbel gudgeon (*Gobio uranoscopus*), occurred only in locations 3 and 4. The faunistic similarity of individual sampling locations reached 100 % between locations 1 and 2; 66.6 % between locations 2 and 3; 60 % between 3 and 4; and 57 % between 4 and 5.

Species spectrum of parasites

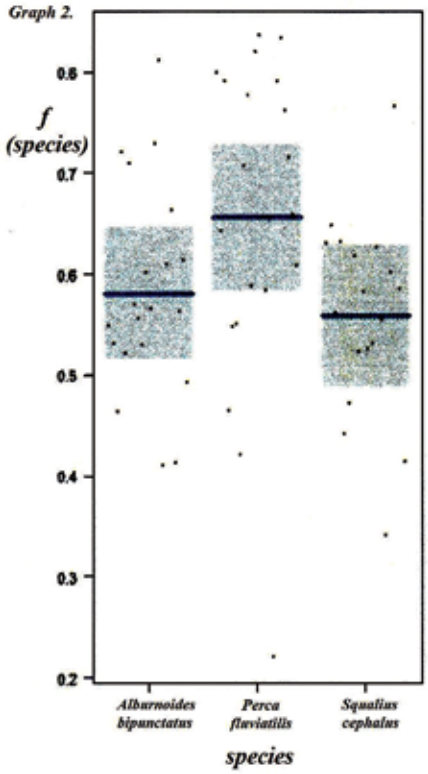
The helminths most frequently found in the fish were the thorny-headed worms (*Acanthocephala*), which by

Fig. 1. Perforation of the intestinal wall caused by massive multi-infection by species P. laevis, P. tereticollis, A. anguillae and A. lucii

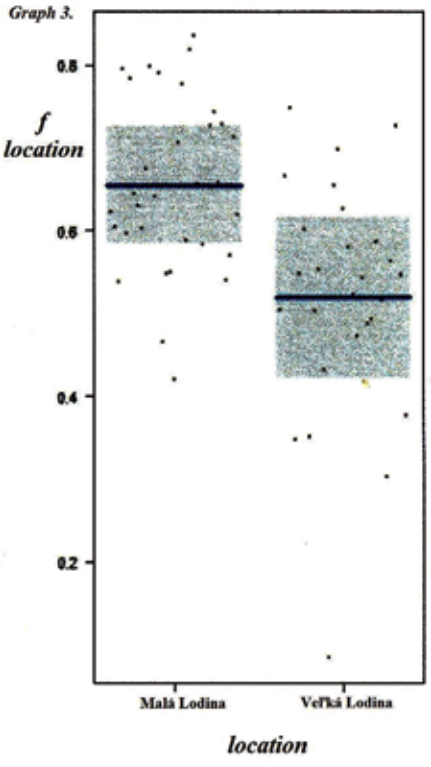
Fig. 2. Perforation of the intestinal wall caused by massive multi-infection by species P. laevis, P. tereticollis, A. anguillae and A. lucii



Graph 1. Relationship between the intensity of infection by thorny-headed worms and the coefficient of condition (CC) in the infected fish



Graph 2. Comparison of the CC of the riffle minnow (ALBI), the European perch (PF) and the European chub (SQCE) throughout the investigated river sector



Graph 3. Comparison of the CC of fish among locations with the highest faunistic similarity of fish:
1 — Malá Lodina; 2 — Veľká Lodina

their prevalence and intensity of infection were an important component of parasitofauna of fish living in the river Hornád. The following species were found: *Pomphorhynchus laevis Rudolphi* 1809; P*omphorhynchus tereticollis Rudophi* 1809; *Acanthocephalus lucii Müller*, 1776; and *Acanthocephalus anguillae Müller*, 1780. The parasites were isolated either as adult helminths from the intestines or as juvenile forms migrating from the intestine and populating the serosa of body cavity organs, mostly at high intensity of infections. The presence of metacercaria of digenetic trematodes on the body cavity serosa was observed only in one location in European perch, at a low intensity of infection. The highest prevalence of thorny-headed worms was observed in the burbot (100 %) followed by the European perch (93.26 %) and the Carpathian barbel (77.41 %). The localization of parasites in the gastrointestinal tract was typical of the riffle minnow (100 %) and the European perch (84.04 %). On the contrary, in the juvenile burbots, the helminths were more frequently found on the serosa of the internal organs (63 %). The influence of thorny-headed worms on the condition of fish depended on the intensity of the infection. The high mean intensity of infection (41.62), in the Carpathian barbell, which prefers benthic invertebrates, is related to the feed spectrum of this species, as is the preferred feed related to the low epizootological indices in the bleak and the riffle minnow.

The tested model identified any significant two way interactions between condition of fishes, number of parasites at the interaction with the effect of locality and fish species for a given number of observations. The parasite number had no significant influence on Clark's coefficient of condition (GLM; d. f. = 1, Chi-square = 0.7565; $P > 0.05$) (Graph 1)

There was a significant relationship between fish species and CC (GLM; d. f. = 2, Chi-square = 10.53; $P < 0.05$) (Graph 2) and between locality and CC (GLM; d.f. = 1, Chi-square = 14.081, $P < 0.001$) (Graph 3).

The condition was lower in the European perch, the European chub and the Carpathian barbell which exhibited the highest intensity of infection (mostly by the species *P. laevis* and P. tereticollis). A worse condition resulted from the inflammatory processes which developed after the mechanical damage and multiple micro-perforations of the mucosa by the parasites (Fig. 1 and 2); in extreme cases, even intestinal perforations may occur. The broad species spectrum of hosts indicates that the observed species of acanthocephalans do not belong among "specialists" preferring certain species. However, the different localisation of parasites also indicates a different role of individual fish species in the life cycle of the genus *Pomphorhynchus*. The body size of fish is an important factor deciding whether it acts as a definitive or paratenic host [12]. The lowest prevalence of acanthocephaloses in the European perch and the European chub was observed in location 1, which was also reflected in the values of the CC in comparison with other locations (Graph 3). This was probably due to a lower concentration of the intermediate hosts caused by changes in the physico-chemical properties of water in this section [11] and discharge of water from the dam [10]. The presence of a suitable intermediate host species is a limiting factor for thorny-headed worms. Relevant studies indicate a relationship between a high prevalence and the intensity of infection by *Pomphorhynchus tereticollis* in fish in the Danube basin and the spreading and high infection of the intermediate host, an invasive crustacean species Dikerogammarus villosus (*Amphipoda*) [4].

CONCLUSION

Fish and their parasites are an important component of water ecosystems capable of very sensitive response to any disturbances of balance and other related changes. The study of their mutual interactions allows one to assess better strategies of parasite transfer, the direct and indirect influence on the hosts and the associated risks.

ACKNOWLEDGEMENTS

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Selected papers from the 58th STUDENT SCIENTIFIC CONFERENCE, Section I, held at the University of Veterinary Medicine and Pharmacy in Košice on April 15, 2015.

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SURVEILLANCE OF RABBIT PARASITOSES ON SELECTED FARMS IN EASTERN SLOVAKIA

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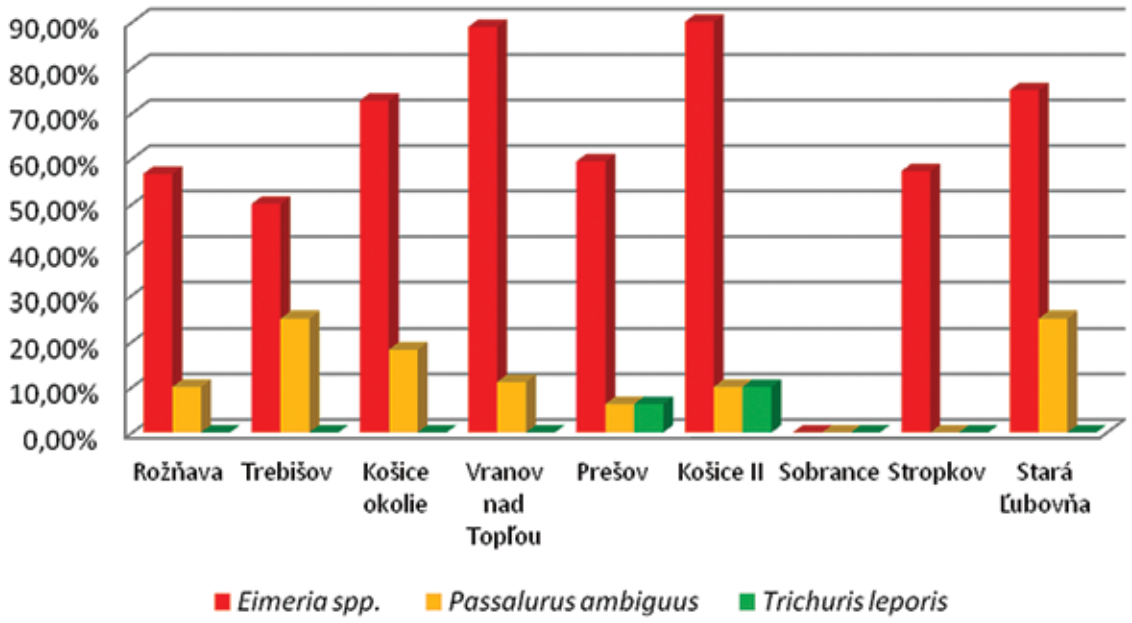
ABSTRACT

Parasitoses of rabbits are a frequent cause of their decreased performance, mainly on small farms. For this reason, we focused on species composition of parasites on selected farms in eastern Slovakia. We examined samples from 137 farms, most of them in the districts of Košice and Prešov. As many as 72 % of the farms were positive, most frequently for *Eimeria* spp. (63.5 % prevalence). The mean intensity of infection with *Eimeria* spp. was high, reaching 4,341 oocysts per gram (OPG) (range 100—194,700 OPG). The presence of nematodes, *Passalurus ambiguous*, was detected on more than 10 % of the farms (range 100—1,400 eggs per gram — EPG) and *Trichuris leporis* was found on 2 % of the farms at low intensity (100, EPG). Parasitological dissection confirmed the occurrence of *P. ambiguous*; 196 females and 56 males were isolated from the large intestine. In one rabbit, *E. stiedai* was found in the liver and the larva-cysts of tapeworms (*Cysticercus* spp.) were observed on the serosa of two rabbits. Examination for ectoparasites allowed us to diagnose *Psoroptes cuniculi* in the ear swabs on one farm (3.85 %).

Key words: *Eimeria* spp.; intensity of infection; *Passalurus ambiguus*; prevalence; *Psoroptes cuniculi*; *Trichuris leporis*

INTRODUCTION

The history of rabbit keeping in Slovakia indicates their continued popularity. Breeders raise them for several reasons which include obtaining high quality diet meat and economical profits. The mean annual consumption of rabbit meat in SR is about 2 kg per person [3]. However, rabbits are the hosts of a large number of parasites, mostly those causing coccidiosis, psoroptosis, and passalurosis, but also of other parasites, which are economically important due to their negative effects on rabbit performance. Other parasites, such as, encephalitozoonosis, toxoplasmosis, sarcocystosis, and cysticercosis, are transmissible to humans. This is the reason why, with an increasing number of rabbit breeders, one should pay increased attention to the prevention of parasitoses.



Graph 1. Species composition and prevalence (%) of parasites in rabbits on selected farms in Eastern Slovakia

Fig. 1. *Cysticercus* spp. (author A. Kočišová)

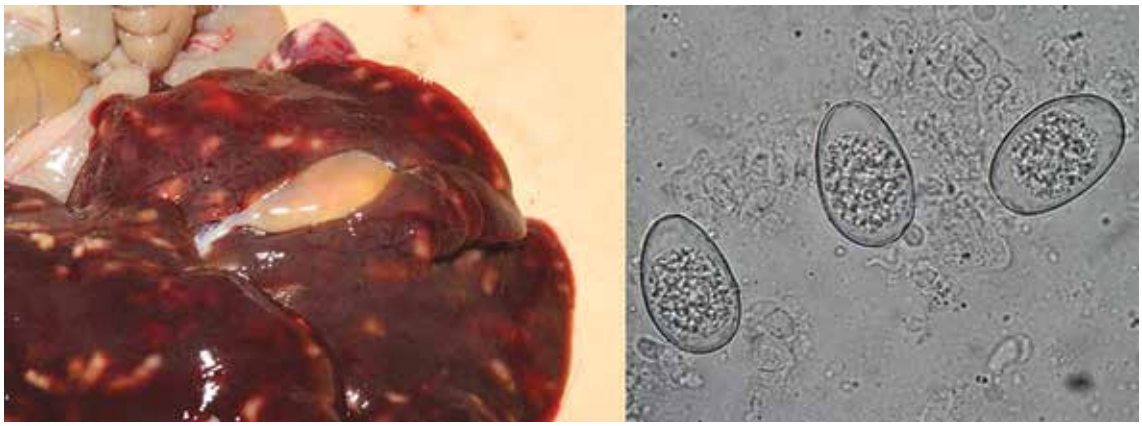


Fig. 2. Liver infected with *Eimeria* stiedai (left); oocysts (right) in smears of liver lesions (author A. Kočišová)

The aim of this study was to determine the species composition of parasites and assess their prevalence and mean intensity of parasitic infection on selected farms.

MATERIALS AND METHODS

To assess the prevalence of internal parasites we processed and examined samples of faeces, the digestive tracts of rabbits slaughtered for meat; also ear swabs and skins of rabbits were examined for the presence of ectoparasites. We examined a total of 137 faecal samples. To diagnose endoparasites we collected 39 digestive tracts from the rabbits of 23 breeder farms. Ear swabs were obtained from 26 farms and the total number of samples examined for ectoparasites was 46.

The faeces were examined by flotation and McMaster's methods. The ear swabs were examined microscopically after cold maceration and the content of the digestive tract, by partial parasitological dissection [2].

RESULTS AND DISCUSSION

To determine the species composition of parasites, we examined 137 samples of faeces from individual farms and observed that 95 (72.5 %) of them were positive. In the positive samples we found oocysts of *Eimeria* spp. and eggs of *Passalurus ambiguus* and *Trichuris leporis* (Graph 1). The mean intensity of infection of *Eimeria* spp. was high (4,341 oocysts per gram — OPG; range 100—194,700 OPG). *P. ambiguus* eggs ranged between 100—1,400 eggs per gram (EPG) and the *T. leporis* egg numbers indicated a low intensity of infection (100 EPG). From 7 (17.95 %) digestive tracts we isolated 196 females and 56 males of *Passalurus ambiguous*. In 2 rabbits (5.13 %), we found *Cysticercus* spp. (Fig. 1) and the liver of one rabbit was infected with *Eimeria* stiedai (2.56 %) (Fig. 2). *Psoroptes cuniculi* was detected in one sample (3.85 %) from a farm in the Spišská Nová Ves district.

The present results indicate that the prevalence of oocysts of *Eimeria* spp. reached 63.5 % which is comparable with the results obtained in Poland [5], Austria and the Czech Republic [1]. Nematodes *Passalurus ambiguus* reached 10.2 % prevalence which was almost double in comparison with Polish farms (5.8 %), but much lower in comparison with Italy [4] where the prevalence reached 82.3 %.

Trichuris leporis eggs were found only on one farm (2.2 %) which is comparable with the study conducted in Poland where the prevalence of *Trichuris leporis* reached 3.64 %. However, studies in Austria [5] and the Czech Republic [1] reported prevalences as high as 39.1 % and 31.4 %, respectively.

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FOLIA VETERINARIA, 59, 2: 77—80, 2015

DETECTION OF PROTOZOA FROM THE GENUS *CRYPTOSPORIDIUM* IN LEOPARD GECKOS BY PCR METHOD

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ABSTRACT

The present study focused on the prevalence of cryptosporidiosis which occurs in at least 57 reptile species. It is one of the most frequent causes of death of the young of leopard geckos (*Eublepharis macularius*), the presently very popular lizards. Cryptosporidiosis has been associated with a wasting syndrome in geckos and it is a serious emerging problem in reptile medicine because there is no specific treatment for this disease. We examined 35 leopard geckos from various breeders in Slovakia by the nested PCR using specific primers for amplification of the gene SSU rRNA. After electrophoretic visualisation, the positive samples were sent for sequencing and comparison of the obtained sequences with those from the GenBank which allowed us, not only to diagnose the presence of *Cryptosporidium* spp., but also the species of this parasite. The detection and identification of zoonotic species is important with regard to the fact that under certain conditions, geckos can become the source of infections which can cause cryptosporidiosis in humans.

Key words: *Cryptosporidium* spp.; lizards: PCR method; reptiles

INTRODUCTION

Cryptosporidia have become adapted to a wide range of hosts, particularly in vertebrates. Further, they have extended their host range to many amphibians, reptiles, birds and mammals. Cryptosporidiosis of reptiles has been studied most extensively in snakes in which it occurs in two forms, clinical and subclinical [4]. This disease may be fatal, particularly in immunosuppressed individuals [1]. The clinical form in snakes is always fatal but the subclinical one may not always be a real infection, as the majority of snakes feed on rodents which may be infected by *Cryptosporidium parvum* or *Cryptosporidium muris*. Oocysts of these species may pass directly through the gastrointestinal tract [2], [12]. Cryptosporidial infections in geckos are localised more frequently in the small intestine than in the stomach. In leopard geckos, it also involves the small intestine and manifests as hyperplastic lymphoplasmatic enteritis [11],

[7]. The gastrointestinal passage of cryptosporidia oocyst has been demonstrated experimentally. To make a correct diagnosis, it is important to distinguish pathogenic species taken up by the pray (pseudoparasites), from those eliminated as intact oocysts.

Currently, a number of different methods are used for the detection of cryptosporidia. However, many of them can diagnose cryptosporidia only at the level of the genus and do not allow one to distinguish individual species.

The polymerase chain reaction (PCR) enables the detection and subsequent sequencing of cryptosporidium species, generally on the basis of relatively long amplicons [10]. Studies based on PCR and rRNA sequence analysis have shown considerable genetic diversity of *Cryptosporidium* spp. isolated from reptiles living in captivity [6].

MATERIALS AND METHODS

Samples

Samples of faeces were collected from 35 leopard geckos which received no therapeutic preparations. The animals were from 2 to 6 years old and their gender was determined. They originated from different breeders from western, central, and eastern Slovakia. The body condition of the geckos ranged from physiological, through moderate emaciation, to pronounced cachexia. Samples were collected over three days due to the irregular elimination of cryptosporidium oocysts to the environment, and stored in Eppendorf tubes at 4 °C (refrigerator temperature) or stored at –18 °C.

Isolation of DNA

DNA-Sorb-B kit (manufactured by Ecoli, Bratislava) was used to isolate DNA from the samples of faeces according to the procedure provided by Ecoli, AmpliSens, Russia.

Nested PCR, gel electrophoresis

VKSS-F1 and VKSS-R1 primers were used in primary PCR and VKSS-F2 and VKSS-R2 primers in nested PCR. The secondary PCR product was visualised electrophoretically by means of UV radiation (312 nm) in 1.5 % agarose gel containing RED gel stain of size 245 bp.

Sequencing

The samples positive in gel electrophoresis were sent for sequencing. The sequences were compared with sequences SSU of rRNA locus of *Cryptosporidium* spp., which are at disposal in GenBank.

RESULTS AND DISCUSSION

The results of the examination of the leopard geckos for the presence of *Cryptosporidium* spp. are presented in Table 1.

Table 1. Number of positive leopard geckos in groups from different locations

Location	Number of animals in group	Negative	Positive
Košice	3	3	0
Prievidza	2	2	0
Košice	5	2	3
Zvolen	3	3	0
Košice	4	4	0
Košice	5	5	0
Zvolen	8	8	0
Košice	5	5	0

In the third group, consisting of 5 geckos from one breeder kept in 5 individual terrariums, three samples were positive upon sequencing. The agents causing the infections were *C. muris* (2) and *C. suis* (1). According to BLAST, there was a 99 % match for *C. muris* (KJ 469984.1) and 98 % for *C. suis* (AB 449822.1).

Our study investigated the prevalence of cryptosporidiosis in routine breeders of leopard geckos in Slovakia. Of the 35 samples examined, only 3 were positive for cryptosporidial infections, i. e. a prevalence of 8.6 %. Although they originated from one breeder they were not kept in one terrarium. The PCR method identified the species as *C. suis* and *C. muris*.

According to Pedraza-Díaz et al. [8], the majority of geckos are fed insects, which means that infections can be introduced by the ingested insects or the insects can serve as vectors of cryptosporidia. Additional sources of infection may be contaminated bedding or other infected animals kept in a household together with the geckos [9].

In an American study conducted by Deming et al. [3], the prevalence of cryptosporidiosis in geckos kept in captivity reached 9.8 %. The authors observed two groups of geckos, each consisting of 20 individuals. The study lasted two months and included repeated examination of the faeces and later all geckos were sacrificed and examined histopathologically. Only one, of the 40 geckos, was negative for infection caused by *Cryptosporidium* spp. The course of the infections were subclinical in the majority of the animals examined [3].

The genetic diversity of cryptosporidia in reptiles was analysed by the PCR method in the study conducted by Xiao et al. [13]. A total of 123 samples were analysed, of which, 48 snake samples, 24 lizard samples, and 3 tortoise samples were positive for *Cryptosporidium*. Nine different types of *Cryptosporidium* were found in snakes, lizards and turtles kept in captivity. The most common species were *C. serpentis* and a desert monitor genotype, both detected in snakes and lizards. Two additional species, *C. muris* and *C. parvum* mouse genotype, were found in some snakes and one lizard [5].

The low prevalence of cryptosporidia found in our study, which differs from results reported abroad, may be ascribed to an increased hygiene of keeping, enlightenment of breeders, and also to increased sensitivity and specificity of the PCR compared to other methods.

Despite the considerable efforts of the pharmaceutical industry to develop an effective antiparasitic preparation for the treatment of cryptosporidiosis, this infection and its clinical consequences is still a risk to public health.

CONCLUSIONS

Our study, based on the examination of samples from 35 leopard geckos from Slovakia showed that their body condition ranged from physiological to pronounced cachexia. The nested PCR method used for the examination of the samples revealed that the prevalence of cryptosporidium infection was only 8.6 %. This suggests good care of the animals, the enlightenment of the breeders, and appropriate measures at their purchase (for example quarantine).

Another important fact supporting the validity of our results, is the high sensitivity and specificity of the PCR. This method appears very suitable for the detection of *Cryptosporidium* spp., provided that all of the relevant instructional handling and manipulations of the samples are observed properly.

The use of more species-specific primers in the future will further simplify the determination of the extent of infections with cryptosporidia species, as a considerable portion of their genome is similar to that of other parasites.

One of the objectives of our study was to reveal the occurrence of zoonotic species, such as *C. muris*. Breeders can be infected not only by contaminated water or feed, but also during manipulation with geckos, cleaning of terrariums, or other exposures.

Because of the lack of effective specific therapy of cryptosporidiosis, it is very important to ensure that the breeders are well informed about the potential transfer of this infection and the ways of its prevention.

Routine methods of treatment may eliminate only a portion of cysts and oocysts. This also explains the increasing interest in the development of a molecular method capable of detecting the parasite with higher sensitivity and specificity.

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COMPARISON OF THE INCIDENCE OF ENDOPARASITES IN CERVIDS FROM THE INDUSTRIALLY ACTIVE ENVIRONMENT OF RUŽOMBEROK AND THE NATIONAL PARK TANAP

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ABSTRACT

Ružomberok is an important crossroads town in northern Slovakia and a centre of industrial activities. The limit values for dust particles of PM10 and PM2.5 in the air for a period of one year set by legislation have been exceeded in this area on a long term basis. The situation is aggravated by frequent inversions which prevent vertical dispersion of air contaminants. The aim of our study was to determine the prevalence of individual families of endoparasites in the faeces of red deer (*Cervus elaphus*) from an industrially active environment and compare it with the results of examination of deer faeces from National Tatra Park (TANAP) where any human activities damaging the natural environment have been banned since 1949. Examination of 211 samples of faeces from the industrially affected area of Ružomberok, showed that the prevalence of the family *Protostrongylidae* reached; almost 70 %, of *Trichostrongylidae* 63.5 %, of *Eimeriidae* 21.3 % and of the family *Capillariidae* 6.2 %. The prevalence of other families of endoparasites that were not found in the samples from TANAP was as follows: *Dictyocaulidae* 33.7 %; *Trichuridae* almost 10 %; *Strongylidae* 6.6 %; *Paramphistomidae* 5.2 %. In 154 samples collected in TANAP the highest prevalence was observed also for the family *Protostrongylidae* (51.3 %). The prevalence of the family *Trichostrongylidae* (11 %) fell considerably behind that in the Ružomberok area (63.5 %) and the prevalence of *Eimeriidae* (2.6 %) in samples from the protected area was more than 8-fold lower compared to the industrial area. The eggs of the family *Capillaridae* were found only in one sample (0.65 % prevalence). On average, in a hundred samples from TANAP, we found all together 600 larvae from the family *Protostrongylidae*, almost 18 oocysts from the family *Eimeriidae*, 59 *Trichostrongylidae* eggs and almost 2 eggs of *Capillaridae*. Hundred mean samples collected from the Ružomberok area contained almost 4300 larvae from the family *Protostrongylidae*, 331 oocysts, 346 trichostrongyle eggs and 10 capillaria eggs. The prevalence and number of parasitic stages in the samples from the Ružomberok area was significantly higher compared to samples from TANAP.

Key words: endoparasitoses; red deer (*Cervus elaphus*); Ružomberok; TANAP

INTRODUCTION

The rapid advancement of technology makes our life easier but, on the other hand, it disrupts the relationship between man and nature. The more intensive exploitation of natural resources goes hand in hand with negative influences on the environment and increased production of pollutants and wastes. The environment affects populations of all living organisms by its abiotic and anthropogenic factors, thus forcing animals to initiate adjustments to changes occurring in their ecosystems.

Ružomberok is one of the more important industrial centres of Slovakia. It is also one of the areas where the limit values for dust particles PM_{10} and $PM_{2.5}$ in the air for a period of one year set by relevant legislation have been exceeded on a long term basis. The main reason is that the climatic conditions of this region were not respected in the process of granting permission for activities affecting significantly air dustiness to continue. On the other hand, environmental protection has been implemented in the TANAP area since 1949, which became part of UNESCO World Network of Biosphere Reserves; since 1993 together with Polish Tatrzański Park Narodowy [3].

The red deer (*Cervus elaphus*) is a ruminant mammal from the family *Cervidae* which lives irregularly throughout Europe from floodplain to mountain forests. It is part of TANAP fauna and also occurs in forests in the Ružomberok area. With the exception of the oldest stag and during the rut, these animals form stable mother and calf or young stag groups. They feed on the plants, leaves and bark of the trees, various fruits and field crops. Deer can serve as an important bioindicator group of animals as they live in an outdoor environment. All their physiological functions are interconnected and can be affected by toxicants [1].

MATERIALS AND METHODS

Between September 2013 and December 2014, samples of faeces of red deer (211 samples from industrially active Ružomberok area and 154 samples from TANAP) were examined at the Institute for rearing and diseases of game and fish of the UVMP in Košice for the presence of parasites.

Fresh faeces were collected from the ground into plastic bags and refrigerated. All collected samples of faeces were examined for the presence of eggs, oocysts and larvae of parasites using qualitative concentration methods: flotation method with the use of Breza solution (of density 1.292 $g.cm^{-3}$); sedimentation method based on rapid sedimentation of heavy eggs of trematodes to the bottom of sedimentation vessel in water; larvoscopic method which uses positive thermo- and hydrotropism of lung strongyles [5].

RESULTS AND DISCUSSION

The total prevalence of endoparasites in the samples of faeces of cervids was 82.47 %. The prevalence in samples from Ružomberok area (98.10 %) differed significantly from that in samples collected in TANAP (61.04 %). The proportion of individual families also differed (Table 1).

The highest prevalence was observed for the class *Nematoda* (81.10 %). A significant difference was observed between deer from the Ružomberok area (97.16 %) and those from TANAP (59.09 %). Parasites of the class *Protozoa* were detected in 49 samples (13.43 %), the majority of them (45) originating from Ružomberka region (21.33 %). Their prevalence was 2–7-fold higher than the prevalence reported in studies conducted in neighbouring countries [6, 7]. Representatives of the class *Trematoda* were found only in 11 samples from the industrially active area (3.01 %). No parasites of the class *Cestoda* were found in the samples.

A broader spectrum of parasitofauna was detected in samples from the industrially active environment, namely representatives of 8 families, while in samples from TANAP only endoparasites from 4 families were present.

In both locations, lung worms from the family *Protostrongylidae* were most prevalent which correlates with results obtained in Poland [2] and Austria [4]. *Eimeriidae* oocysts, *Capillariidae* and *Trichostrongylidae* eggs and the larvae of lung worms from the family *Protostrongylidae* were found in much higher prevalence in samples from the Ružomberok area compared to the samples from TANAP.

One sample from the protected region contained on average 6 larvae from the family *Protostrongylidae* which was 7-fold less compared to the Ružomberok region. On average, in a hundred samples from TANAP, we found almost 18 oocysts from the family *Eimeriidae*, 59 *Trichostrongylidae* eggs and almost 2 eggs of *Capillaridae*. Hundred mean samples collected from Ružomberok area contained 331 oocysts, 346 trichostrongyle eggs and 10 capillaria eggs (Fig. 1).

Table 1. Prevalence of endoparasites in faeces of red deer

		Ružomberok		TANAP		Total	
		N/n	P [%]	N/n	P [%]	N/n	P [%]
Protozoa	*Eimeriidae*	211/45	21.33	154/4**	2.60	365/49	13.43
Trematoda	*Paramphistomidae*	211/11	5.21	154/0	0	365/11	3.01
Nematoda	*Trichostrongylidae*	211/134	63.51	154/17**	11.04	365/151	41.37
	Capillariidae	211/13	6.16	154/1*	0.65	365/14	3.84
	Strongylidae	211/14	6.64	154/0	0	365/14	3.84
	Trichuridae	211/21	9.95	154/0	0	365/21	5.75
	Dictyocaulidae	211/71	33.65	154/0	0	365/71	19.45
	Protostrongylidae	211/147	69.67	154/79**	51.30	365/226	61.92

N — number of examined samples of faeces; n — number of positive samples; P — prevalence; * — $P < 0.05$; ** — $P < 0.001$

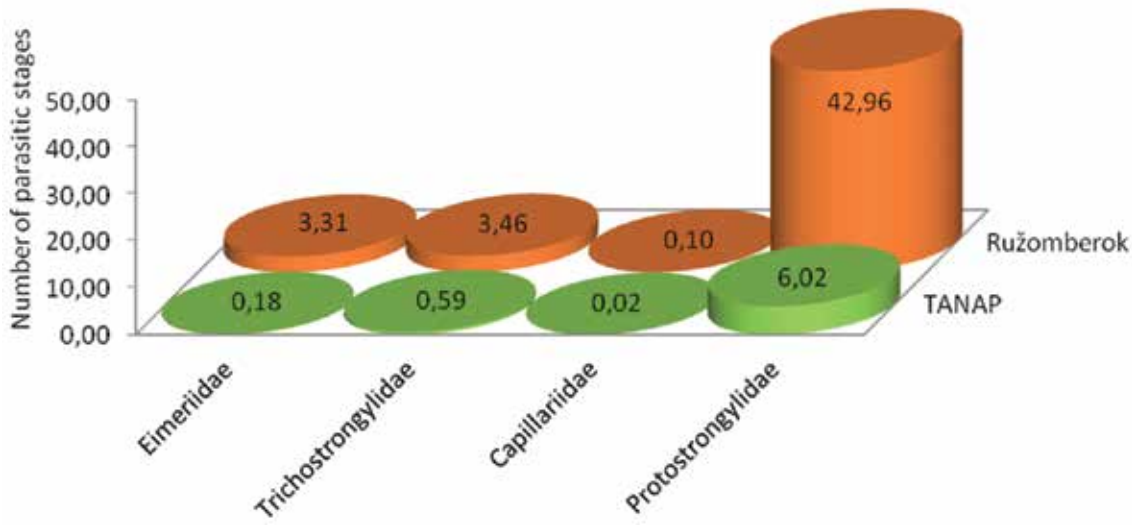

Fig. 1. Mean number of parasitic stages in samples of faeces collected in the Ružomberok and TANAP regions

CONCLUSIONS

The parasitological examination of samples of faeces revealed the parasitofauna of red deer from the industrially active Ružomberok region and from the protected TANAP region. Our results demonstrated that parasitoses of free living animals can present a problem regarding overall health of game and potential transfer of parasites to farm animals as they frequently share the same meadows and pastures. Industrial contaminants probably affect both the endoparasite spectrum and intensity of invasion as indicated by comparison of results from the industrially active region with samples from TANAP which showed considerably lower prevalences and number of detected parasitic stages of the investigated families of endoparasites.

ACKNOWLEDGEMENT

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FOLIA VETERINARIA, 59, 2: 85—87, 2015

THE INFLUENCE OF T-2 TOXIN AND QUERCETIN ON THE STRUCTURE OF THE RABBIT LIVER

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ABSTRACT

In this study we observed morphological changes in the liver of rabbits which were administrated T-2 toxin and antioxidant quercetin. Adult rabbits were divided into two experimental groups (E1 and E2) and a control group. Quercetin was administered intramuscularly to animals in E2 group at a dose of 1000 µg.kg^{-1} for 90 days, 3 times per week. T2 toxin was administered to groups E1 and E2 at a dose of 0.08 mg.kg^{-1} b. w. 72 hours before slaughter. In E1 group we observed inflammation in the portal spaces. The ultrastructure of hepatocytes was not altered markedly. However, pronounced changes were observed in the bile canaliculi. They were dilated with reduced microvilli. Changes in E2 group were moderate in comparison with those observed in E1 group. The examination by light microscopy revealed only slight inflammation in the portal spaces. The electron microscopic study detected increased amount of lipid droplets but the bile canaliculi were unchanged.

Key words: liver; quercetin; rabbit; structure; T-2 toxin

INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi. They contaminate animal feed, food and food products. T-2 toxin is one of the most toxic mycotoxins of the type from Atrichothecenes, which is produced mainly by Fusarium species. The effect of this mycotoxin on humans and other animals differs. Severe poisoning results in; prostration, weakness, ataxia, collapse, reduced cardiac output, shock and death [8]. T-2 toxin is an inhibitor of DNA and RNA synthesis and synthesis of proteins in several cellular systems. It acts as an immunosuppressive agent, induces lesions in hematopoietic, lymphatic and digestive systems [5]. The toxic effects of T-2 toxin were studied in experimental animals (poultry, cattle, sheep and pigs) all of which appeared sensitive to this mycotoxin [3].

In the present study, we observed structural and ultrastructural changes in the liver of rabbits which were administrated T-2 toxin and the antioxidant quercetin.

MATERIALS AND METHODS

Animals and diets

Adult rabbits of meat line M91(n = 18) were used in the experiment. Animals were divided into two experimental groups (E1 and E2) and a control group, balancing the rabbits for age (150 days) and body weight (4 ± 0.5 kg). Rabbits in the experimental group E2 received quercetin in an injectable form at a dose of 1000 μg.kg^{-1} for 90 days, 3 times per week. T-2 toxin (Romer Labs Division Holding GmbH, Tulln, Austria) was administered (E1 and E2) at a dose of 0.08 mg per kg body weight 72 hours before slaughter. In this study, institutional and national guidelines for the care and use of animals were observed, and all experimental procedures involving animals were approved by the State Veterinary and Food Institute of the Slovak Republic, No. 3398/11-221/3 [7].

Histological samples of the liver for light microscopy were processed by a common histological technique. They were fixed in 4 % neutral formaldehyde and embedded in paraffin. Then 7—12 μm thick slides were stained with haematoxylin and eosin and photographed under a Jenamed light microscope.

The liver samples intended for transmission electron microscopy were fixed in 3 % glutaraldehyde, postfixed in 1 % OsO_4 (both in a cacodylate buffer pH 7.2—7.4), dehydrated in acetone and embedded in Durcupan ACM (Fluka). The ultrathin sections were cut on a Tesla BS 490 ultramicrotome, stained with uranyl acetate and lead citrate and evaluated under a Tesla BS 500 transmission electron microscope.

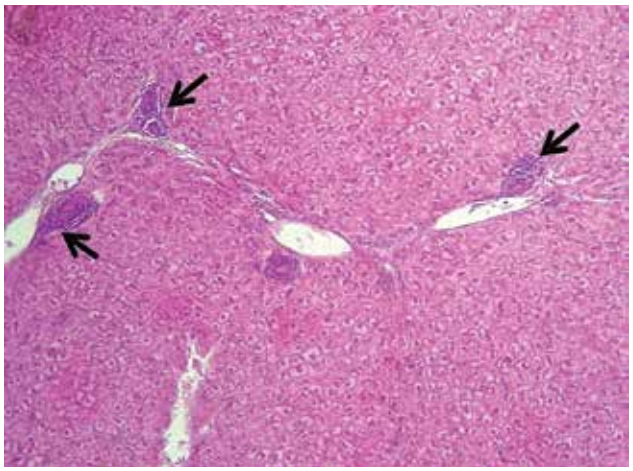

Fig. 1. Light microscopy of E1group
Liver lobuli separated by connective tissue septa. Inflammation in portal spaces (arrows). Magn. ×200

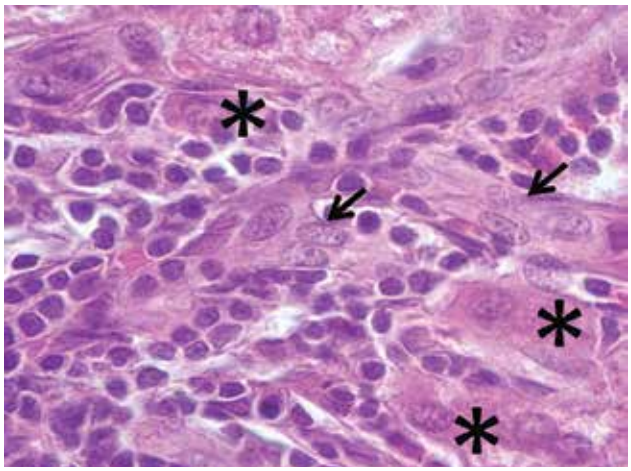

Fig. 2. Light microscopy of E1group
Portal space infiltrated with inflammatory cells. Oval cells (arrows) and necrotizing hepatocytes (asterisk). Magn. ×1,000

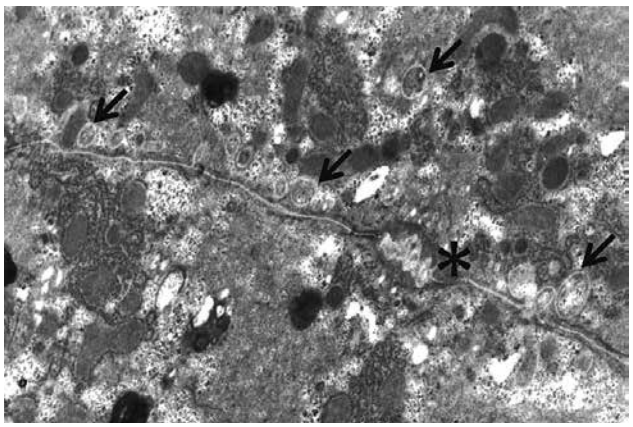

Fig. 3. Electron microscopy of E1group
Changes in the bile canaliculus with reduced microvilli (asterisk). Vesicles in hepatocytes (arrows). Magn. ×12,857

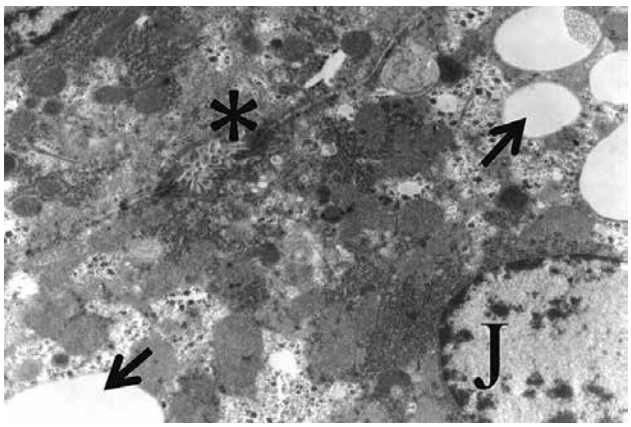

Fig. 4. Electron microscopy of E2 group
Hepatocytes with euchromatic nucleus (J), unchanged bile canaliculus (asterisk), and lipid droplets (arrows). Magn. ×7,000

RESULTS

In the control group, the liver displayed a normal structure and ultrastructure. In the E1 group, structural changes were observed in the portal spaces. They were oedematous and contained inflammatory cells. Individual portal spaces were interconnected with thin connective tissue septa. Oval cells were observed in the portal spaces and in the connective tissue septa. They had light-staining cytoplasm with oval nuclei. Necrotizing hepatocytes were observed at the periphery of the portal spaces. Their nuclei had condensed chromatin and their cytoplasm was markedly acidophilic. The ultrastructure of hepatocytes was not altered markedly. Their cytoplasm contained only small amount of lipid droplets. More pronounced changes were observed in the bile canaliculi. They were dilated with reduced microvilli. Vesicles were clearly visible at the periphery of the bile canaliculi (Fig. 1, 2, 3).

Changes in the E2 group were moderate in comparison with changes observed in the E1 group. Only slight inflammation in the portal spaces was observed. The number of oval cells and amount of connective tissue was decreased considerably. The ultrastructure of the organelles in the hepatocytes was comparable with that in the control group. We observed an increased amount of lipid droplets in the cytoplasm but the bile canaliculi were unchanged (Fig. 4).

DISCUSSION AND CONCLUSION

In this study, we investigated structural changes in rabbit livers exposed to T-2 toxin. The changes described above resembled those observed by other authors. Hepatotoxic and nephrotoxic effects were observed in mice fed wheat contaminated with T-2 toxin. Examination of the liver by light microscopy revealed enlargement of hepatocytes and infiltration of mononuclear cells in and around congested blood vessels [1]. Daraeia et al. [2] observed a significant increase in alanine aminotransferase and aspartate aminotransferase activity and increased lipid peroxidation in the rat liver. However, in our experiment the negative effects of T-2 toxin (inflammation and necrotizing cells) were suppressed by flavonoid quercetin. Flavonoids are a group of naturally occurring compounds widely distributed as secondary metabolites in the plant kingdom. They have interesting clinical properties, such as anti-inflammatory, anti-allergic, antiviral, antibacterial, and antitumour activities [6]. Quercetin prevents oxidative injury and cell death by several mechanisms [4], which were investigated in many studies. Petruška et al. [7] observed changes in the content of bilirubin and albumins in rabbits which were exposed to different concentrations of quercetin and T-2 toxin.

Our observations demonstrated that T-2 toxin caused structural and ultrastructural changes in the rabbit liver. However, quercetin had a hepatoprotective effect on liver injuries induced by T-2 toxin, which could be associated with its antioxidant potential.

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INTESTINAL DIGESTIBILITY OF RUMEN-UNDEGRADABLE PROTEINS IN RUMINANT FEEDSTUFFS

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ABSTRACT

During lactation the content of nitrogen compounds (NC) is the most important parameter affecting the production of milk proteins. An *in situ* method is suitable for determination of NC rumen degradability in housed fistulated animals. A modified three-step *in vitro* method employing Daisy II incubator allows one to determine intestinal digestibility of NC under laboratory conditions. The use of these methods and the results obtained, enable the correct and effective control of total milk production and the correct formulation and balancing of rations for high-yield dairy cows in their peak of lactation. This study investigated the intestinal digestibility of proteins in selected bulk and concentrate feeds for ruminants.

Key words: Daisy incubator; intestinal digestibility; rumen; rumen-undegradable proteins

INTRODUCTION

The protein nutrition of high-yield dairy cows, from the point of view of, nitrogen compounds (NC), proportion and rate of rumen degradation, and the amount of rumen-undegradable proteins (RUP), significantly affects the productive, health and reproductive parameters of the herd. The lower usability of nitrogen (approximately at the level of 25 % in dairy cows) is the limiting factor of protein nutrition [5]. The balancing of the diet during its formulation is very important in order to ensure the sufficient supply of proteins in the form of rumen-degradable and undegradable proteins. RUP as the second main source of metabolisable amino acids (AA), affecting the proportion of digestible and absorbed proteins and AA at the small intestinal level, are limited particularly by their intestinal digestibility depending on the type and way of feed treatment. The intestinal digestibilities of RUP and their amino acids are variable factors in the evaluation of protein nutrition of high-yield dairy cows [4]. To optimally balance AA in rations, one must have sufficient knowledge of the ruminal degradability and intestinal digestibility of proteins

and individual AA in the feed. The assessment of the AA digestible in the small intestine (AADI) was introduced within the evaluation of Proteins Digestible in the Intestine (PDI = PDIE) (INRA 2001) for nine essential amino acids.

The aim of this study was to determine the intestinal digestibility of RUP in selected feedstuffs analysed for their content of nutrients and to evaluate the suitability of the system PDI for assessment of digestibility of AA in the small intestine (AADIˇ) as a part of the above system.

MATERIALS AND METHODS

The analysis and evaluation of nutrients in protein feedstuffs was carried out by conventional methods [2]. Rumen degradability of NC was determined by the *in situ* method [6] in fistulated dairy cows. The concentrate feed was incubated in the rumen for 12 h and bulk feed for 16 h. The intestinal digestibility of RUP was determined by a modified three-step method [3]. This modified *in situ*/*in vitro* method uses the ANKOM Daisy incubator and solutions containing pepsin and pancreatin, the main enzymes participating in the digestion of proteins in the small intestine. The content of digestible AA was calculated by means of regression formulas according to AADI (INRA 2001, 2007) within the PDI system. The rumen degradability, proportion of individual AAs, and intestinal digestibility was calculated for each AA.

RESULTS AND DISCUSSION

NC content

The analysis of nutrients and the calculation of the nutrient value of the analysed concentrate feeds showed that the mean NC content for the group of concentrate feeds was 387.8 ± 143.6 g.kg^{-1} dry matter (DM), ranging from 205.7 g.kg^{-1} DM (Amygold) to 748.39 g.kg^{-1} DM (maize gluten — Albumex). In alfalfa bulk feeds, the mean NC was 182.6 ± 39.94 g.kg^{-1} (120.2 g.kg^{-1} – 247.2 g.kg^{-1} DM). The NC content in feed is the most important parameter in relation to the production of milk proteins, especially at the peak of lactation. The highest NC content was determined for maize gluten which is highly resistant to rumen fermentation and the lowest for Amygold, which is subjected to rumen fermentation due to low proportion of starch and high proportion of fibres. The proportion of NC in the bulk feed is most affected by the vegetation phase at harvest, reflected in an increase in neutral-detergent fibres and a decrease in NC.

Rumen degradability of proteins

Our analyses showed the influence of the type and treatment of feed on rumen degradability of proteins and the time of the incubation of the feed in the rumen. The most common treatment is thermal treatment based on various processes. The mean value for the entire group of concentrate feeds was 51.5 ± 16.64 % and for alfalfa bulk feeds 72.3 ± 10.31 %. Thermal treatment is the dominant factor affecting the level of rumen degradability of the analysed protein feeds and side-products from the food industry used for feeding. While thermally treated feeds are resistant to rumen digestion and almost pass unchanged into the small intestine where they are digested, the untreated ones are degraded in the rumen and pass to the small intestine in small proportions. The most important factors affecting the degradation of proteins in the feed include; their solubility and the prevailing microbial population, depending on the type and proportion, passage rate and rumen pH [10].

Intestinal digestibility of RUP

The intestinal digestibility of RUP determined by the three-step method ranged between 55.39 and 96.59 % for concentrate feeds, with a mean value of 81.0 ± 12.4 %, and reached the mean level of 66.2 ± 5.27 % in alfalfa bulk feeds.

Assessment of intestinal digestibility of AA (AADI) as a part of the system PDI

The assessed digestibility of methionine in the small intestine according to calculations in the system AADI in feed was in the range of 1.4—1.7 g.100 g^{-1} PDIE. The calculated digestibility of lysine in the small intestine was in the range 2.8—7.2 g.100 g^{-1} PDIE. The resistance to rumen degradation increased in heat treated feeds so they pass almost unchanged into the small intestine, where the digestibility of proteins may vary in a range of 70—90 %, in comparison with untreated feeds the intestinal digestibility of which ranges between 60 and 70 %. The variations in intestinal digestibility depend on the way of the treatment and processing of feed. The factors affecting the digestibility of proteins in the small intestine include: source of proteins, their composition and structure, processing of feed (e. g.

heat treatment) and the presence of anti-nutritional factors (e.g. inhibitors of trypsin) [7]. The values of the intestinal digestibility of RUP and the amount of absorbable AA in the feed are suitable indicators for the selection of protein components and help to develop models for optimization of AA in dairy cow nutrition.

In conclusion, it should be stressed that the milk yield and total production of milk proteins by high-yield cows is limited by the insufficient intake of some AA. The modified three-step method for the evaluation of the intestinal digestibility of RUP, helps to control the quality of protein nutrition in ruminants. The analysis and evaluation of the content and usability of proteins and AA in feed and their absorbability serve as a suitable tool when deciding about the incorporation of RUP in rations for high-yield dairy cows. The assessment of the intestinal digestibility of RUP and the digestibility of AA (AADI) is important for the development of systems and models intended for optimization of amino acid nutrition of dairy cows.

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TREATMENT OF SPONTANEOUS CHRONIC CORNEAL EPITHELIAL DEFECTS (SCCEDS) IN DOGS WITH DIAMOND BURR DEBRIDEMENT

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ABSTRACT

Dogs with corneal epithelial defects are being presented more frequently in small animal practices. The Diamond Burr Debridement (DBD), as a preliminarily investigative tool, is proving to be also an extremely effective treatment, therefore providing a valuable method in ophthalmology today, being both uncomplicated to perform and relatively inexpensive. The objective of this study was to evaluate the outcome of five SCCEDS (spontaneous chronic corneal epithelial defects), in five individual patients, before and after treatment with DBD. In this study, no subsequent Bandage Contact Lenses (BCL) were placed in any of the subjects. Alternatively, autograft techniques were used with nictitans flaps were performed in all, but one, of the cases as a supportive healing measure. Five eyes of five dogs were studied after being presented to a single private practice in Kosice, the Slovak Republic. Cases were eligible for inclusion into the study if they were diagnosed with SCCEDS by a veterinary ophthalmologist and the decision was to treat with DBD. DBD was performed using a battery powered, handheld motorized burr. In each case, the time taken for complete resolution of the defect was recorded along with any complication or subsequent treatment with DBD. The results suggest that DBD is a safe and effective method of treatment for canine spontaneous chronic corneal epithelial defects.

Key words: cornea; epithelial defect; diamond burr debridement

INTRODUCTION

Spontaneous chronic corneal epithelial defects (SCCEDS), with no apparent underlying cause, are observed frequently in small animal veterinary practices, and the resulting corneal ulcers are the most common disease of the cornea [14], [28]. Regardless of the initial cause, all ulcerative defects of the cornea have the potential to progress into severe endophthalmitis if left untreated. Endophthalmitis is an inflammatory complication affecting the internal structures of the eye with potential blindness or complete

loss of the eye itself. Therefore, this study, and current parallel studies, are vital in exploring and, potentially, supporting diamond burr debridement (DBD) as a new method of treatment for SCCEDS [3], [7], [19].

The main clinical signs recorded in the development of SCCEDS include; ocular discharge, blepherospasm, hyperaemia of the conjunctiva, and epiphora [15], [29]. The presentation of some or all of these clinical signs can be useful when diagnosing the severity of the defect, along with a full history obtained from the client or previous practice records [20], [22].

Depending on the severity and location of the corneal defect, the current methods of treatment used are topical antibiotics and other ocular drop medications such as growth factors [1], [8], [9]. The surgical procedures, including keratotomy/keratectomy, *tarsorrhaph*y or debridement, will also be discussed in this comparative study of DBD with other current treatment methods [2], [4]. The main aim of this study is to link what is already known about SCCEDS to their healing with DBD, in five clinical cases of dogs in Slovakia, and to establish the efficacy of DBD as a treatment method.

MATERIALS AND METHODS

The patients for this study were selected after a full ophthalmic examination and a diagnosis of SCCEDS with no apparent underlying cause was established. The breeds, ages and sexes of the dogs were recorded and along with the follow up information were collected and evaluated statistically. A full ocular examination included a hands off examination and a hands on exam using an ophthalmoscope and slit lamp. One percent sodium fluorescein ophthalmic dye was used to detect the corneal defects. In all cases, the procedure was carried out using a handheld, battery operated diamond burr unit (Alger brush II) with a medium round head. The patients ocular surface was prepared with 0.5 % iodine disinfectant solution and a 0.4 % topical anaesthetic solution was administered before the debridement process. The patients were given sedation premedication and maintenance anaesthesia throughout the procedure. The eye palpebra were fixed gently with eye retractor forceps or physical manipulation. The burr was moved over the defect in multiple even movements removing all non-adherent or unstable epithelium in the surrounding area.

RESULTS

The mean age of the dogs was 6.8 years old with a standard deviation of ±3.8 and a range of 1—11. The breeds represented were; a mixed breed, a Shi Tzu, a Weimaraner and two English bull dogs. None of these patients had any other concurrent ocular disease. The diameter of the lesions seen, upon initial clinical examination, ranged from 3 mm to 5 mm. Prior to DBD treatment, the median duration of clinical signs was 3 days with a range of 1—9 days. The most common presenting signs noticed by the owners were; blepherospasm, excessive lacrimation and ocular discharge consistent with epiphora. The corneal oedema ranged from slight to moderate and the neovascularisation ranged from mild to severe. Upon clinical examination, hyperaemia of the conjunctiva, miosis, and redundant, non adherent epithelial margins surrounding the defect were noted. The median time to first recheck examination after the first DBD treatment was 10 days with a range of 7—12. A total of 4/5 (80 %) of the cases were resolved by the first recheck examination. Only one case was subjected to a second recheck examination, with additional surgical intervention required in this particular individual case. None of the dogs required a second DBD treatment. Four of five cases had a nictitans flap performed to support the corneal healing. The success rate of the nictitans flaps was 100 % with all sutures being removed at first recheck examination. Clinical signs present at the final recheck examination included corneal haze in 2/5 cases and mild vascularisation in 1/5 cases. The only significant complication encountered in this study was the development of a desmatocele in a 1 year old male bulldog. A deeper corneal erosion with 80% corneal oedema and deep vascularisation occurred. Further intervention with conjunctival pedicle graft and topical antibiotics successfully resolved the issue and the SCCED healed within 3 months with minimal resulting corneal scar tissue.

DISCUSSION

This study represents one of the first reports of the use of diamond burr debridement. Gosling et al, [14] published the first report of DBD treatment in conjunction with the placement of a bandage contact lens. The decision not to place a bandage contact lens in the present study was

based on the scientific support of nictitans flaps aiding in the corneal healing of ulcers [5], [6]. Combined with DBD we wanted to see if this combination would affect the rate of healing in a positive or similar way to BCL placement [10].

It is well reported that surgical intervention of SCCEDS produces the highest success rates of complete resolution [31].

The mean age of dogs included in the current study was 6.8 years old with a range of 1—11, indicating a predilection of SCCEDS in older animals [11], [26]. This correlates with previous studies presenting results mainly found in typically middle aged dogs published by Gosling et al, [14], and Garcia et al [12], in which the corresponding mean ages were 8.9 and 9.5 years old respectively. Stanley et al [27] reported on the nine most prevalent breeds presenting with SCCEDS. Other studies have concluded that some breeds are over-represented and therefore more likely to be predisposed to SCCEDS including English Bull terriers, Boxers, Corgis and mixed breeds [17], [30].

The clinical symptoms of SCCEDS include; redundant, nonadherent epithelial margins in conjunction with varying degrees of corneal vascularisation and ocular pain. Ocular pain was commonly manifested as epiphora and blepherospasm within our study. The vascularisation of the superficial cornea was a common denominator in all of the cases included in our study, as was the presence of nonadherent epithelium associated with the defects reported by others [13], [16].

The exact mechanism of the diamond burr debridement method and its influence on the healing of the cornea is not fully understood [14], [18]. Previous studies have suggested that DBD creates micro erosions of the basement membrane which alters the corneal topography and improves the epithelial adhesion complexes that form in normal corneal healing. It is also thought that DBD causes the expression of proteins by the surrounding extracellular matrix. These proteins may contribute to fibrosis and an increased strength of epithelial adhesions. There is reason to believe that by exposing the normal basement membrane adjacent to the corneal defect, this also positively influences epithelial adhesion [21]. Therefore, during treatment with DBD, usually the area of debridement significantly exceeds that of the initial defect and in some cases it can be indicated to remove the entire surface of the cornea. In this study there was no significant difference between healing time and the size of the debrided area although it is one factor of DBD that may be explored further in future studies as suggested by Trbolova [28].

The results of this study demonstrate that DBD is an effective treatment for canine SCCEDS. The overall healing after a single treatment was 80% and no case included in this study required a further debridement procedure. Morgan and Abrams [21] reported the mean healing times for punctate keratotomy (25.1 days), third eyelid flap (17.9 days) and bandage contact lens (24.8 days). In a study by Champagne and Munger [10], healing time for multiple punctate keratotomy was 12.25 days. The treatment with other pharmaceutical procedures, for example, Substance P and Insulin-like growth factor, took much longer with an average of 2.5 weeks [23].

One advantage of DBD is that it can be repeated and it causes minimal post treatment scarring. One of the main limitations in this and other studies regarding DBD is the lack of control on the time taken for owners to present their dog for rechecks. All of the owners included in the study were required to bring their animals back for a recheck in 10 days for removal of nictitans flap [24], [28]. However, the variance in time to first recheck is solely reliant on owner compliance. It is, therefore, possible that the healing times presented in this study may not accurately reflect the true healing time. Similarly, it is vital that DBD is carried out before any secondary infection or complications arise in the case of SCCEDS. The first recognition of the presence of clinical signs of SCCEDS by owners varies a great deal and thus, their decision to bring their dog to the veterinary doctor is not consistent; some catch the SCCEDS early and some later.

CONCLUSIONS

On the basis of the results of this study, a number of factors relating to SCCEDS and the treatment with DBD can be surmised. English bull dogs may be a breed predisposed to develop SCCEDS. Corneal ulcers occur in middle aged dogs. The length of time for DBD to successfully heal the erosion is approximately 12 days. It can be hypothesised that DBD treatment for SCCEDS because of its low cost and high effectivity will become widely used in the coming years.

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ULTRASONOGRAPHIC EXAMINATION OF THE THORACIC REGION IN HORSES

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ABSTRACT

Ultrasonographic (USG) examination of the thoracic region (TR) allows one to carry out a dynamic assessment of physiological processes, particularly in the lung region and cardiac compartment throughout the entire cardiac cycle. Physiological reference values serve as a basis for the identification of pathologically altered organs and parts of organ systems, and any dynamic deviations. TR sonography enables one to carry out; biometric measurements, investigate contractility, compliance, volumes of the compartments rates of body fluids flow, and thus, also derive the pressure in the fluid-filled compartments. In addition, the USG output reflects the effects of medicament therapy on TR organs.

Key words: echocardiography; horse; thoracic ultrasonography

INTRODUCTION

The technique of using an X-ray examination for the thoracic region, presents some problems. The quality of radiographs obtained by X-ray equipment commonly used in an equine practice is low. The superimposition of the thoracic cavity organs precludes a correct diagnosis, because many pulmonary diseases result in the same X-ray image and it is very difficult to determine whether the left or the right lung is affected. Ultrasonographic (USG) examination allows one to; obtain an image of almost the entire thoracic region (TR), easily identify which lung is affected, and locate the lesions with accuracy [8]. It is also possible to examine and locate free fluid in the thoracic region [3]. Information about cardiac function can be obtained only by echocardiography. This technique is important for distinguishing physiological and pathological murmurs and can also detect abnormalities which are not audible upon heart auscultation [5]. USG is an irreplaceable component of a complex examination of sport horses [8]. The M-mode echocardiography allows one to measure the; mean diameter of aorta and valves, diameters of the left and right ven-

tricles and left atrial appendage, and the thickness of the interventricular (IVS) septum, as well as the posterior wall of the left ventricle [7].

MATERIALS AND METHODS

Our study was carried out on 8 horses: five Slovak warmblood (SW), one English thoroughbred (A1/1), and two Haflings. The horses were 5—15 years old; 5 were geldings and 3 were mares. The body weight of the horses was as follows: (1) 755 kg; (2) 678 kg; (3) 515 kg; (4) 693 kg; (5) 592 kg; (6) 641 kg; (7) 385 kg; and (8) 567 kg.

M-mode echocardiographic measurements

The ultrasonography measurements were made using a SonoScape SH apparatus, equipped with a microconvex transducer (2—4 MHz), and a linear transducer (6—10 MHz). Individual acoustic windows or approaches for ultrasonography of the internal structures, were defined according to Reef and Virginia [8]. We identify the best location for distinguishing the parenchymatous and tubular organs.

The horses were examined from the short parasternal axis view and the M-mode cursor was positioned across the left ventricle. The measurements at the end of diastole were obtained on the basis of the Q wave of the electrocardiogram image, which was displayed simultaneously during the echocardiographic examination. The measurements at the end of systole were carried out during the widest interventricular septal thickness. The mean left ventricular internal diameter at the end of diastole (LVIDd), mean left ventricular internal diameter at the end of systole (LVIDs), IVS at the end of diastole (IVSd), IVS at the end of systole (IVSs), left ventricular posterior (free) wall thickness at the end of diastole (LVPWd or LVFWd), as well as, the LVFW at the end of systole (LVFWs), were measured from the same echocardiograph [2].

Pharmacological modulation of the heart function during sedation

The 8 clinically healthy horses included in this study were examined before and after sedation. The medication was administered 5—10 minutes prior to the USG examination. Xylazin (Rometar 20 mg.ml^{-1}) at 0.6 mg.kg^{-1} b. w. i. v.: (1) 22.7 ml; (2) 20.3 ml; (3) 15.5 ml; (4) 20.8 ml; (5) 17.8 ml; (6) 19.2 ml; (7) 14.5 ml; and (8) 17 ml. Detomidine (Domosedan inj.) 10—20 μg.kg^{-1} b. w. i. v. in combination with butorphanol (Butomidor) 0.02 mg.kg^{-1} b. w. i.v. was administered at a dose of 0.5 ml detomidine and 1 ml butorphanol to all horses.

Functional parameters of the myocardial wall and mass of the left ventricle

Calculation of fractional shortening:

$$(\text{FS }\%) = \frac{\text{LVIDd} - \text{LVIDs}}{\text{LVIDs}} \times 100$$

Calculation of ejection fraction:

$$(\text{EF }\%) = \frac{\text{LVID} - \text{LVIDs}^3}{\text{LVIDd}^3}$$

Calculation of left ventricle mass:

$$\text{LVmass} = 1.04 \times \{(\text{LVIDd} + \text{LVFWd} + \text{IVSd})^3 - \text{LVIDd}^3\} - 13.6$$

The results obtained in this study were processed statistically using the Student *t*-test and $P < 0.05$ as the level of significance.

RESULTS

Window for cardiac USG examination

The heart was examined between the 3rd and 5th intercostal spaces (ICS) on both sides of the thorax, approximately in the centre, between the level of the elbow and shoulder.

The USG cardiac images are presented in Figures 1 to 4.

Pharmacological modulation of the cardiac function during echocardiographic examination

After administration of xylazin or detomidine with butorphanol, we observed a significant increase ($P < 0.01$) in LVIDd and LVIDs and a significant decrease ($P < 0.01$) in IVSs and LVFWs, compared to non-sedated patients. Other echocardiographic parameters of the left ventricle showed no significant changes. The values of FS and EF decreased significantly ($P < 0.01$) after the administration of xylazin or detomidine with butorphanol, but the LV mass showed no significant change.

Table 1. Echocardiographic parameters in non-sedated patients

	Non-sedated patients								Mean	σ
	1	2	3	4	5	6	7	8		
LVIDd [cm]	11.5	11.4	11.35	11.28	11.48	11.89	11.17	11.56	11.454	0.20
LVIDs [cm]	7.75	7.6	7.38	7.19	7.82	7.95	7.22	7.84	7.5938	0.28
IVSd [cm]	2.96	2.9	2.83	2.65	3.18	3.25	2.89	3.02	2.96	0.26
IVSs [cm]	4.3	4.36	4.11	4.07	4.41	4.79	3.89	4.5	4.3038	0.26
LVFWd [cm]	2.44	2.32	2.22	2.19	2.38	2.4	2.17	2.39	2.3138	0.10
LVFWs [cm]	3.92	3.85	3.78	3.69	3.97	4.12	3.4	3.98	3.8388	0.21
Number of pulses	30	32	26	38	34	32	28	30	31.25	3.46

σ — standard deviation

Table 2. Echocardiographic parameters in patients sedated with xylazin

	Patients sedated with xylazin								Mean	σ
	1	2	3	4	5	6	7	8		
LVIDd [cm]	11.76	11.69	11.74	11.72	11.85	12.33	11.53	11.99	11.826	0.23
LVIDs [cm]	8.61	8.52	8.24	8.11	8.79	8.78	8.07	8.6	8.465	0.27
IVSd [cm]	2.85	2.8	2.78	2.55	3.12	3.13	2.74	2.98	2.8688	0.19
IVSs [cm]	4.02	3.94	3.74	3.62	4.03	4.16	3.43	4.07	3.8763	0.24
LVFWd [cm]	2.36	2.25	2.15	2.04	2.19	2.33	2.07	2.24	2.2038	0.11
LVFWs [cm]	3.64	3.44	3.36	3.28	3.59	3.78	3.01	3.57	3.4588	0.23
Number of pulses	26	26	20	30	24	22	20	24	24	3.16

σ — standard deviation

Table 3. Echocardiographic parameters in horses sedated with detomidine and butorphanol

	Patients sedated with detomidine and butorphanol								Mean	σ
	1	2	3	4	5	6	7	8		
LVIDd (cm)	11.88	11.85	11.7	11.64	11.95	12.1	11.57	11.95	11.83	0.17
LVIDs (cm)	8.58	8.52	8.3	8.22	8.93	9.01	8.85	8.47	8.61	0.27
IVSd (cm)	2.87	2.79	2.81	2.58	3.09	3.16	2.77	2.9	2.8713	0.17
IVSs (cm)	4.13	4.06	3.95	3.74	3.87	4.42	3.45	4.08	3.9625	0.27
LVFWd (cm)	2.37	2.26	2.14	2.1	2.25	2.27	2.09	2.28	2.22	0.09
LVFWs (cm)	3.47	3.39	3.51	3.38	3.65	3.62	3.05	3.49	3.445	0.17
Number of pulses	24	28	22	30	24	22	20	28	24.75	3.31

σ — standard deviation

Table 4. Echocardiographic parameters in non-sedated patients

	Non-sedated patients								Mean	σ
	1	2	3	4	5	6	7	8		
FS [%]	32.6	33.3	35	36.3	31.9	33.1	35.4	32.2	33.725	1.52
EF [%]	69.4	70.4	72.5	74.1	68.4	70.1	73	68.8	70.838	1.97
LV mass [g]	3424.6	3220	3197.3	2850.1	3558.6	3850.3	2983.2	3462.3	3318.3	284.52

σ — standard deviation

Table 5. Echocardiographic parameters in patients sedated with xylazin

	Patients sedated with xylazin								Mean	σ
	1	2	3	4	5	6	7	8		
FS [%]	26.8	27.1	29.8	30.8	25.8	28.8	30	28.2	28.413	1.64
EF [%]	60.8	61.3	65.4	66.9	59.2	63.9	65.7	63.1	63.288	2.51
LV mass [g]	3377.5	3203.6	3121.3	2824.4	3511	3892.4	2929.5	3495	3294.3	324.79

σ — standard deviation

Table 6. Echocardiographic parameters in horses sedated with detomidine and butorphanol

	Patients sedated with detomidine and butorphanol								Mean	σ
	1	2	3	4	5	6	7	8		
FS [%]	27.8	28.1	29.1	29.4	25.3	25.5	23.5	29.1	27.225	2.04
EF [%]	62.3	62.8	64.3	64.8	58.3	58.7	55.2	64.4	61.35	3.30
LV mass [g]	3461.1	3275.7	3121.1	2866.8	3587.1	3746.4	2988.2	3439.3	3310.7	283.64

σ — standard deviation

Echocardiographic parameters in the non-sedated patients and patients sedated with xylazin or detomidine with butorphanol are summarised in Tables 1 to 6.

Window for USG examination of the lungs

Lung compartment ventral arc is a line of points connecting 17th ICS and coxal tuber, 15th ICS and ischiatic tuber, 13th ICS and the thorax centre, 11th ICS and brachial joint, 9th ICS and the elbow on both sides of the thorax.

DISCUSSION

The measurements of USG biometric parameters of the wall and compartment of the left ventricle decrease the risk of undetected states manifested by a decreased cardiac function or performance of the heart muscle, frequently in horses subjected to sport-related loads [1]. Biometric measurements provide information about the relevant heart function and its performance particularly in horses with unexplainable reduction in performance in the absence of musculoskeletal or respiratory diseases [6]. The microcon-

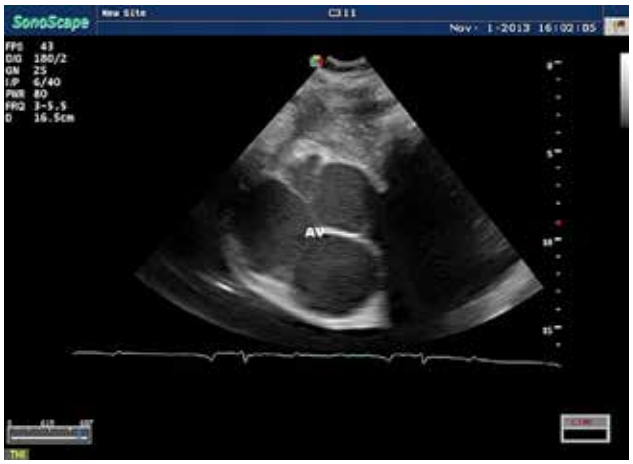

Fig. 1. M-mode echocardiograph of the left ventricle

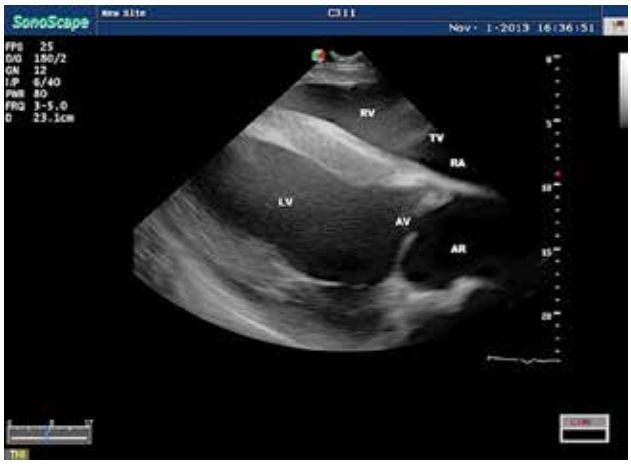

Fig. 2. USG of the heart, right aspect, long axis
LV — left ventricle; RV — right ventricle; TV — tricuspid valve
RA — right atrium; AV — aortic valve; AR — aortic root

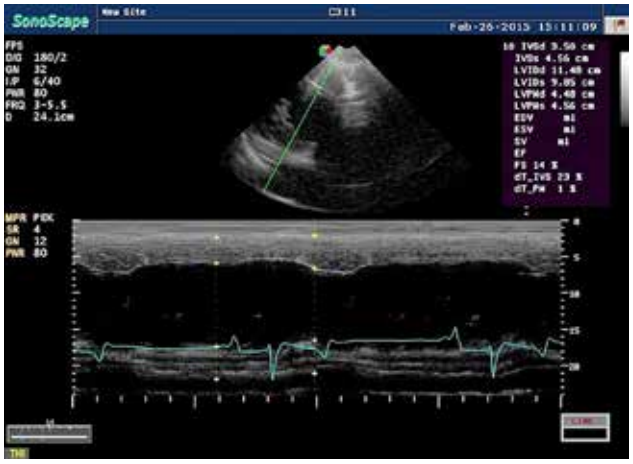

Fig. 3. USG of the heart, right side, short axis, aortic valve with three cusps; AV — aortic valve

Fig. 4. USG of lungs, arrows point to reverberations

vex 2—4 MHz probe employed in this study was suitable for biometric measurements of the wall and compartment of the left ventricle in horses with a large body frame using the left acoustic window which differs from observations of Reef and Virginia [8] and Marr and Bowen [6], who recommended to use the window from right 3—5 (ICS). Our USG measurements showed that the concentration of xylazin and detomidine with buthorphanol intended for the sedation of the horses affected the biometric parameters of the left ventricle involving; the thickness of the free wall, interventricular septum, and the ventricle compartment at the end of systole and at the end of diastole (LVIDd, LVIDs, IVSd, IVSs, LVFWd, LVFWs, FS, EF, LV mass). After the administration of xylazin, as well as, detomidine with butorphanol, we observed a significant increase in the USG biometric values of LVIDd and LVIDs, and a significant decrease in the; IVSs, LVFWs, FS and EF. Other parameters were not affected significantly. Therefore, if the echocardiographic examination cannot be carried out without sedation, one must consider changes in the measurements of the individual parameters related to sedation. In the case of the combination of several clinical signs, such as; aug-

mented respiratory murmurs, reduced performance, nasal discharge, and cough, we recommend to carry out the USG examination of the entire pulmonary compartment in both B-mode and M-mode with a focus on the pulmonary tissue and pleura in inspiration and expiration dynamics [4]. The changes in the respiratory mechanics during the physiological and pathological states, were manifested by the typical character of the M-mode signal (seashore sand, flood tide, the stratosphere or bar code) with the dynamic pulmonary USG which was in agreement with Cavaliere et al. [4]. Healthy pulmonary tissue is characterised by thin hyperechogenic lines of varying thickness and, as a rule, of varying echogenicity — reverberations. The thoracic USG enables a rapid assessment of the health of the pleura and pulmonary tissue. It, however, cannot replace a conventional physical examination of the patient and a thorough auscultation of the pulmonary compartment. The thoracic USG provides to clinicians, complementary information supporting a provisional clinical diagnosis made on the basis of an anamnesis and clinical examination. The successful therapy of the patient is an ideal way of verification of the correctness of the sonographic diagnosis.

Our USG measurements of biometric parameters of the wall and compartment of the left heart ventricle showed significant differences in some parameters, after the administration of xylazin or detomidine with butorphanol. The thoracic USG provides swift images of the following: small amount of effusion in the thoracic cavity; consolidation of the lungs; pleural fibrin deposits; and multifocal lung abscesses. Because we are unable to estimate the presence of fluid in the thoracic cavity, changes in the density of the pulmonary tissue or lung echogenicity, the USG examination is an irreplaceable part of the clinical examination.

ACKNOWLEDGEMENT

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TIME DEPENDENCE OF OVULATION ON PRE-OVULATORY FOLLICLE IN MARES AFTER ADMINISTRATION OF hCG

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ABSTRACT

The aim of our study was to determine the relationship between the time interval from the administration of human chorionic gonadotropin (hCG) up to ovulation and the size of the pre-ovulatory follicle at the administration of hCG. This relationship was investigated in a group of 25 mares of various ages and breeds during 4 years, by ultrasonography during rectal examination. In addition to the time interval, we observed also the development of follicles and changes in their size before ovulation. We also compared the course of ovulation with repeated use of hCG during different oestral cycles in 4 of the 25 mares. Our observations showed that the size of the pre-ovulatory follicle is related to the ovulation time; the smaller the follicle on the ovary at the time of the administration of hCG, the longer the time interval from this moment up to ovulation. It was also observed that the repeated administration of hCG during two different oestral cycles had no unambiguous influence on the course and time of ovulation in the respective cycles.

Key words: follicle; hCG, mare; ovulation

INTRODUCTION

Human chorionic gonadotropin (hCG) is frequently administered to mares to speed up ovulation. The injection of hCG suffices to induce ovulation of pre-ovulatory follicles bigger than 35 mm in mares with regular oestrous cycles. The administration of hCG during the second or third day of the cycle, results in approximately 65 to 70 % ovulations occurring within 36 to 48 hours [4]. In follicles that should ovulate within 36 hours, the administration of hCG will not accelerate the process. According to Evans et al. [3], after the administration of hCG, the growth rate of follicles slows down from 2.25 mm.day^{-1} to 1.7 mm.day^{-1} [3].

The disadvantage of repeated administration of hCG is that it may induce the production of antibodies. However, it is not clear whether these antibodies decrease the successfulness of hCG therapy, or delays ovulation during a cycle not affected by HCG. The effect of serum hCG antibodies on subsequent spontaneous ovulations remains controversial. Their functional life may last from one, up to several months [4].

The aim of our study was to observe the relationship between the size of pre-ovulatory follicle at the administration of hCG and the ovulation time. We made an effort to

determine the specific time of ovulation for various sizes of follicles, and to find out whether there is a significant difference in the relevant time intervals in individual cases. In four mares we also investigated the differences in ovulation time after administration of HCG during different oestrous cycles.

MATERIALS AND METHODS

The research was carried out on 25 mares of various breeds and ages, housed at the Equine Clinic of the UVMP in Košice and the Riding establishment of UVMP in Košice during the period from 2010 to 2014. All mares were in fertile age and observations were carried out during the mating season.

The ultrasonographic examination of ovaries was conducted using a USG apparatus Aloka SSD-500, Tokyo MURE HITAKA-SH CO, Ltd., Japan, equipped with a linear rectal probe UST – 588 U, 5.0 MHz. The USG apparatus used, allowed us to determine the size of the pre-ovulatory follicles, as a mean of two subsequent measurements, conducted during one examination. The ovulation time was determined on the basis of changes observed on the ovary upon rectal examinations in 6-hour intervals and when the follicle was no more present and a yellow body was produced in its place.

The hormonal preparations used in the study depended on their availability in the respective years, and contained hCG Pregnyl and Werfachor. The administered dose of the human medicine Pregnyl inj., was 5000 IU hCG. The dose of the veterinary preparation Werfachor inj. ad. us. vet. was 3000 IU. The hormonal preparations were administered i.v. into the vena jugularis, after previous disinfection of the injection site.

Statistical processing of the data used, the time interval between the two last examinations as the so-called mean time of ovulation. By subtracting three hours from the time of the last examination, we fell exactly into the centre of this time interval, which allowed us to determine the ovulation time with the deviation of ±3 hours. The results were evaluated on the basis of an arithmetic mean using the Student *t*-test and $P<0.05$ as the level of significance.

RESULTS

Our investigations showed that out of 33 cases of ovulation recorded in the 25 observed mares, 85% of the mares ovulated within 48 hours after the administration of hCG. The mean time of ovulation was 37±3h. The mares with follicles of size up to 4cm ovulated on average 48±3h after the administration of hCG; those with follicles of size 4—5cm, after 39.82±3h; and mares with follicle size exceeding 5cm, after 34.55±3 h (Table 1). The mean increase in follicle size between the administration of hCG and ovulation was 0.18cm. Only the difference between groups with follicle size up to 4 cm and above 5cm was significant ($P<0.05$).

Observation of differences between individual ovulations while administering hCG during different oestrus cycles showed, that, the interval between the administration of hCG and ovulation increased in 50% of cases, decreased in 25% of cases and remained unchanged in the remaining 25%. The mean time up to ovulation was 43±3h after the first administration of hCG and 49±3h after the second ($P>0.05$). The mean difference in the time of ovulation for all mares was 15 hours.

The dependence of ovulation time after the administration of hCG on pre-ovulatory follicle size for individual ovulations is shown in Fig. 1.

Differences in ovulation time after the administration of hCG during two different oestrous cycles in four mares are shown in Table 2. Mares 2 and 4 were administered hCG during two subsequent cycles, while the interval between the administrations of hCG to mares 1 and 3 was 4 and 1.5 years, resp.

DISCUSSION

The results of our investigations showed that after the administration of 3000—5000 UI hCG to mares with a follicle size >3.5 mm, 85% of the investigated mares ovulated within 48h after the administration of hCG. This agrees with the observations of Evans et al. [3] who reported that 96% and 95% mares ovulated within 48h after administration of 2500 IU and 1500 IU hCG, resp.

In our study on mares with a follicle size >3.5 cm, 15% of them ovulated within 24h, 70% in 24—48h and 15% after 48h. This is comparable with the research of Barbacini et al. [1] who described that after administration of 2000 UI hCG to mares with follicle size 3.5cm and more, 16% of them ovulated within 25h, 76% within 25—48h and the remaining 9% after 48h.

Table 1. The time dependence of ovulation on the size of follicle following the administration of hCG

Follicle size	Number of ovulations	Mean time up to ovulation ± 3 [h]	Mean size of follicle [cm]	< 24 h	24—36 h	36—48 h	> 48 h
< 4 cm	2	48	3.75	0	0	1	1
4—5 cm	11	39.82	4.41	0	4	6	1
5 cm and more	20	34.55	5.33	5	5	7	3
			Σ =	**5**	**9**	**14**	**4**

Table 2. Difference in the time of ovulation in four mares following the administration of hCG during two different oestral cycles

Mare No.	Time interval between administration of hCG	Differences in ovulation time [h]	Ovulation time after 1st administration [h]	Ovulation time after 2nd administration [h]	Difference in changes in follicle size [cm]
1	May 2010/ June 2014	18	36	54	0
2	July 2013/ July 2013	0	40	40	0.2
3	Sept. 2010/ May 2012	24	36	60	0.6
4	June 2010/ August 2010	18	60	42	0.7

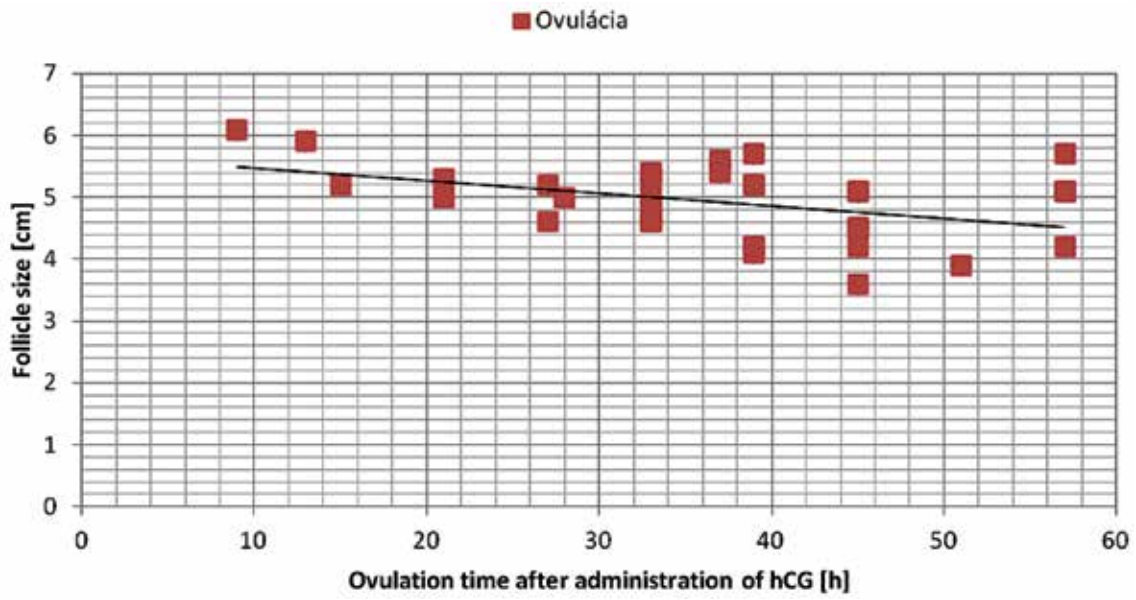

Fig. 1. The dependence of ovulation time after the administration of hCG on pre-ovulatory follicle size at individual ovulations

According to Samper et al. [5], the administration of 2500UI hCG to mares with follicles bigger than 4cm resulted in ovulation of 83% of them within 48h. Our study showed that the administration of hCG of follicles of size 4cm and more, induced ovulation within 48h in 88% of the mares.

According to Sauberli [6], the difference in the time of ovulation after the administration of hCG for follicles of size 3.5—3.9cm and 4.0—4.4cm was 6h. In our study, the difference for follicles of size 3.5—5cm and 5cm was, also 6h.

The mean growth of follicle between the administration of hCG and ovulation observed in our study was 0.18cm while Evans et al. [3] recorded 3mm growth within 42h and Cuervo-Arango and Newcomb [2] reported only minimum growth between the administration of hCG and ovulation.

Our research also showed that the mean difference in ovulation time between the first and second administration of hCG in different oestral cycles over several years, was 15h in one mare, but the delay in the second ovulation compared to the first one, was observed in only 50% of the mares (the mean delay was 21h). This delay, is considerably lower than the delay by 28.8hours observed by Sullivan et al. [7], however we observed altogether only 4 mares and the interval between the subsequent administrations of hCG differed. Additional research showed that repeated treatment of one mare with hCG during individual oestral cycles may delay considerably the onset of ovulation. According to Wilson et al. [8], during the first year of five repeated administrations of hCG, the onset of ovulation was not affected, however, in the following year, the interval between hCG treatment and ovulation increased to 52h, 66h and 88h at sixth, seventh and tenth administrations (including the previous year), respectively. Our study, involving the period ranging from one month to four years, does not suffice to evaluate, objectively, the influence of repeated administration of hCG in individual oestral cycles. To answer this question, it is necessary to observe repeated treatments in the same mare with hCG during longer time interval and thus obtain sufficient data for evaluation.

CONCLUSIONS

Our results indicate that the size of the pre-ovulatory follicles, at the time of the administration of hCG, affects the time of ovulation. The difference in the time of ovulation between mares with follicles of size 3.5—5 cm and 5 cm and more was evident, although not significant. We also observed that repeated administration of hCG during different oestral cycles may result in prolongation of the interval between the administration of hCG and ovulation in mares, although the results were ambiguous.

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DETERMINATION OF PHYSICAL AND PHYSICO-CHEMICAL PARAMETERS IN GREEK CHEESES

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ABSTRACT

Some physical and physico-chemical parameters of 15 different Greek cheeses were assessed. The cheeses differed as to their origin (cow, sheep and goat), they had different texture (soft, semi-hard and hard), and they were from different regions in Greece. The a_w values in the observed samples ranged from 0.799 (in semi-hard cheese Kefalotiri) to 0.889 (in soft cheese Katiki Domokou), while the average value of the water activity in the samples was 0.851. The water content of the samples was 24.0 to 73.6 %. The dry matter in the cheese thus constituted from 26.4 to 76 %. The fat content in dry matter was from 34.27 to 62.11 %. The NaCl concentration in cheeses ranged from 0.82 to over 5.15 %. The colour data for the monitored cheeses were analysed colorimetrically and expressed by the CIELab color range as parameters L*, a*, b* and as parameters L*, C*, h° in cylindrical colour space CIELCh.

Key words: colour; dry matter; fat; Greek cheese; NaCl; pH; water activity

INTRODUCTION

Cheeses belong to traditional Greek food and their production has a long tradition and was described already by Homer [7]. In cheese consumption, Greece ranks third in the world (behind France and Iceland), with a consumption of 23.4 kg per capita per year (it is more than twice as much as Slovakia with 10.3 kg per capita) [3]. Greek terrain and climate conditions are suitable for breeding sheep and goats as well as cattle, which also contribute to the wide variety of cheeses produced. The most popular Greek cheese is a soft white cheese called Feta, which is ripened and stored in brine. It is well known because Greek cheese and the name of Feta are included in the register of Protected Denomination of Origin (PDO) of the European Union [5]. In general, the quality of the cheese is affected by a number of parameters and depends primarily on their basic composition: water content, fat content, pH, salt content and water activity. Water in cheese plays a relevant role for the curd consistency and the bacterial metabolism, and consequently for the processes during cheese ripening. The influence of the water content and the water

activity on the cheese quality is very complex. Cheese contains besides high molecular proteins, also low molecular compounds which are partly produced during ripening or, as in the case of NaCl, are added during manufacturing. The low molecular soluble compounds (especially sodium chloride) have the biggest influence on the water activity in cheese [9]. Determining the values of water activity, pH and salt content belong to relevant data necessary for assessing the health safety and quality of the cheese. The fat content in cheese is an important parameter, because in addition to significant effect on the sensory quality of the cheeses, it affects also its thermo-physical properties (meltability, flowability, stretchability and oiling-off) especially in a semi-hard cheese.

The natural colour of the fattiest cow's cheeses, and those from cows grazed on open pasture tends to be more yellow than cheese made from winter milk because of beta carotene. Beta carotene is a fat-soluble yellow pigment contained in grasses. Some cheeses made from other animals' milk are white because they don't store beta carotene in their fat the way cows do, but they convert it to a colourless vitamin A. One of the validation criteria for milk products made from sheep and goat's milk can also be the colour [2]. Variations in quality may also be reflected in the colour of the cheese.

The aim of the presented work was to estimate some physical and physico-chemical properties of typical Greek cheeses, in order to: assess the results obtained in relation to the values given for certain cheeses by the Greek Codex, compare parameters of Greek cheeses with the Slovak ones, and determine the differences in their properties.

MATERIALS AND METHODS

Fifteen kinds of different cheeses were assessed, which differed by the origin (cow, sheep and goat), region where they were produced in Greece and the texture (soft, semi-hard and hard). Cheeses (commercially produced) were purchased in Greece in the period of October—November, 2013. They were transferred to Slovakia in a portable refrigerator kept in original packaging (vacuum-packed) and kept in a refrigerator until analysis. Two samples of each product were analysed for the value of; water activity, pH, water content (moisture), fat content and salt concentration. The determinations on all samples were carried out in triplicate and the results were reported as a mean value.

The water activity of cheese was determined by a non-destructive method using the LabMaster-aw apparatus with electro-resistive sensor (Novasina, Switzerland). Finely chopped cheese samples were sequentially measured in duplicate in plastic containers (10 ml) along with preheating of the next sample. The resulting a_w value, the selected measurement temperature (25 °C) and time measurements were read from the device's LCD display.

The pH values were measured with a sharp needle Sen Tix pH-electrode (WTW, Germany) directly in the cheese. The measurement was repeated consecutively using three different test points. Between measurements, the electrode was wiped to remove the cheese, soaked in ethanol/ether (50/50), rinsed with water and wiped again.

The water content in cheese was determined by the method of drying to a constant weight at 102 °C (±2 °C) in a drying oven UFP 500 (Memmert, Germany). Grated cheese (5 g) (m_c) was uniformly distributed over the surface of an aluminium dish containing sand (20 g) both being dried in the oven until a constant weight (24 hours) at 102 ± 2 °C (m_1). The dish with cheese was then dried and weighed in the same way (m_2):

$$\text{Dry matter (\% cheese)} = ((m_2 - m_1)/m_c).100$$

$$\text{Moisture (\% cheese)} = 100 - \text{dry matter}$$

The fat content was determined by a simple and rapid Gerber-Van Gulik method based on the digestion of proteins and non-fat components by concentrated sulphuric acid and the separation of fat from the aqueous phase after centrifugation in a special glass butyrometers (Van Gulik). The butyrometer was charged with 3.0 g of grated cheese, the 3 ml of distilled water and 10 ml of sulphuric acid (density 1 522 kg.m^3) were added. After centrifugation, the weight percentage of the fat present in the cheese was read on the butyrometer scale [1].

The NaCl content was determined by Mohr argentometric method [8]. Grated cheese (10.0 g) was stirred in the mortar with 10 ml of distilled water, transferred quantitatively into a 100 ml volumetric flask and filled up to the mark. The obtained homogenous solution was filtered. In the titrimetric flask, 10 ml of filtrate was diluted with 30 ml water and 1 ml of potassium chromate indicator was added. The mixture was titrated with silver nitrate ($c = 0.1$ mol.l^{-1}) until an orange colour appeared. The percentage mass fraction of sodium chloride content was calculated from the

volume of the silver nitrate solution in millilitres according the formula:

NaCl (% cheese) = 0.585. a. f

where:

a = ml of 0.1 mol.l^{-1} $AgNO_3$

f = factor of 0.1 mol.l^{-1} $AgNO_3$)

The colour of the cheeses was measured by a colorimeter CM410 (Konica Minolta, USA) with C type illumination, and expressed by rectangular CIELab colour range as 3 parameters L*, a*, b* which can accurately characterize various shades and brightness of colour. In the space CIELab L* expresses the brightness values, a* and b*are the chromaticity coordinates. L* takes the value from 0 to +100 (from black to white). The values of a* and b*range from -60 to +60, where –a* direction is greenish, +a* represent red direction, –b* is the blue direction and +b* direction is yellow. Values of difference *Δ E* parameters, C* and h° were calculated according to the formulas:

$\Delta E = \sqrt{L^2 + a^2 + b^2}$ $C^* = \sqrt{a^2 + b^2}$ and

$h° = \tan^{-1}(a/b)$

Chroma, C*, is colour saturation and h° gives hue angle degree in CIELCh colour range [6].

RESULTS AND DISCUSSION

We analysed two samples of 15 different Greek cheeses and each analysis was done in triplicate. Mean values of water activity, pH and sodium chloride are given in Fig. 1—3. Determining the values of water activity, pH and salt content belong to the relevant data necessary for assessing the health safety and quality of the cheese. The water activity is an important parameter in food technology optimisation to provide the microbiological, chemical and physical stability of food. The a_w values in the observed samples ranged from 0.799 (in semi-hard cheese Kefalotiri) to 0.889 (in soft cheese Katiki Domokou); while the average value of water activity in the samples was 0.851. The pH of the samples ranged from 4.317 to 6.350. The NaCl concentration in the cheeses ranged from 0.82 to over 5.15 %. Most of the samples (73.3 %) had salt contents between 2.0 and 4.0 %. The salt content was higher in 2 samples and lower in another two. In the sample with the lowest salt content and the highest a_w value, we detected the lowest pH which just ensured the health safety of the product. The level of fat in the samples ranged between 12.0 and 43.5 % (Fig. 4).

The results presenting the mean levels of water content and fat in cheese dry matter in the samples are shown in Fig. 5. Water content (moisture) in the cheeses ranged from 24.0 to 73.6 %. Dry matter in the cheeses thus constituted from 26.4 to 76 %. The fat content in dry matter was from 34.27 to 62.11 %. Our results (in brackets) were compared with the values given for certain cheeses by the Greek Codex Alimentarius [5].

The permitted maximum moisture content in Feta is 54 % (5—51.6 % was in both our Feta samples, No. 4) and minimum fat content in dry matter is 46 % (51.6 % in No. 4; 61.9 in No. 5). In Mizitra from the whey of Feta or hard cheeses (e. g. Kefalotyri, Graviera) the permitted maximum moisture content is 60—70 % (in Dry Mizitra, No. 11 we determined 35.6 %) and the minimum fat content in dry matter is 50—70 % (62.1 % in No. 11). In Katiki (spread acid-curd soft white cheese) the permitted maximum moisture content is 75 % (73.6 % in sample No. 15) and minimum fat content in dry matter is 40 % (45.4 % in No. 15), pH should be in a range of 4.3—4.5 (pH 4.31 in No. 15). The cheeses analysed in our study met the requirements of the Greek Codex Alimentarius.

The colour characteristics of all samples were monitored in the inner part of cheeses. Mean L*, a*, b* parameters of the cheeses are presented in Table 1. The colour of cheese depends on the type of milk and fat content. L* parameter values ranged in 79.87—94.08. Standard deviations (ΔL) in individual samples were in the range of 0.02 – 1.64. The intensity of greenish colour was expressed by a* values which ranged from –5.76 to –1.55 (Δa from 0.01 to 0.16). The values of the parameter b*, which reflects the intensity of the yellow colour ranged in assessed cheeses from 11.91—30.67 (Δb from 0.01 to 0.56). Chroma parameters C* were from 12.17 to 30.87. The hue angle degree h° was in a range 94.61—104.15.

Comparison of the measured parameters with those published for various Slovak cheeses showed the biggest differences in water activities (0.909—0.998). Greek cheeses a_w values were generally lower (0.799—0.899) compared with similar Slovak cheese samples [4].

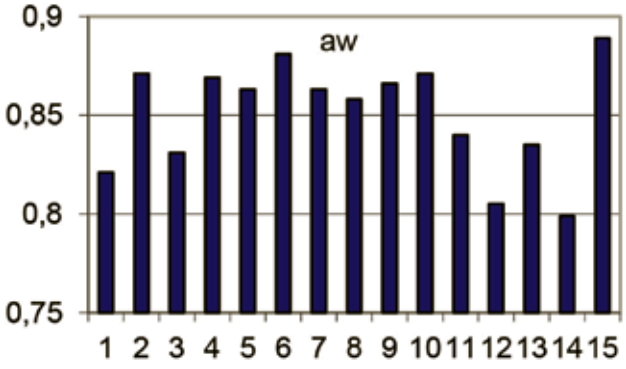


Fig. 1. Water activities in cheese

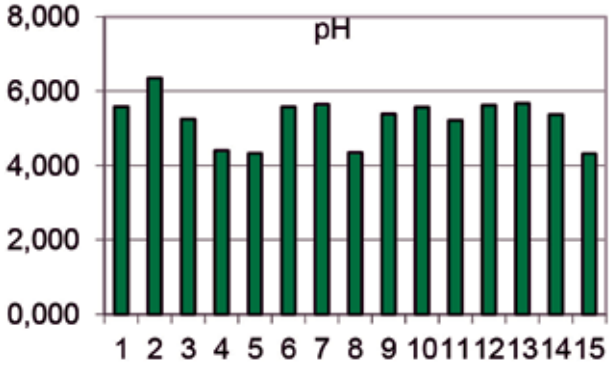


Fig. 2. pH values in cheese

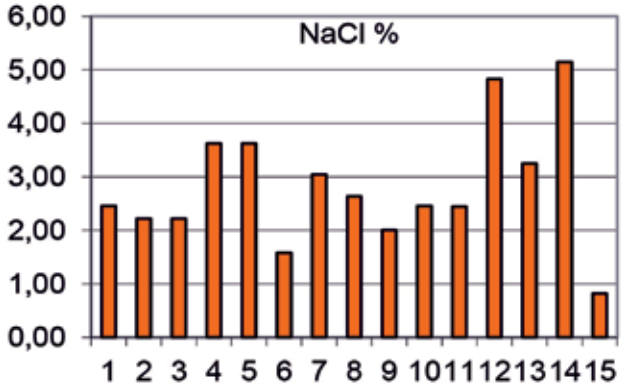
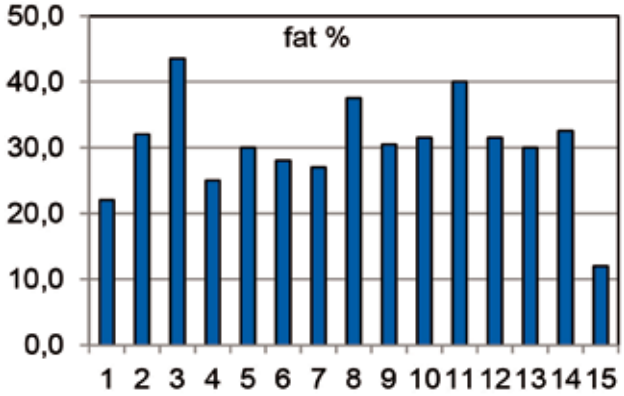


Fig. 3. Salt content in cheese



4. Fat content in cheese

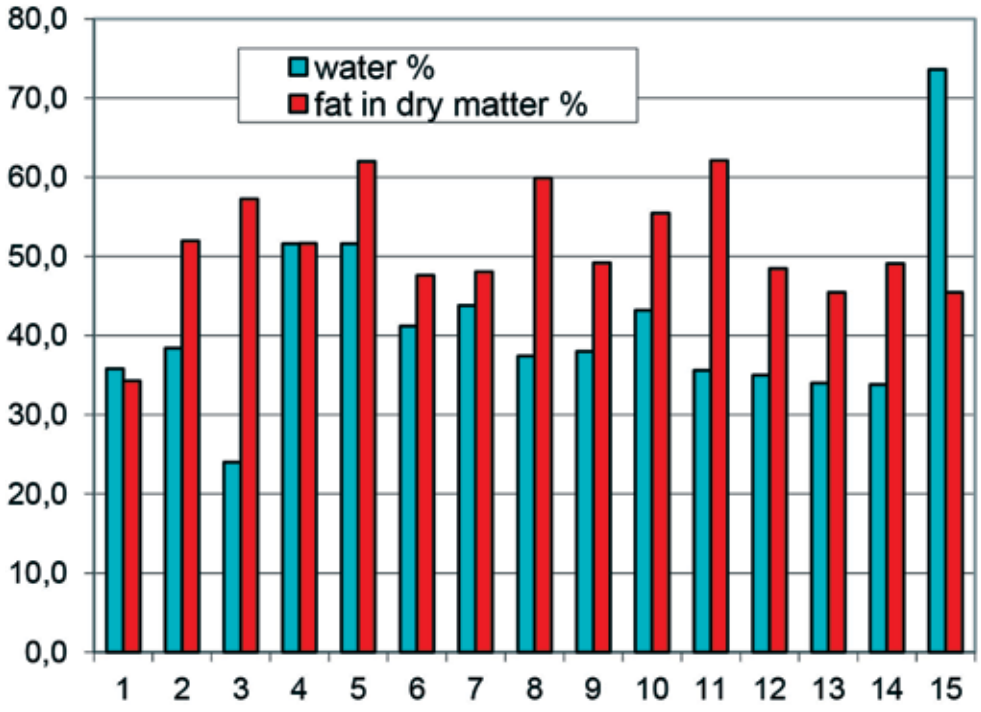


Fig. 5. Fat in dry matter and water content in cheese

Table 1. Colour parameters of inner parts in assessed cheese samples

No.	Name	L*	a*	b*	ΔE	C*	h°
1	Graviera fromCreta	80.01	–3.46	30.67	85.76	30.87	96.44
2	Mastelo from Chios	87.21	–3.71	18.74	89.28	19.1	101.2
3	Ladotiri from Mytilini	87.61	–5.76	28.67	92.36	29.24	101.36
4	Feta from sheep	91.34	–2.44	14.47	92.51	14.67	99.57
5	Feta from goat	94.08	–2.5	11.91	94.86	12.17	101.86
6	Chaloumi monastiriou	90.93	–4.09	25.63	94.56	25.95	99.07
7	Chaloumi from goat and sheep	88.74	–2.77	18.28	154.39	18.49	98.62
8	TirakiTiniako	92.76	–1.86	18.79	94.66	18.88	95.65
9	Kaseri from Mitilini	79.87	–3.19	25.16	83.80	25.36	97.23
10	Talagani	92.36	–4.21	17.23	95.94	17.74	102.73
11	Dry Mizithra from Creta	82.45	–2.83	24.06	85.93	24.23	96.71
12	Pecorino Karali	84.87	–3.77	22.21	87.81	22.53	99.63
13	Gaviera from Tinos	82.27	–1.75	21.72	85.11	21.79	94.61
14	Kefalotiri Karali from Epirus	87.57	–1.,55	13.63	88.64	13.72	96.47
15	Katiki Domokou	93.95	–3.75	14.88	95.19	15.35	104.15

ACKNOWLEDGEMENT

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ARE THE E-SUBSTANCES AN INVENTION OF CHEMISTS OR DO THEY REPLICATE THE WISDOM OF NATURE?

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ABSTRACT

Currently we are witnessing a certain fear (even hysteria) about food additives. We certainly can question the anxiety about some of them. The overall benefits of many of them are greater than the problems associated with their use. It is especially wrong to raise doubts about the use of preservatives. Many food preservatives are naturally present in some foods, sometimes at higher amounts than those we commonly add during processing. Benzoic acid (E 210), currently one of the most frequently used preservatives, is present, for example, in cranberries (up to 0.5 %). Who dares to question the beneficial effects of cranberries on health? Another preservative, sorbic acid (E 200), is naturally a part of rowan berries from which it is extracted. Propionic acid (E 280), an excellent inhibitor of the growth of moulds and some bacteria, is naturally present in various types of starter cultures and ensures the prolongation of shelf life of products of propionic fermentation. This effect is used also in obtaining winter feed (silage for animals). By its preservative effects, propionic acid prolongs, together with other carboxylic acids, also the shelf life of products of lactic fermentation, such as milk products and fermented vegetables. Additive substances with conservative effects include such carboxylic acids as; lactic acid (E 270), acetic acid (E 260), citric acid (E 330), malic acid (E 296) and fumaric acid (E 297). These are compounds that are produced by metabolic processes in all living organisms. Despite that, as soon as their names or numeric codes occur of food labels, the majority of people think that they are buying "pure chemistry". Perhaps one should realise that nature is also chemistry and there is not life without chemistry.

Key words: additives; natural occurrence; preservatives

INTRODUCTION

One of the most important challenges of the present is supplying sufficient food for the increasing population. Food preservatives guarantee good selection of products

on the store shelves, their longer storage life, and availability of some types of food throughout the year in a way we became used to in our fast paced life. Food preservatives are defined as chemicals added to food to prolong their shelf life and prevent them from spoilage by the action of micro-organisms. Many of these preservatives are found naturally in food, some at higher amounts than those commonly added to conserve food.

E numbers are codes for substances that can be used as food additives within the Europan Union and Switzerland. The "E" stands for "Europe". They are commonly found on food labels throughout the European Union. The negative image of food additives among consumers has been due to emotional articles and entries into the popular press and on pseudoscientific internet pages. This "anti-campaign" causes consumers to be afraid of E-substances present in one food according to its label and they are not aware of ingesting them as natural components in other foods. However, the additives include also compounds that bring more harm than benefit and we should learn to recognize them and become well informed about our daily consumed foodstuffs.

This study focused on preservatives that constitute the group of additives most needed to ensure quality and long shelf life of food.

THE EFFECT OF ADDITIVES ON HEALTH

The negative effects of additives are thoroughly investigated during the approval process and toxicological tests, however already in this stage there are some challenges we face.

The toxicological tests are conducted using pure substances, while in the actual processing, no producer of additives is capable of producing high purity additives. The negative effects on health, of even minimal amount of contaminants, can be more serious than those of the additive itself. Moreover, several additives are mostly used in parallel and there is not enough information on their synergistic effects in the consumer's body [4]. Toxicological tests are conducted on animals. The mechanism of effect, distribution and metabolism of the additives in the human body may not be the same as in the experimental animal.

On the other hand, tests are conducted with considerably higher doses of additives than those present in foodstuffs. However, in some cases we ingest the additives throughout our life. Laboratory tests cannot ensure long-term observation of the effects of the respective substances before they are permitted for use. The effects are observed for several generations of laboratory animals with short life spans. Due to their character, many additives can accumulate during a life time and we lack sufficient knowledge on that related risk.

PRESERVATIVES

Benzoic acid (E 210) and its derivatives (E 211—E 219)

Benzoic acid is one of the oldest and most frequently used chemical preservatives. This acid and its salts are found in many natural plant raw materials, but its use as an additive is based on synthetic compounds. Benzoic acid has a very strong antimicrobial effect on bacteria, yeasts and moulds, particularly in acidic environment [4]. It inhibits the utilization of amino acids by micro-organisms, by inhibiting enzymes which participate in the metabolism of acetic acid, oxidative phosphorylation and the Krebs cycle [6].

Benzoic acid and its derivatives, which belong to the group of phenolic acids, occur naturally in small amounts of food, either as free acids or in the form of esters. Their concentration in fruits and vegetables is generally very low (around 0.05 %), with the exception of some red fruits (particularly cranberries containing up to 0.5 %), black radishes and onions, where some tens of mg per kg of fresh weight can be found [3]. The well known derivatives of benzoic acid, include gallic acid and ellagic acid, and the components of tannins. Phenolic acids are present in our everyday cup of coffee (70—350 mg per dose) or in apple cider (up to 100 mg per dose).

The acute toxicity of benzoic acid, as an additive, is low. However, in sensitive individuals, daily intake of doses lower than 5 $mg.kg^{-1}$ body weight can cause a non-immunological response (pseudoallergies) [8]. Such symptoms can occur more frequently in people with aspirin sensitivity. Some studies suggested that a very high intake of benzoic acid may have negative effects such as; metabolic acidosis, hyperpnoea, and spasms. Allergic reactions have been observed to benzoic acid and benzoates, such as urticaria, rhinitis and pruritus [1] or contact eczema.

Benzoic acid, its salts and esters, are frequently used in combination with sorbic acid owing to its synergistic action.

Sorbic acid (E 200) and sorbates (E 202—E 203)

Sorbic acid and sorbates are extensively used preservatives just as those from the previous group. The salts are preferred due to their better solubility in water. Sorbic acid is a component of rowan berries from which it is extracted. The preservative effect of sorbic acid in a slightly acidic environment is about 3—5-fold stronger than that of benzoic acid. It is a selective preservative compound acting particularly on yeasts and moulds with low effectiveness against bacteria. This results in its use as an additive to fermented food because it does not inhibit the beneficial lactic bacteria. Sorbic acid and its salts are considered safe preservatives [7]. Sensitivity to sorbic acid is rare.

Citric acid (E 330)

Citric acid is an important part of intermediary metabolism in all organisms. It is abundant in fruits and vegetables, particularly in citrus fruit. On an industrial scale, it is obtained from lemon juice or by fermentation of molasses. Citric acid prevents the growth of bacteria, yeasts and moulds in fruit syrups and soft drinks [7]. In order to ensure low pH, it is added to many products subject to the risk of excessive growth of *Clostridium botulinum*. Botulotoxin is the strongest toxin implicated in fatal cases of food intoxication [2]. The problem is that citric acid is added to too many products. Frequent consumption of high doses of citric acid may result in; dental erosion, cause muscular spasms, swelling, weight gain, mood swings and rapid shallow respiration. The unlimited addition of citric acid in food production may result in premature birth or abortions, so its use should be reassessed and restricted [5].

Lactic acid (E 270)

Lactic acid, the main product of lactic acid fermentation performed by lactic bacteria, is one of the most important preservative agents. It naturally preserves milk products in which it is found in concentrations of 0.5—1 %. In meat, it is produced from glucose after the degradation of glycogen. Fresh beef contains 0.2—0.8 % lactic acid. Lactic fermentation is an ancient way of preserving vegetables. The content of lactic acid in sauerkraut reaches 1.5—2.5 % and in pickled cucumbers 0.5—1.5 %.

Acetic acid (E 260)

Acetic acid is the monocarboxylic acid most commonly found in food. It is present in fruit (free acid and its esters) and foods produced by using the fermentation processes. For example, beer contains up to 155 $mg.dm^{-1}$ acetic acid. It also develops as a product of degradation of sugars and other components during thermic processes.

Propionic acid (E 280)

Propionic acid develops as a side product of lactic fermentation or the main product of propionic fermentation. It is also used in the production of certain cheeses (e. g. Emmenthaler). Due to its strong preservative effects, either propionic acid or bacteria of the genus *Propionibacterium* producing this acid are added to silage [6]. Certain amounts of propionic acid naturally occur in bakery products as a result of the fermentation processes. Propionic acid is added mainly to packaged and sliced baked goods in order to prolong the shelf life.

Other acids

Higher saturated aliphatic dicarboxylic acids, such as malonic acid, succinic acid, glutaric acid and other higher homologues of oxalic acid, occur in many raw materials used by the food industry and in foodstuffs, but as a rule in smaller amounts. Only succinic acid occurs in higher amounts in some types of fruits (currant, strawberry). All of the above dicarboxylic acids are present in small amounts in the wine and beer. Of the unsaturated acids, fumaric acid (E 297*)* is present in practically all products of animal and plant origin (in higher concentration in some mushrooms). Fumaric acid develops also during thermic processes, such as the roasting of coffee beans. Malic acid (E 296) is naturally present particularly in fruits and vegetables [6].

It is not necessary to present on foodstuff labels all of the naturally produced preservatives, even if they are present at higher concentrations. Labels must contain only added food preservatives, although this may involve the same compounds.

CONCLUSIONS

Some foodstuffs consumed today are literally full of additives which are in fact unnecessary. We could live well without colourings and flavour enhancers. However, omission of preservatives can bring up much bigger problems than their use. They are frequently naturally present in foods and no one is afraid of them in such cases. We should

realise that it is not necessary to fight against the use of all food additives. Some caution should be issued for their use, but it should be based on relevant knowledge of their benefits and potential health consequences.

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THE USE OF RAPID SCREENING METHODS FOR DETERMINATION OF COCCIDIOSTATS IN FEED

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ABSTRACT

Premi®Test and Total Antibiotics are rapid broad-spectrum microbiological screening tests intended for the determination of antibiotic residues in food. This study compares the results of the determination of coccidiostats in animal feed obtained by a widely recognised and used Premi®Test and the later developed Total Antibiotics test. Fourteen samples of commercial mixed feeds for poultry served as a matrix. They were analysed primarily for coccidiostats by a confirmation method. The Premi®Test provided 8 positive, 5 dubious and 1 negative result. By using the Total Antibiotics test, 7 samples tested positive, 4 were dubious and 3 negative. Comparison with the confirmation analysis showed higher accuracy with the Total Antibiotics. Our results indicated that both tests (Premi®Test and Total Antibiotics) are suitable for the determination of coccidiostats not only in food but also in feed.

Key words: coccidiostats; determination; feed

INTRODUCTION

Coccidiosis of poultry is a serious disease causing enormous economic losses in intensively produced flocks due to acute enteritis. The infective agents are protozoan parasites from the genus *Eimeria*. The highest coccidial challenge occurs in broiler flocks kept on deep bedding where these parasites find suitable conditions for sporulation and they can survive in the external environment for several months. Chickens are infected by the alimentary tract when sporocysts are ingested and subsequently eliminated in faeces [3].

Coccidiostats are used for the treatment and prevention of coccidiosis. Currently, 11 approved coccidiostats are available for the prevention of coccidiosis in poultry [4]. Coccidiostats are subject to official controls in Agreement with Regulation of the Government of SR No. 320/2009, Coll. For the sake of public health, the quality of analytical results must be ensured by using relevant screening and confirmation methods [5].

The aim of our study was to compare the suitability of two screening tests for the determination of coccidiostats in poultry feed. The Premi®Test is a recognized test for the determination of residues of inhibitory substances in food and raw materials of animal origin, developed by R-Biofarm AG (Germany). It allows one to detect β-lactam antibiotics, cephalosporins, macrolides, tetracyclins, sulphonamides and aminoglycosides. The tested sample is dosed into vials containing a solid nutrient medium with the test strain, *Bacillus stearothermophilus* var. *calidolactis*. They are heated to 64 °C, to allow the spores to germinate. In the absence of inhibitory substances, the germination of the spores is manifested by a change of colour, from violet to yellow. If the residues exceed the threshold of determinability, the spores will not germinate and no colour change will be observed [1]. Total antibiotics is a new microbial inhibitory test introduced by EuroClone S.p.A. (Italy) functioning on the basis of the same principle as the Premi®Test [2].

MATERIALS AND METHODS

We tested 14 commercial mixed feeds intended for broilers (HYD 01, 02, 03 and BR 2, 3, 4), layers (HYD 10) and meat chickens (HYD 04). The primary determination of coccidiostats in feed was carried out by the State Veterinary and Food Institute (SVFI) in Košice.

The Premi®Test was used to analyse 10 g of each homogenised sample, to which we added 30 ml of freshly demineralised water. The mixture was then agitated in titration flasks for 30 minutes on a shaker, 100 µl of supernatant was transferred to a vial and pre-incubated at 80 °C for 10 min.. The pre-incubation was followed by incubation at 64°C for about 3 hours. After incubation, we recorded the results on the basis of the medium colour change. A yellow colour indicated a negative result, a violet colour was positive and a light violet colour indicated a dubious result [1].

The preparation of sample for the Total Antibiotics test consisted in homogenization of 2.5 g feed with 10 ml extraction solution prepared by mixing a stock solution with demineralised water at a ratio 1 : 9. This way, the prepared sample was pre-incubated at 37 °C for 2 hours. Then 200 µl of supernatant was transferred to a vial and incubated at 65 °C for 3 hours. After incubation we obtained results which declared presence or absence of coccidostats in feed [2].

RESULTS

The results obtained in our study are presented in Table 1.

Table 1. Results of the determination of coccidiostats in feed using the Premi®Test and the Total Antibiotics test

Feed (sample)	Declared content [mg.kg^{-1}]	Premi®Test	Total Antibiotics	Confirmed content [mg.kg^{-1}]
HYD 02 (5)	NAR/NIK (80)	+	+	NAR/NIK (32.5/40)
BR2 (7)	NAR/NIK (90)	+	+	NAR/NIK (46.7/41.7)
BR2 (16)	SAL (60)	+	+	SAL (58)
HYD 02 (18)	SAL (70)	+	+	SAL (66)
HYD 10 (27)	0	+	±	0
HYR 02 (28)	0	+	+	NAR (65.7)
HYD 10 (29)	0	±	-	NAR (0.4)
HYD 01 (38)	ROB (100)	+	+	MON (96.3)
BR 2 (32)	MON (125)	+	+	MON (112.3)
HYD 10 (22)	0	±	–	0
BR 4 (24)	0	±	±	0
HYD 03 (35)	0	–	–	0
HYD 04 (14)	0	±	±	NAR/NIK (0.5/0.15)
BR 3 (12)	0	±	±	SAL (3.1)
Control	0	–	–	0

+ — positive sample; ± — dubious sample; – — negative sample
NAR — narasin; NIK — nicarbazin; SAL — salinomycin
ROB — robenidin; MON — monensin

The Premi®Test provided 8 positive results, 5 dubious results and 1 negative result. By using the Total Antibiotics test, 7 samples tested positive, 4 were dubious and 3 were negative.

CONCLUSIONS

The comparison of our results, with those declared by producers of mixed feeds, and the primary results of SVFI in Košice, showed that both Premi®Test and Total Antibiotics confirmed the declared presence of coccidiostats in the feed. The comparison with the results of the confirmation analysis indicated 100 % successfulness of both tests. This preliminary test therefore, confirmed the suitability of both tests for the determination of coccidiostats in the mixed feed for poultry, although for the validation of this conclusion, more samples should be tested for a wider range of coccidiostats.

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CONTAMINATION WITH RADIONUCLIDES IN THE DISTRICT TURČIANSKE TEPLICE IN SLOVAKIA

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ABSTRACT

With regard to the migration time of radionuclides, the most important for radioecology are the medium- and long-lived ones, which due to their similarity with biologically important elements, enter the food chain with man on its top. Twenty nine years have passed since the Chernobyl nuclear power plant accident and the ^{137}Cs half-life is about 30 years. The radioactive cloud reached not only Czechoslovakia, but also other very distant places of Europe. Our study investigated the radiocaesium activity concentrations in the district of Turčianske Teplice in the Slovak Republic, in 38 samples of several important components of the food chain. It was revealed that none of the concentrations exceeded the limit for radiocaesium activity.

Key words: contamination; monitoring; radiocaesium

INTRODUCTION

Radionuclides present potential risk to humans due to their similarity with some biologically important elements which are part of the food chain. One of the most important contaminants is ^{137}Cs because of its long half-life and potential accumulation in the muscles of man and other animals. ^{137}Cs binds strongly to the soil and is soluble in water. Its hygienic importance is related to the fact that its metabolism resembles the metabolism of potassium.

Contamination of the Slovak Republic by radionuclides produced by anthropogenic activities has originated from two sources. The first was the global atmospheric fallout after tests of nuclear weapons that were conducted in the 1950's. Today, ^{137}Cs from this source is almost completely immobilized in the clayey portions of the soil and thus barely available to most plant roots [4]. The second source of horizontal contamination of the Slovak Republic was ra-

dioactive fallout after the accident at nuclear power plant in Chernobyl in 1986. It was a single contamination and the intensity of the deposit was affected by meteorological conditions at the time of spreading of the radioactive cloud above the respective locations. ^{137}Cs showed high mobility in the soil which, however, began to decrease immediately after deposition. In non-cultivated soil there were differences between the forest and meadow soils, related to the depth in the soil horizon. Under certain conditions the content of radiocaesium available to plants in mineral soils can even increase [11].

The aim of our study was to investigate radiocaesium activity concentration in the district Turčianske Teplice of Slovakia, namely in several important components of the food chain.

MATERIALS AND METHODS

Collection and processing of samples

Altogether, 38 different types of samples were collected and investigated from the period between 2012 to 2014.

One sample (0.5 kg) of each sample (apples, pears and onions) was collected from the location of Diviaky in the Turčianske Teplice district. The samples were dried at 105 °C and homogenized.

The honeybee honey sample originated from Sklené. One sample (0.5 kg) was obtained and measured without further processing.

A total of 28 samples of; muscle, skin, stomach and stomach contents, were obtained from 1 red deer, 1 roe deer and 5 wild boars hunted in the locations of; Dubové and Turčianske Teplice. Three brown trout (1 pooled sample) caught in the river Turiec were also examined.

Four samples of soil were obtained from different locations where the investigated game was hunted. From each location, 0.5 g of soil was collected from a depth of 5 to 10 cm. After removal of roots and larger stones, the samples were dried under laboratory conditions.

One sample of mushrooms originated from the location of Sklené, specifically from Biely Potok area. Whole mushrooms were collected (0.5 kg of Summer Bolete mushrooms *Boletus reticulatus*), cut into small pieces and allowed to dry.

Measurement of radiocaesium activity concentration

Samples were placed into a Marineli vessel and the activity of ^{137}Cs was measured by a gamma-spectrometric system (Canberra) consisting of a Ge detector (GC 3520; effectiveness 35 %; 2.0 keV resolution) and a multichannel analyser DSA 1000. The results were read by means of a software system Genie 2000 (Canberra). The equipment used was verified by the Slovak legal metrological authority.

RESULTS AND DISCUSSION

The activity of ^{137}Cs in the samples of apples, pears and onions was in the range of 0.43—0.81 Bq.kg^{-1}. Low activity concentrations in farmed vegetables and fruit have been reported for a long time [5]. The activity of ^{137}Cs in honey, reached 0.12 Bq.kg^{-1}, which could be ascribed to the fact that we examined flower honey. Some authors indicated a higher radiocaesium activity concentration in forest honey compared to meadow honey [2].

In the 1950's, the activity of radiocaesium in the meat of hunted game gradually decreased, but after floods in the north-eastern Moravian area in 1997, it began to increase again. The legal limit of 600 Bq.kg^{-1} was exceeded in some samples of meat from wild boars. This involved mainly the age category up to one year. The radiocaesium activity concentration decreased to the level before the floods after the year 2000 [10]. Seasonal variations in the activity of ^{137}Cs were recorded in meat from wild boars hunted in south Rhineland forests. The median value in muscles was 129 Bq.kg^{-1} and the maximum reached 5,573 Bq.kg^{-1} [7]. In the period between 1998 and 2008, altogether 656 samples of muscles originating from wild boars hunted in the district Ravensburg (southern Germany) were analysed. The radiocaesium activity in these samples varied from less than 5, up to 8,266 Bq.kg^{-1}. These variations were affected by the season and climatic conditions and the related changes in the feeding habits and the availability of feed [12]. Latini [9] called attention to the above limit contamination of wild boar meat in the area of Šumava. The activity of ^{137}Cs in wild boar muscles reached up to 10,699 Bq.kg^{-1}. The highest activity of the concentration in this area was measured in 2012 when it reached 14,252 Bq.kg^{-1} [8]. One of the ways how the radiocaesium gets into the meat of wild boars is consumption of Deer Truffle (*Elaphomyces granulatus*)

which the boars may find by rooting [7]. This mushroom does not grow in the locations investigated in our study.

The results of ^{137}Cs activity in our meat samples differed (roe deer, red deer, wild boar). The highest ^{137}Cs activity measured in our study was 0.8 Bq.kg^{-1} and was determined in a sample originating from a young boar (25 kg) which corresponded with the observations of Obzina [10]. The results of the analysis of the stomach muscles indicated the considerable importance of supplementary game feeding in the nutrition of wild boars [6]. This is related to lower than minimum detectable activity of ^{137}Cs in the stomach muscles in our study.

The activity of ^{137}Cs in fish meat was below the minimum detectable limit.

Soil is one of few environmental components in which we can detect ^{137}Cs even today. Retention of radiocaesium is the highest in clay minerals. The residual fraction of this radionuclide is the most important one [3]. The highest activity of ^{137}Cs in the samples of soil collected in our study, were from locations where individual animals were hunted, reached 85.2 Bq.kg^{-1}. This can be explained by the clayey bedrock of the Turčianska fold and the considerably higher mobility of radiocaesium in forest ecosystems compared to soil used for intensive agriculture.

Another important environmental component capable of accumulating a larger part of ^{137}Cs is mushrooms. Mushrooms growing in conifer forests demonstrated a higher level of contamination than those growing in deciduous forests [1]. Various types of mushrooms exhibited different abilities to accumulate radionuclides from the environment. This corresponds to observation that increased accumulation of ^{137}Cs in Bay Bolete mushroom (*Boletus badius*) is caused particularly by norbadion A, which is present in the brown pigment of the mushroom capsule. In the sample of the Summer Bolete mushroom (*Boletus reticulatus*) investigated in our study, we measured an activity of ^{137}Cs, equal to 38.8 Bq. kg^{-1}.

None of the 38 various types of samples collected in the district of Turčianske Teplice exceeded the limit for radiocaesium activity concentration. This indicates a low contamination with radionuclides in this area and no increased risk to human health.

ACKNOWLEDGEMENT

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CHARACTERISATION OF SELECTED TUMOUR LINES WITH RESPECT TO HYALURONAN BIOLOGY

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ABSTRACT

Hyaluronan (HA) is a high molecular weight polysaccharide and the principal component of intercellular mass (extracellular matrix). Its main role is to hydrate and lubricate a large number of tissues, particularly of; cartilage, skin, and vitreous humour of the eye. After binding to relevant receptors, hyaluronan changes and affects some of the processes in the organism. It participates in many physiological and pathological processes. It affects inflammatory processes and plays an important role in the development of tumours. This study characterizes selected tumour lines from the point of view of production and degradation of hyaluronan. It involved the preparation of cell cultures and observation of the influence on these cultures by modification of the medium, from serum-containing to serum-free medium. The RT PCR method was used to determine the expression of hyaluronan receptors, binding proteins and compounds needed for its degradation and metabolisation. The hyaluronidase activity of media obtained from individual tumour line was determined.

Key words: cancer; hyaluronan; hyaluronidase

INTRODUCTION

One of the most common hyaluronan (HA) receptors is the CD44 receptor which mediates cell interactions with hyaluronan [4]. These interactions are very important for the correct course of some physiological functions, such as cell aggregation, migration, proliferation and cell activation, adhesion of cells and HA endocytosis. Hyaluronan synthetases, Has1, Has2 and Has3 play an important role in HA synthesis. These enzymes are involved in physiological and pathological processes throughout life [3]. Hyaluronidase mediates the degradation of HA, and thus increases the permeability of the connective tissue, reduces the viscosity of body fluids, and is involved also in bacterial pathogenesis and the spreading of toxins and poisons.

HA closely participates in the biology of cancer [2]. It accumulates in the stroma of various human tumours and modulates; intracellular signal pathways, cell proliferation, motility, as well as the invasive properties of malignant cells. The high content of stromal HA is associated with poorly differentiated tumour and is responsible for the aggressive behaviour of adenocarcinomas. The HA receptors, hyaluronan synthases and hyaluronan-degrading en-

zymes affect progress of carcinomas depending on the type of tumours [1]. HA affects the development of tumours also by its fragments which participate in intracellular signalisation and angiogenesis.

MATERIALS AND METHODS

In our study, mRNA was isolated from cells incubated in two medium variants. One portion of cells was incubated in serum containing (10 % denaturated foetal bovine serum) medium and the second portion, in a serum-free medium for 24 hours. Transcription of mRNA to cDNA was achieved by means of a kit, High Capacity RNA-to-cDNA (Life Technologies Corporation), using a thermocycler Bioer GENE PRO and the following thermal and temporal protocol: I. 37 °C/59 min; II. 95 °C/5 min; III. 4 °C/ up to the removal of the samples. Samples of cDNA were diluted with water for injection at a ratio of 2 : 198. The reaction mixture for the PCR (Taqman® Fast advanced master mix) was prepared by mixing the probe (10 µl and 1 µl) with 9 µl of the sample. The following probes were used: CD 44, HYAL-1, HYAL-2 a HYAL-3. The samples (10 µl) were pipetted to a plate in doublet, vortexed and spinned. The RT PCR method temperature/time programmed (Stage Holding) was used for measurement; 95 °C/20 s, II (Cycling Stage) 95 °C/1s, 60 °C/20s (cycles are repeated for approximately 40 min). The results were evaluated on the basis of the highest gene expression in a medium containing 10 % of the serum which was selected as a calibrator.

The samples for measurement of the hyaluronidase activity were prepared by mixing 1 % hyaluronan in 1.5 MDav acetate buffer and 1 % agarose (10 mg.ml^{-1}) in an acetate buffer at a ratio of 1 : 1. Bovine testicular hyaluronidase (6.25 U.ml^{-1}) was used as a positive control, and sodium aurothiomalate (100 mg.ml^{-1}) as a negative control. The samples of the conditioned media which were prepared by aspiration of media from tumour cell lines after 24 hour incubation, were applied to the gel. The samples, as well as the negative and positive controls were used in 50 µl amounts. The gel, with samples, was incubated for 24 hours, after which time, the solution above the gel was removed by a pipette, and the gel was washed with isopropyl alcohol. The high molecular mass hyaluronan (non-degraded) was precipitated with 1 % cetylpyridium chloride. The hyaluronidase activity of the conditioned media was determined by the measurement of the transmittance of the samples by an ELISA reader at a wavelength of 595 nm. Table 1 shows the tumour lines used in these experiments.

RESULTS AND DISCUSSION

Expression of genes CD44, Hyal-1, Hyal-2 and Hyal-3

Transcription of CD 44 gene (Fig.1) showed that cells of the colon tumour lines (HCT-116, HT-29, Caco-2) and melanoma line, expressed CD 44 gene more intensively in serum-free medium, while the expression of this gene by the mammary and pulmonary cell lines (MDA-MB-231, A-549) was decreased in this medium.

The mammary line MCF-7 showed almost zero expression of CD44 gene. The transcription of the Hyal-1 gene (the so-called serum or lysozomal hyaluronidase), occurred in cell lines HCT-11 and Caco-2, and to a smaller extent, also in A-549, however not in such intensity as observed for fibroblasts (Fig. 2). It is interesting that the expression exhibited by these lines increased during cultivation in the serum-free medium. With regard to the presence of Hyal-1 in the serum, it is possible that this increase constitutes a compensation reaction to the deficiency of the degradation enzyme. Such an increase was not observed in fibroblasts, whereas, on the contrary, complete inhibition of the Hyal-1 expression was observed after withdrawal of the serum.

Contrary to Hyal-1, another form of hyaluronidase, Hyal-2, was detected in all tested types of cells. This enzyme is essential for the degradation of the extracellular matrix containing high molecular mass hyaluronan and produces larger hyaluronan fragments which are subsequently subjected to final cleavage by lysozomal Hyal-1. It was interesting that the HCT-116 tumour line exhibited a 5-fold higher presence of Hyal-2 gene in serum-free medium compared to serum containing medium (Fig. 3).

Surprisingly, Hyal-3 gene was also detected in all tested cells, although its expression is mostly described in the bone marrow and testes. The Hyal-3 gene was expressed more intensively in serum-free media, with the exception of NHDF and THP-01 cells. The Caco-2 line expressed the Hyal-3 gene more in the serum-free medium. Expression of the Hyal-3 by the leukemia line THP-01 was about 60 % lower than the expression of this gene by the fibroblasts (Fig. 4).

Table 1. List of tumour cell lines used in the study

Caco-2	Colorectal adenocarcinoma	A-2058	Melanoma
HCT-116	Colorectal adenocarcinoma	A-549	Alveolar adenocarcinoma
HT-29	Colorectal adenocarcinoma	NHDF	Normal dermal fibroblasts
MCF-07	Mammary adenocarcinoma	THP1	Promyelocytic leukemia
MDA-MB-231	Mammary adenocarcinoma		

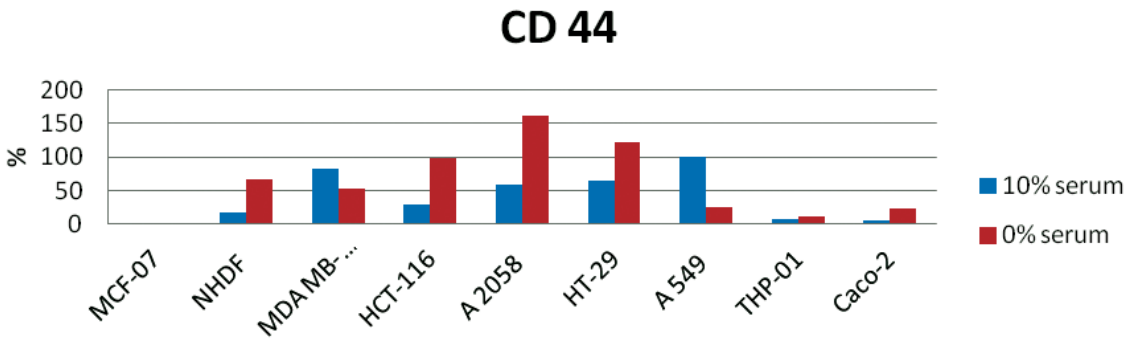

Fig. 1. Expression of CD 44 gene

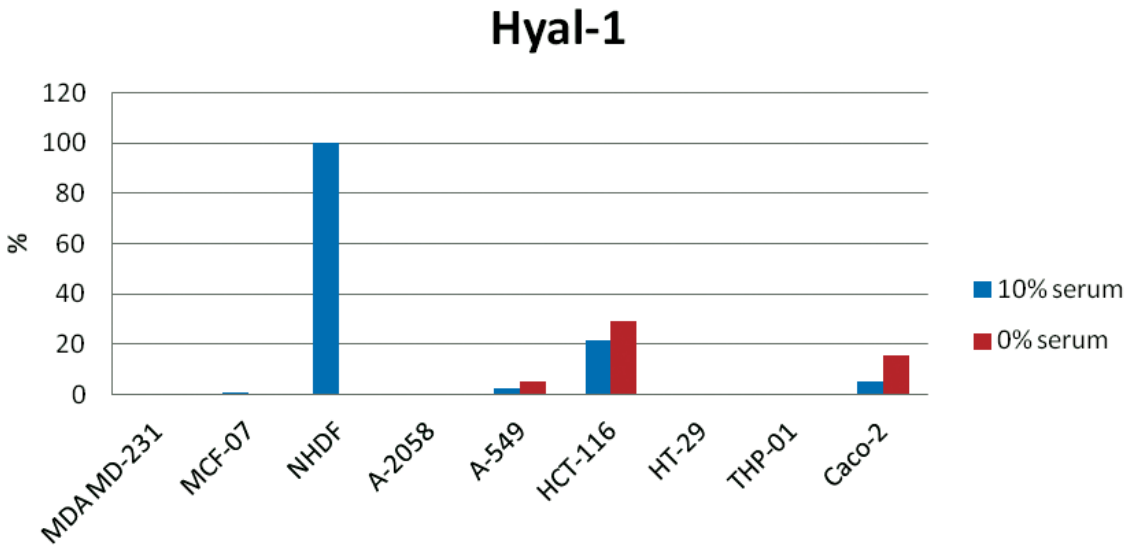

Fig. 2. Expression of Hyal-1 gene

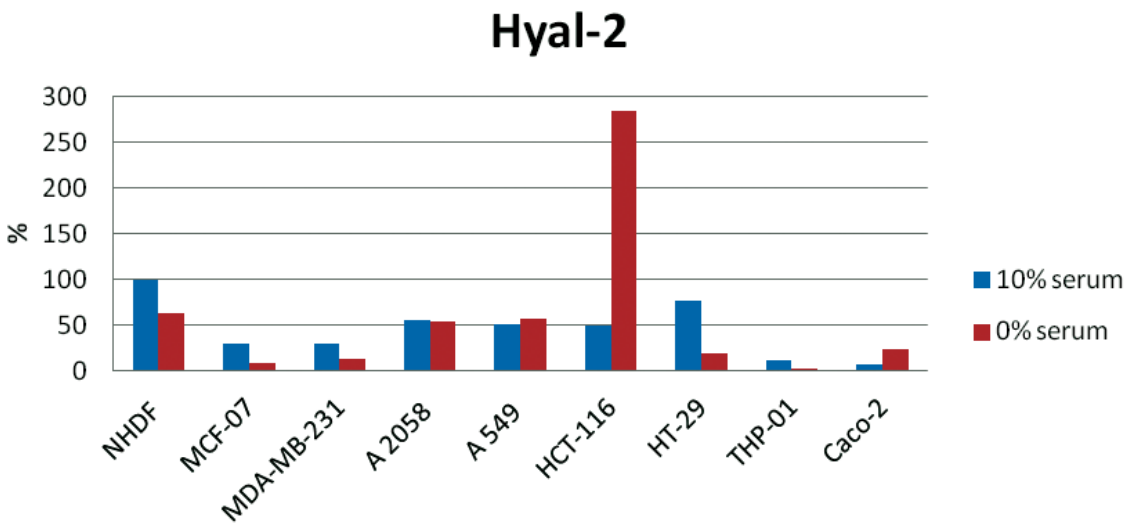

Fig. 3. Expression of Hyal-2 gene

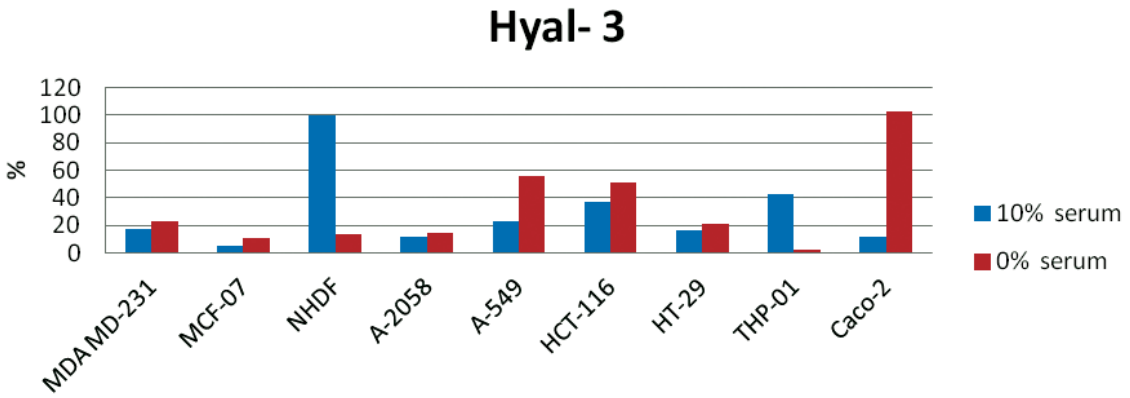

Fig. 4. Expression of Hyal-3 gene

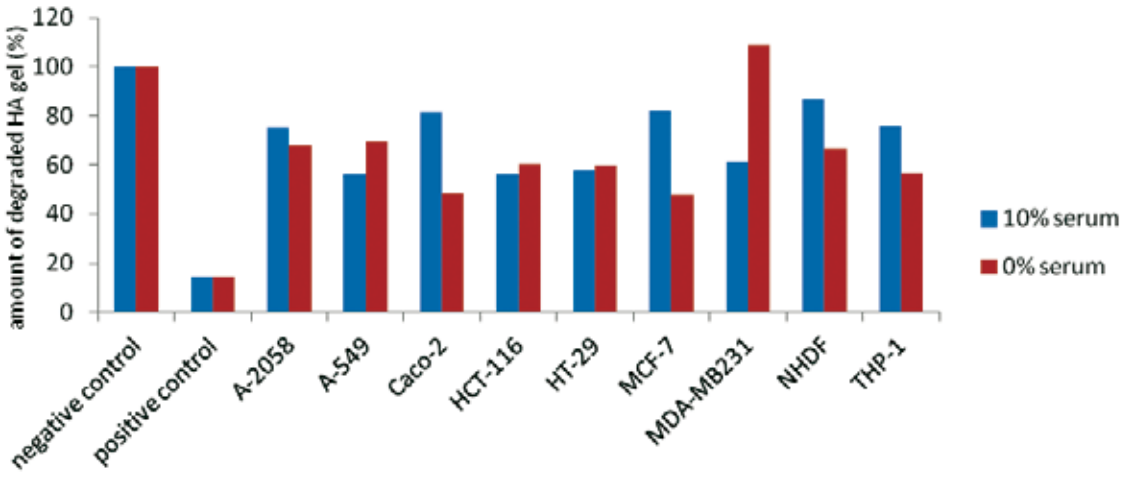

Fig. 5. Hyaluronidase activity of conditioned media

Hyaluronidase activity of conditioned media

The hyaluronidase activity of cell culture media depends on the content of serum in these media. While cells A-2058, Caco-2, MCF-07, NHDF and THP-01 showed lower activity in serum media, which degraded lower quantity of HA, with cells A-549 and MDA-MB 231, the quantity of degraded gel was higher for serum media. In the case of the intestinal cells, Caco-2 increased activity correlated with the increased expression of all measured hyaluronidases by means of the PCR. This trend was, however not observed for the line HCT-116, which also at the RNA level, showed higher an expression of Hyal in serum-free medium (Fig. 5).

CONCLUSIONS

The change of the medium, from serum containing medium to serum-free, affects cells from many aspects. The deficiency of growth factors present in the serum caused a slower growth of the tumour cell lines. The gene expression for cell surface receptors and enzymes needed for metabolism of HA also changed.

The comparison of mammary tumour lines MCF-07 and MDA- MB 231 with regard to transcription of the CD44 gene, showed that the tumour line MCF-07 expressed this gene to a lower extent (5 %) compared to MDA-MB 231 (80 %).

The expression of genes associated with the metabolism of HA, either synthesis or degradation, is an indicator of the invasiveness of the mammary tumour lines. While the less invasive line (MCF-07) showed a low expression, the expression in MDA-MB 231 was higher.

Hyaluronidase activity was determined in the media which degraded the HA gel. The influence of serum on this activity was considerable in cells MDA-MB 231, whereas, in accordance with PCR results, the serum containing medium degraded 40 % of the hyaluronan. The tumour lines HCT-116 and HT-29 degraded the same amount of HA in the serum containing and serum-free media. With the Caco-2 cells, the degradation of HA gel was lower in the serum containing medium. The media from the cells of MCF-07 degraded HA gel more in the serum-free medium. The degradation of the HA gel by the cell cultures; THP-01, A-549, A-2058 and NHDF, was lower than 40 %.

Our results confirm the cardinal influence of cultivation conditions and their importance for good characterisation of in vitro cell models intended for basic research or as a tool for the development and testing of new biomaterials, or medicinal products based on specific interaction of hyaluronan with cell receptors or enzymes.

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MONITORING OF THE NEPHROTOXICITY OF SELECTED ANTIMYCOTICS USING A REAL-TIME CELL ANALYSER

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ABSTRACT

Antimycotics commonly used in the therapy of yeast infections are known for their nephrotoxic and hepatotoxic effects. The aim of this study was to test the effect of selected antimycotics (amphotericin B, fluconazole and itraconazole) on a model rabbit kidney RK13 cell line. The viability of cells after their exposure to antimycotics was evaluated by two methods. The first method monitored the cells in real time using xCELLigence system (Real-TimeCellAnalyser RTCA). For 60 hours in 1 hour intervals the analyser observed the response of cells treated with antimycotics, expressed as the cell index (CI). Twenty four hours after the treatment of the cells by antimycotics, the MTT assay (the second method) was used to confirm their toxic effects. The MTT colorimetric test serves to determine the metabolic activity of the cells. The first method confirmed nephrotoxicity of all three antimycotics and significant differences (P < 00001) were observed with all tested concentrations with the exception of itraconazole at 0.05 mg.l^{-1}. The MTT test confirmed the decreased metabolic activity of the treated cells for all three antimycotics. Significant differences (P < 0.0001) in comparison with the control were observed regarding the effect of all three medicines at all tested concentrations, with the exception of itraconazole at the lowest tested concentration of 0.005 mg.l^{-1}.

Key words: antimycotics; MTT; nephrotoxicity; RTCA

INTRODUCTION

Despite advanced pharmaceutical research, mycotic infections have spread considerably over recent years. This is ascribed to; a higher frequency of surgical interventions, occurrence of the HIV virus, use of immunosuppressives and antibiotics, generally decreased immunity, and the increased possibility of becoming infected [4].

Besides the beneficial action of medicines one must consider also their undesirable effects. Kidneys are the most important elimination organ and the nephrotoxicity of some antimycotics is so serious that it requires attention in order to prevent their fatal consequences. Many

methods are available for the determination of cytotoxicity. Currently, there is an effort to prefer in vitro tests over in vivo tests, for ethical and economic reasons, but also for the rapidity of the former.

MATERIALS AND METHODS

Three antimycotic medicines were tested in our study, amphotericin B, itraconazole and fluconazole (Sigma Aldrich, Germany). Their stock solutions were diluted with 1 % DMSO (dimethyl sulfoxide) to individual concentrations presented in Table 1. Suspension of 24-hour cell cultures of rabbit kidney cells RK13 with a density of 15,000 cells per well, were used as a model cell line.

Real-time monitoring of cells using the Real-TimeCellAnalyser (RTCA)

The RK13 cells were seeded in 16-well E-plates with gold electrodes (Roche AppliedScience, Germany) at a density of 15,000 cells per well. After 24-hour cultivation, tested concentrations (Table 1) of antimycotics were added to the cells. The monitoring of the cell response was carried out by the xCELLigence system (Acea Biosciences Inc., Canada) throughout the experiment (60 hours). Electric resistance, which changes with the intensity of adherence of cells to the plates with gold micro-electrodes, is expressed as cell index (CI) and plotted into curves; the bigger the resistance recorded by the system, the more cells have adhered to the plate surface and the higher the cell index [6]. All changes in the cell index were recorded by the software in the form of curves showing the relationship between the cell index CI and time t.

MTT test

The MTT test was carried out in parallel with the RTCA. The cells were seeded into 96-well micro-plates (Greiner Bio One, Greiner, Germany). After 24-hour cultivation they were treated in the same way with antimycotics (24 hours). The principle of the test is based on the ability of vital cells to metabolize yellow-stained MTT (3-(4,5-dimetyltiazol-2-yl)-2,5-difenyltetrazolium bromide) to blue-stained insoluble formazan. The intensity of staining expressed as an absorbance was observed spectrophotometrically (BioTecReader) at 570 nm wavelength [7].

The results were processed by means of the statistical software Graf PadPrism 4.0, using the one-way Anova test (Dunnet's comparison test) to compare groups of results of individual triplicates with a control group. The differences at the level of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The effects of antimycotics on the proliferation of cells were recorded by the xCELLigence system in the form of graphs. Significant differences ($P < 0.0001$) in CI in comparison with the control were observed for all three antimycotic medicines with the exception of itraconazole at a concentration of 0.05 $mg.l^{-1}$. At the highest concentrations of itraconazole (0.8 $mg.l^{-1}$ and 1.6 $mg.l^{-1}$) we observed a marked decrease in CI soon after 1 hour after the treatment of the cells (Fig. 1).

Amphotericin B in concentrations of 0.8 $mg.l^{-1}$ and 1.6 $mg.l^{-1}$ exhibited such toxicity that the cell index decreased soon after 1 hour of treatment. All tested concentrations of fluconazole were toxic (graphs not shown).

The results of the MTT assay revealed significant differences ($P < 0.0001$) in comparison with the control for all

Table 1. The tested concentrations of antimycotics compared with mean daily dose

Medicine	Mean daily dose [$mg.kg^{-1}$]	Final concentration of antimycotics [$mg.l^{-1}$]				
Amphotericin	1.5	1.6	0.8	0.4	0.2	0.1
Itraconazole	2	1.6	0.8	0.2	0.05	0.005
Fluconazole	0.5	0.512	0.256	0.128	0.0512	0.005

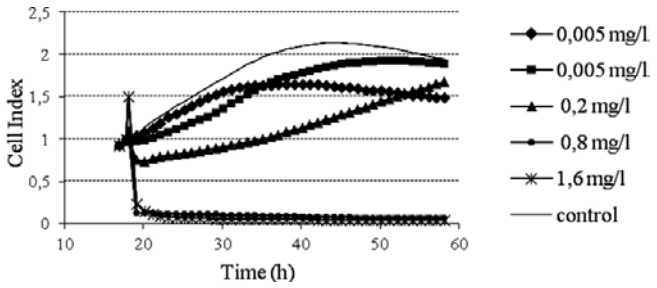

Fig. 1. Real-time changes in the cell index (CI) after treatment with itraconazole

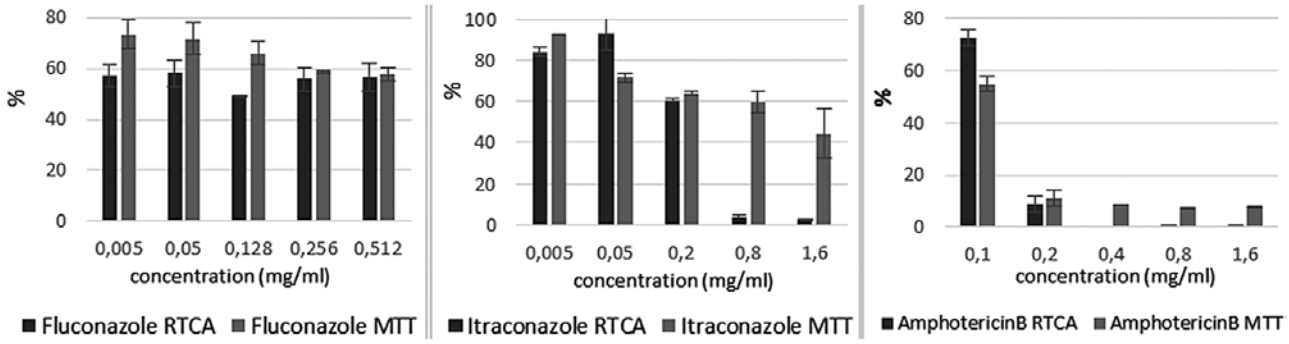

Fig. 2. Comparison of the proliferation and metabolic activity of cells exposed to the tested antimycotics (%)

concentrations of the tested antimycotics with the exception of itraconazole at the lowest concentration (0.005 mg.l^{-1}; P > 0.05). For easier comparison of both methods, the results were expressed as a percentages by means of a formula:

% = value of sample × 100/value of control

Both tests proved the comparable toxicity of amphotericin B, depending on the concentrations. The percentage difference in values between MTT and RTCA were caused by different principles of these methods. The cells exposed to amphotericin B at concentration of 0.1 mg.l^{-1} were still adhered relatively well, but their decrease in metabolic activity was more pronounced. The cells treated with fluconazole and itraconazole showed a contradictory effect, i. e. the cells had decreased ability to adhere and proliferate, but their metabolic activity was somewhat higher (Fig. 2).

The use of antimycotics increased over recent years for several reasons. Increased number of immunocompromised patients, either due to AIDS or use of immunosuppressives, caused an increased prevalence of mycotic infections. Excess broad-spectrum antibiotic prescriptions and more frequent surgical interventions participate also in the reduced immunity of humans. Mycoses belong to troublesome infectious diseases and should not be underrated. They require long-term treatment and considerable patience. In addition, to their specific effectiveness against some mycotic agents, antimycotics also have some serious undesirable side-effects. The most frequent and very serious side-effects are nephrotoxicity and hepatotoxicity. Our study focused on the nephrotoxic effects of three antimycotics of amphotericin B, itraconazole and fluconazole, and the tested concentrations were derived from therapeutically effective concentrations.

Currently, the only over-the-counter (OTC) peroral medicine with antimycotic effects in Slovakia, is MYCOMAX. It contains, in one capsule, 150 mg of the active ingredient fluconazole, and the daily therapeutic dose for adults of this antimycotic, is 50—400 mg. Incorrect dos-

age or repeated use of the recommended dose can result in overdose and damage to kidneys [3]. This was confirmed also by our tests, as fluconazole caused a significant decrease in adherence and metabolic activity of cells even at the lowest tested concentrations in comparison with untreated cells. By substituting the antimycotic therapy, one can affect the influence on renal function. Amphotericin B, a broad-spectrum antimycotic, is currently the treatment of choice for a number of serious mycoses. The nephrotoxicity is the most serious complication of the treatment with amphotericin B and is related to its high potential accumulation or by combining this medicine with diuretics or other nephrotoxic medicines [1]. Lipid forms of amphotericin B, such as liposomal amphotericin B, amphotericin lipid complex and amphotericin B colloid dispersion, are the options for reducing its toxicity. Studies have shown that these new forms of amphotericin, reduced the nephrotoxicity of this antimycotic by 50 % [2]. Another possibility is the alternation with new broad-spectrum antimycotics, such as, caspofungin and voriconazol. By this alternation, the incidence of renal disorders decreased from 10 % to 1 % [5].

CONCLUSIONS

Our results showed that the nephrotoxic effects of amphotericin B were considerable at the lowest tested concentration of 0.1 $mg.l^{-1}$, while the maximum therapeutic daily dose is 1.5 $mg.l^{-1}$. Therefore, it is very important to consider carefully therapy with amphotericin B, particularly in patients with renal disorders. All concentrations of fluconazole (0.005—0.512 $mg.l^{-1}$) resulted in significant cytotoxic effects and the differences in toxicity between individual concentrations were not pronounced. Real-time monitoring of cells (RTCA) confirmed the nephrotoxic effects of all tested concentrations with the exception of the concentration with 0.05 $mg.l^{-1}$. The most pronounced changes were detected in the cells exposed to concentrations of 0.8 $mg.l^{-1}$ and 1.6 $mg.l^{-1}$. The MTT test showed significant differences in comparison with the control, for all tested concentrations of Iitraconazole, with the exception of the lowest one (0.005 $mg.l^{-1}$).

All tested concentrations confirmed the nephrotoxic effects of antimycotics. Thus, the medicines with lower nephrotoxic effects or new medicinal forms, for example liposomal amphotericin B with lower nephrotoxicity, appear to be the most suitable medicines for the treatment of mycoses.

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DETERMINATION OF CAPSAICIN IN FRUITS OF THE *GENUS CAPSICUM* BY HPLC

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ABSTRACT

Capsaicin is a plant alkaloid obtained from plants of the genus *Capsicum* (pepper, family *Solanaceae*). Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin are called capsaicinoids and are produced as secondary metabolites by chilli peppers. At the present, capsaicin and extracts of fruits of the genus *Capsicum* are components in weight loss medicines and in products for local application intended for the alleviation of pain and inflammation. Their prophylactic effect on gastrointestinal mucosa has been confirmed. In this study, we analysed the representatives of 16 varieties: Bhut Jolokia; Habanero; Naga; Trinidad Scorpion Moruga; Fatali; Carolina Reaper; 7Pod; obtained from William ChiliProducts and cultivated on the premises of the University of Veterinary Medicine and Pharmacy in Košice, SR. Methanol extracts of dried powdered drugs were analysed by high-performance liquid chromatography (HPLC). The concentration of capsaicin in fruits of individual varieties ranged from 5.87 mg.g^{-1} DM (Habanero Peruvian White) to 77.70 mg.g^{-1} DM (Trinidad Scorpion Moruga Red). The pungency values (Scoville Heat Units — SHU) for this range, were 94,024 and 1,234,280 SHU, respectively. The varieties with high pungency level have high potential for the use in practice for the extraction of capsaicin.

Key words: capsaicin; *Capsicum*; HPLC

INTRODUCTION

Fruits of the genus Capsicum, family Solanaceae, are a source of capsaicinoids and the best known alkaloid capsaicin. Capsaicin and dihydrocapsaicin, the most abundant and potent analogues in pepper are responsible for 90% of the pungency [1]. The potential of these compounds far exceeds their role as spices used in the food industry. Capsaicinoids are known for their pharmacological properties, for example, as chemoprotectors against the development of tumours [10], antimicrobials [3], antioxidants [6] and analgesics [7]. The anti-tumour effect is closely related to the induction of apoptosis and prevention of proliferation or migration [8].

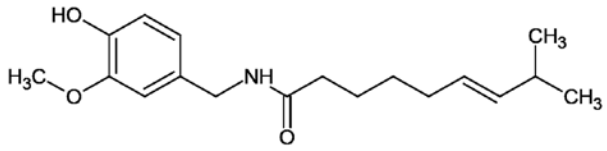

Fig. 1. Formula of capsaicin (8-Methyl-N-Vanillyl-6-Nonenamid)
Source: Author

The aim of this study was to determine the content of capsaicin in 16 Capsicum varieties by HPLC method and compare the content of capsaicin in varieties grown in the Slovak Republic with its concentration in varieties grown abroad.

MATERIALS AND METHODS

The pepper seeds were sown to cultivation containers on February 25. The following cultivation conditions were observed: temperature during germination 26—28 °C and during the growth 18—20 °C; and light regimen 12/12 h, later 16/8 h. (Elektrox 200W GROW). The peppers for analysis was harvested after reaching botanic maturity and were dried at 41 °C for 72 h. Additional material for analysis was provided by Martin Šuran and Miroslav Klen from Villiam Chilli Products Trnava, SR, cultivated, collected and dried under the same conditions. Samples for analysis were prepared by maceration of homogenized (powdered) dried peppers with methanol, 1 : 10, at laboratory temperature in the dark, for 24 h. The obtained extracts were filtered through a microfilter intended for high-performance liquid chromatography (HPLC).

Chemicals and equipment used for HPLC analysis

Acetone p. a.; formic acid p. a.; methanol p. a. (Penta); acetonitrile and capsaicin (Sigma Aldrich) [9]; deionized water for HPLC; microfilter for HPLC 0.2 µm; analytical balance KERN EW 620-3NM (Kern, Germany); apparatus for preparation of water for HPLC Aqua MAX BASIC 360 series, Aqua MAX ULTRA 370 series (ATB Umwelttechnologien, Germany); ultrasonic bath Polsonic; analytical HPLC Agilent 1100, detector DAD UV/VIS (Agilent Technologies, Germany), constituted the chemicals and equipment used for analysis.

Quantitative analysis

The method used for a more rapid HPLC separation, was used under the following conditions: stationary phase – analytical column Ascentis® Express C8 (100 × 2.1 mm, 27 µm); gradient elution (Tab. 1); injected volume 1 µl; column temperature 40 °C; flow rate 0.3 ml.min^{-1}; and UV detection at λ 254 nm, 280 nm.

For calculation, a calibration curve was constructed on the basis of the measurement of a capsaicin standard, using the following injected volumes 0.5 µl, 1 µl, 2 µl, 5 µl, 7.5 µl, 10 µl.

The solutions of samples were prepared in the concentration of 0.1 g.ml^{-1}, so the injected volume of 1 µl contained 1 µg of the sample. The mass of substances in the samples was calculated from the calibration curve on the basis of the peak area corresponding to the respective standard. The calculated mass of capsaicin in the injected volume was recalculated per the volume of extract and then for the content in the entire fruit according to the formulas below.

Table 1. HPCL gradient parameters

Time [min]	Acetonitrile	0.2 % HCOOH
0	20	80
20	64.5	35.5
20.1	100	0
25	100	0

Calculation of capsaicin content

Concentration of capsaicin in the injected volume
(z1) = y × AUC [µg]
Concentration of capsaicin in total extract volume
(z2) = volume of methanol [ml] × 1,000 × z1
Volume of capsaicin in the sample
(z3) = z2/1000 × m sample [g]
Recalculation per Scoville Heat Units (SHU):
SHU = z3/1000 × 16,000,000

Table 2. Concentration of capsaicin in the analysed samples and the values of SHU

Sample No.	Variety	Capsaicin content [mg.g^{-1} DM]	Scoville Heat Units
1	Bhut Jolokia	41.33	661, 360
2	Habanero Red Savina	27.2	435, 328
3	Habanero Giant White	19.69	315, 192
4	Habanero Peruvian White	5.87	94, 024
5	Naga Black	20.93	334, 904
6	Naga King	40.78	652, 560
7	NagaYellow	20.34	325, 568
8	Trinidad Scorpion Moruga Red	77.70	1, 234, 280
9	Trinidad Scorpion Moruga Yellow	20.40	326, 488
10	Carolina Reaper	33.40	534, 432
11	7 Pod BrainStrain	18.04	288, 704
12	7Pod Primo	44.44	711, 144
13	7Pod Congo SR GAG	32.25	516, 072
14	7Pod Barackpore	36.85	589, 720
15	Fatali Brown	46.90	750, 432
16	Fatali Black	24.92	398, 808

RESULTS

The analysis of chromatograms at the wavelengths of 254 nm or. 280 nm confirmed the presence of the analysed component in the samples. Figures 2 to 6 show selected chromatograms of samples arranged according to cultivars. Comparison of sample chromatograms and standard UV spectra showed agreement for all samples. On the basis of the agreement of the UV spectrum and retention time, we concluded that the prepared standard was capsaicin (Fig. 7), and was present in our standard in a 96.8 % concentration. The respective ratio was used also for the calculation of the content of capsaicin in the samples.

Table 2 shows the content of capsaicin in mg.kg^{-1} dry matter (DM) and the calculated Scoville Heat Units (SHU). The table shows that the capsaicin content exceeded 30 mg.kg^{-1} (500,000 SHU) in half of the samples. The lowest value (5.87 mg.kg^{-1}) was measured in Habanero Peruvian White and the highest (77.70 mg.kg^{-1}) in Trinidad Moruga Scorpion Red.

DISCUSSION

A wide range of substances have been isolated from the fruit of Capsicum plants including; flavonoids, vitamin C, vitamin E, many carotenoids (particularly capsorubin) and alkaloids [1]. The most important alkaloid, capsaicin, is present in all varieties but in different concentrations [11]. We selected representatives of the genus Capsicum chinense with the potentially highest contents of capsaicin [2, 4]. Methanol was used as an extraction agent as it ensures better solubility of capsaicin at HPL analysis [5]. The highest concentration of capsaicin was determined in Sample 8

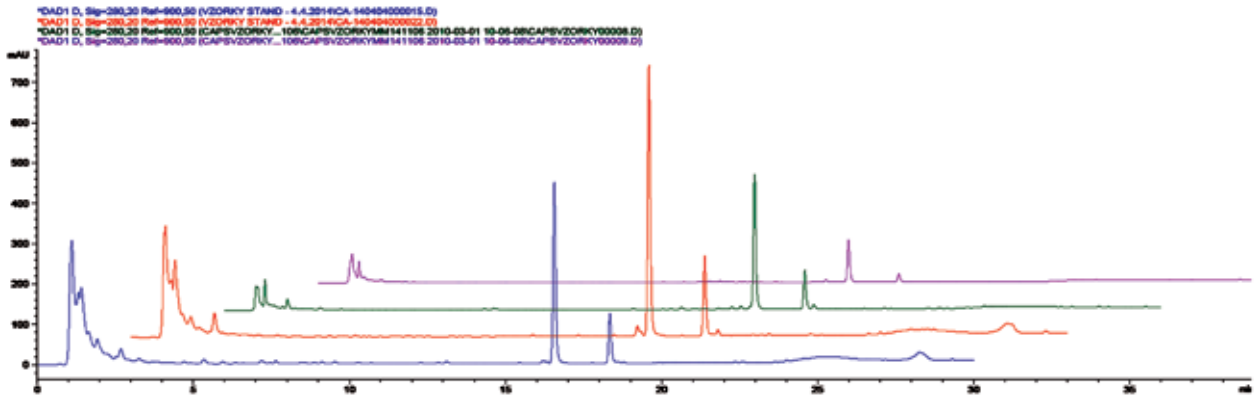

Fig. 2. Chromatograms of the varieties of Habanero Red Savina (blue), Bhut Jolokia (red), Habanero Giant White (green), and Habanero Peruvian White (pink)

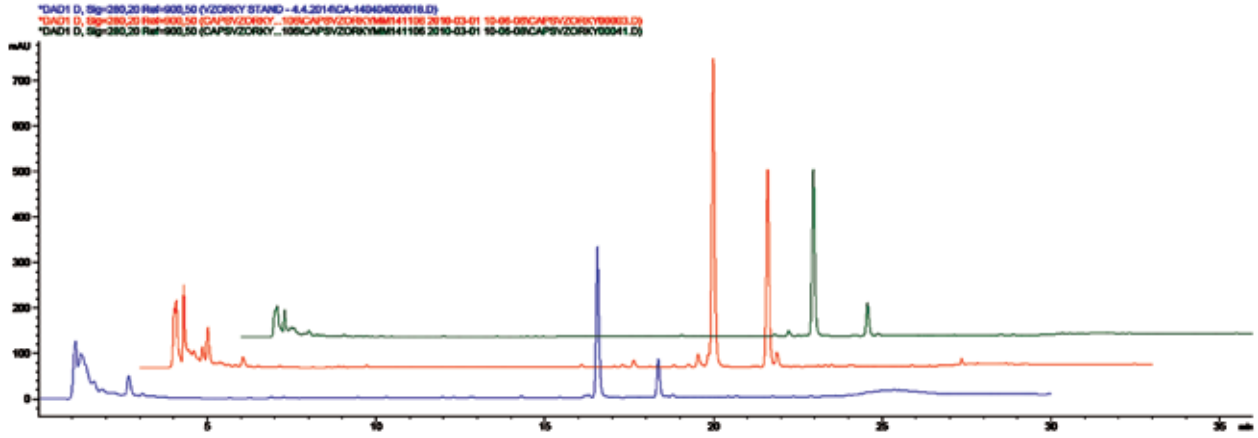

Fig. 3. Chromatograms of the varieties of Naga Yellow (blue), Naga King (red) and Naga Black (green)

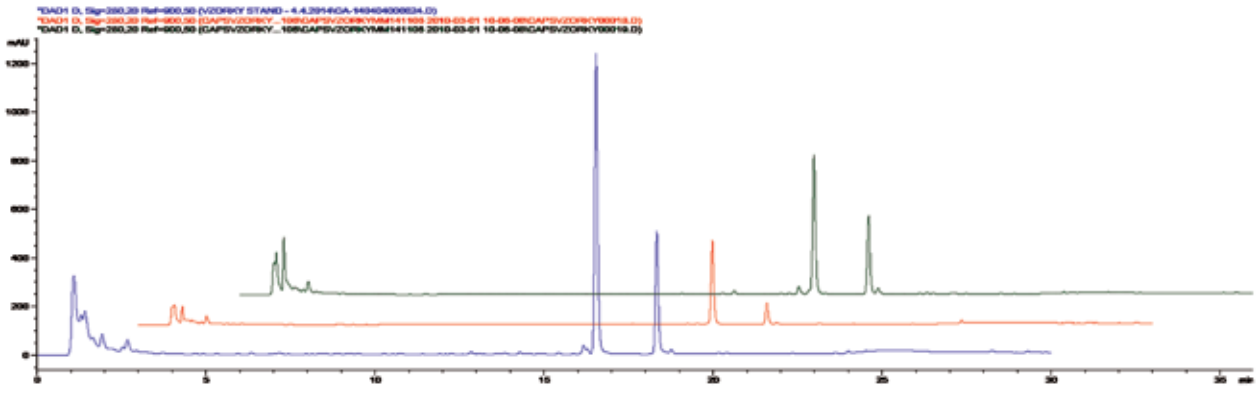

Fig. 4. Chromatograms of the samples of Trinidad Moruga Scorpion Red (blue), Trinidad Moruga Scorpion Yellow (red), and Carolina Reaper (green)

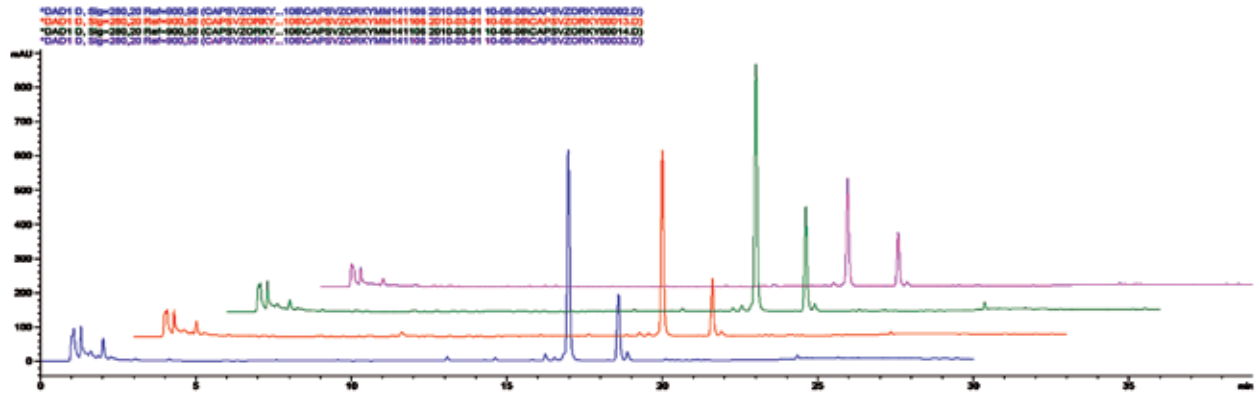

Fig. 5. Chromatograms of the samples of 7 Pod Barackpore (blue), 7 Pod Congo SR GAG (red), and 7 Pod Primo (green)

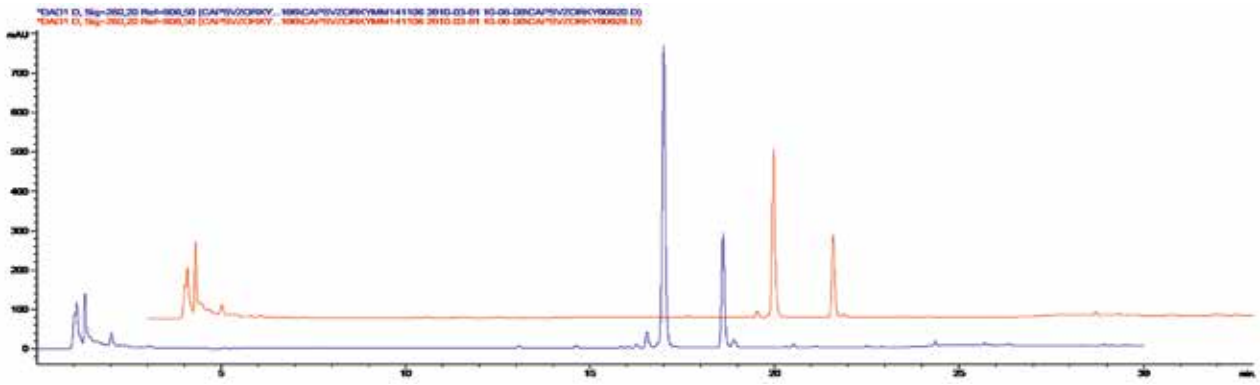

Fig. 6. Chromatograms of the samples of Fatali Brown (blue), and Fatali Black (red)

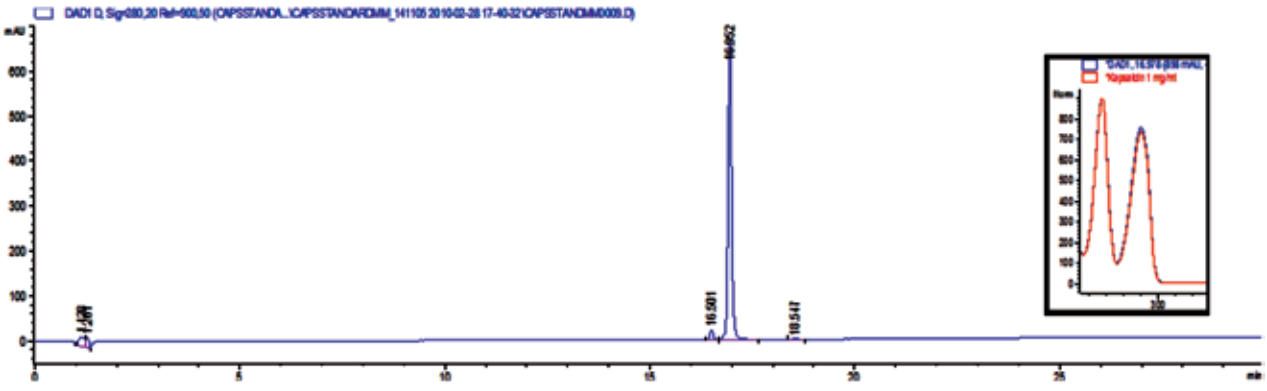

Fig. 7. Chromatogram of the capsaicin standard of concentration 1mg.ml^{-1}
The highest peak (retention time RT = 16.952) represents capsaicin (96.8 %), the small previous peak (RT = 16.501) nordihydrocapsaicin and the last small peak (RT = 18.547) is the second most important capsaicinoid – dihydrocapsaicin

(Trinidad Moruga Scorpion Red, 77.70 mg.kg^{-1}) which reached the pungency level of 1,234,280 SHU [8]. A study conducted in 2012 at the State University in New Mexico reported that the mean of four measurements for this pepper reached 1,463,700 SHU. The difference (229,420 SHU) can be related to the local microclimate which differs from that in Slovakia, particularly by temperatures during summer and also by relative humidity. An important factor affecting the concentration of capsaicin is the daylight length. After publication of the above mentioned results, this variety was recorded in the Guiness Book of World Records as the hottest on Earth. The above test included, also variety Bhut Jolokia which reached 1,019,687 SHU while the Jolokia cultivated in Slovakia reached a pungency level of 661,360 SHU. We can assume again that this was caused by differences in climate [2].

The results obtained in our study showed the highest level of capsaicin in the samples of Trinidad Moruga Scorpion and 7Pod Prim. They are very hot varieties with a high concentration of capsaicin and thus potentially suitable for extraction of capsaicin intended for use in medical preparations and pharmacy. They are also excellent for consumption, due to their high content of vitamin C and capsaicinoids.

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CORDYCEPS SINENSIS: DETERMINATION OF TOTAL ANTIOXIDANT ACTIVITY OF THE ETHANOL EXTRACT

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ABSTRACT

Cordyceps **are exotic rare curative parasitic fungi used in traditional Chinese medicine (TChM) from ancient times. The beneficial effects of several *Cordyceps* species have been known for more than 2000 years. The generic name, *Cordyceps* is derived from the Latin words "cord" and "ceps", meaning "cub" and "head". The shape and importance is related to the origin of the Slovak name "žezlovka" for these fungi. The name used for these fungi in China is "Dong Chong Xia Cao", translated as „worm in winter grass in summer". *Cordyceps sinensis* is an Ascomycetes fungus closely related to the mushroom; although, taxonomically it is not a mushroom. The *C. sinensis* body contains mummified remains of insect larvae, particularly the Himalayan Bat Moth Hepialis armoricanus. In 2008 in San Francisco, the price of naturally collected *C. sinensis* exceeded 75,000 dollars per kilogram. Due to varying proportions of chemical compounds, the best known and most interesting biological effects of this parasitic fungi include; antibacterial, antioxidant, antiviral and antitumour effects. It has a positive effect on; blood pressure, kidneys, liver and lungs, reduces blood sugar level, supports immunity and sexual potency, and acts against stress. The experimental part of our study focused on; extraction of the biologically active organic compounds from raw *Cordyceps sinensis* with ethyl alcohol, preparation of samples for the determination of the antioxidant activity using UV/VIS spectroscopy, chromatographic separation and subsequent determination of the structure in isolated compounds by means of 1D and 2D NMR.**

Key words: antioxidant activity; *Cordyceps sinensis*; DPPH˙; 1D and 2D NMR

INTRODUCTION

Cordyceps sinensis has been used in traditional Chinese medicine (TChM) for more than 2,000 years. In 1726 at a meeting of scientists in Paris, its beneficial action on the human body had been declared based on the scientific evidence [2]. The first knowledge about this fungus was ob-

tained by a Jesuit priest who chronicled his experiences with the *Cordyceps* mushroom during his stay at the Chinese Emperor's court. He brought it later back to France [4]. *C. sinensis* is found freely only at high elevations in the Himalaya mountains and thus its collecting is extremely demanding. It is found in China, Tibetan highlands, Bhutan, Nepal and the north-eastern territories of India, 3,500 to 5,000 metres above sea level [5]. For this reason, it was one of the most expensive "herbs" and thus destined for use mainly by rich families of the emperors. It was this high value, not only financial, of this fungus growing in alpine habitats which laid the basis of the precious core of Chinese medicine [1]. While nature made from this fungus a medicine in short supply, but all the more precious, the modern technologies and procedures of its cultivation strive to make this unique fungus available to the broadest possible spectrum of patients at reasonable prices. However, all of this effort would not make sense, if the health benefits of *C. sinensis* had not been proven [4]. This involves its positive effects in the treatment of; respiratory and pulmonary diseases, kidney, liver and cardiovascular diseases, hyposexuality and hyperlipidaemia. Its positive effects in the treatment of immune disorders and as a useful supplement to modern therapy of cancer have been reported as well [3].

MATERIALS AND METHODS

The analysed material originated from the Technical University in Zvolen, Slovakia. Samples of *C. sinensis* designated CS1 and CS2 were cultivated on the premises of this university. The samples differed by the host plants used for growing *C. sinensis*. The host plant for the cultivation of CS1 was proso millet (*Panicum miliaceum*), while chickpea (*Cicer arietinum* L.) served as the host plant for the cultivation of CS2. The antioxidant activity was determined by the radical scavenging DPPH˙ (1,1-diphenyl-2-picrylhydrazyl) method at λ_{max} 517 nm. The structure of the chemical compounds was determined by NMR analysis. 1H, 13C NMR spectra (ppm) were measured employing a NMR spectrometer Varian Mercury Plus (400.13 MHz) at laboratory temperature in a deuterated dimethyl sulfoxide (DMSO-d6) using tetramethyl-silan as an internal standard ($\delta = 0.00$ ppm). Protons and carbon atoms were assigned on the basis of the analysis of 1H, 13C, COSY, HSQC, HMBC and DEPT spectra. UV/VIS spectra were measured using a spectrophotometer Libra S12, in 1 cm cuvettes in methanol at laboratory temperature.

Samples weighing 10 g (CS1 and CS2) were mixed with 150 ml of ethanol in a 250 ml boiling flasks. In this way, the prepared mixtures were subjected to reflux extractions lasting 3 hours. The ethanol extracts were then obtained by filtration and evaporation of the solvent. After extraction of samples CS1 and CS2, the extracts were properly diluted with methanol to prepare for the measurement of CS1 and CS2 methanol samples of concentrations: 12.50; 6.25; 3.12; 1.56; 0.78; 0.39; 0.19; and 0.10 mg.ml^{-1}. To each of these diluted samples, we gradually added 1 ml of stock solution of DPPH˙ (1,1-diphenyl-2-picrylhydrazyl) of concentration 0.2 mmol.l^{-1}. Before use, its absorbance was measured at 517 nm against a methanol blank. The samples were then incubated in the dark, at laboratory temperature (25 °C) for 30 minutes. After this time, we started to measure the antioxidant activity of the samples. To calculate the antioxidant activity, we used the method of percentage reduction of the activity of the DPPH˙ radical solution by measuring absorbance at λ_{max} 517 nm and using the following formula: $I\ (\%) = (ASL - AVZ/ASL) \times 100$, where I is the inhibition of the activity of the DPPH˙ radical in per cent (%); ASL is the absorbance of the DPPH˙ blank; and AVZ is the measured absorbance of the sample after 30 min of incubation in the dark at laboratory temperature, after adding 0.2 mmol.l^{-1} DPPH˙ solution.

RESULTS AND DISCUSSION

The results of the measurements of antioxidant activity of the samples CS1,CS2 and their total extracts, indicated a higher total antioxidant activity for the sample CS1, compared to the sample CS2 (Fig. 1). This was confirmed by the calculation of the minimum mean inhibitory concentrations, as IC50(CS1)DPPH = 1.76 mg.ml^{-1} was calculated for the sample CS1, and as IC50(CS2)DPPH = 7.93 mg.ml^{-1} for the sample CS2; the latter value being 4.5-fold higher (Fig. 2).

In order to determine which chemical compounds participated the most in the scavenging of free radicals, we carried out separation of ethanol extracts CS1 and CS2 by column chromatography with silica gel as the stationary phase. We were successful in the separation of both extracts to several fractions (on the basis of Rf factor).

Fig. 1. *Cordyceps sinensis*

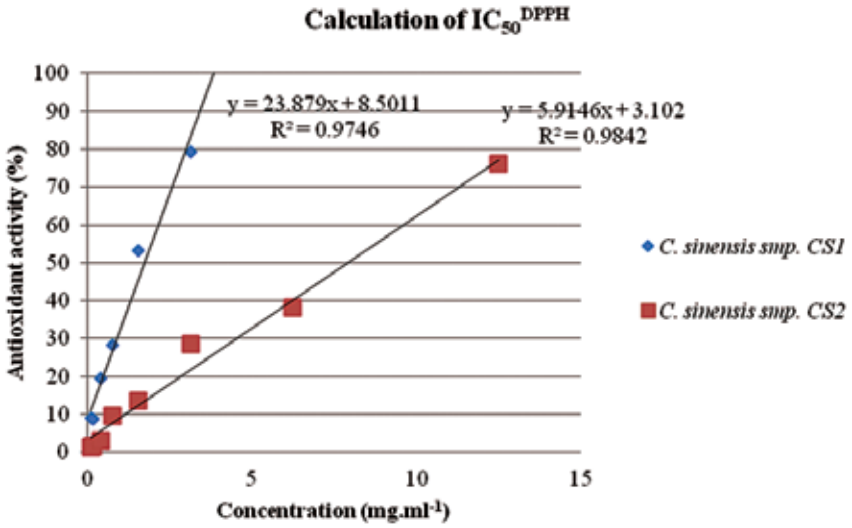

Fig. 2. Comparison of the antioxidant activity of ethanol extracts of *Cordyceps sinensis* (samples CS1 and CS2)

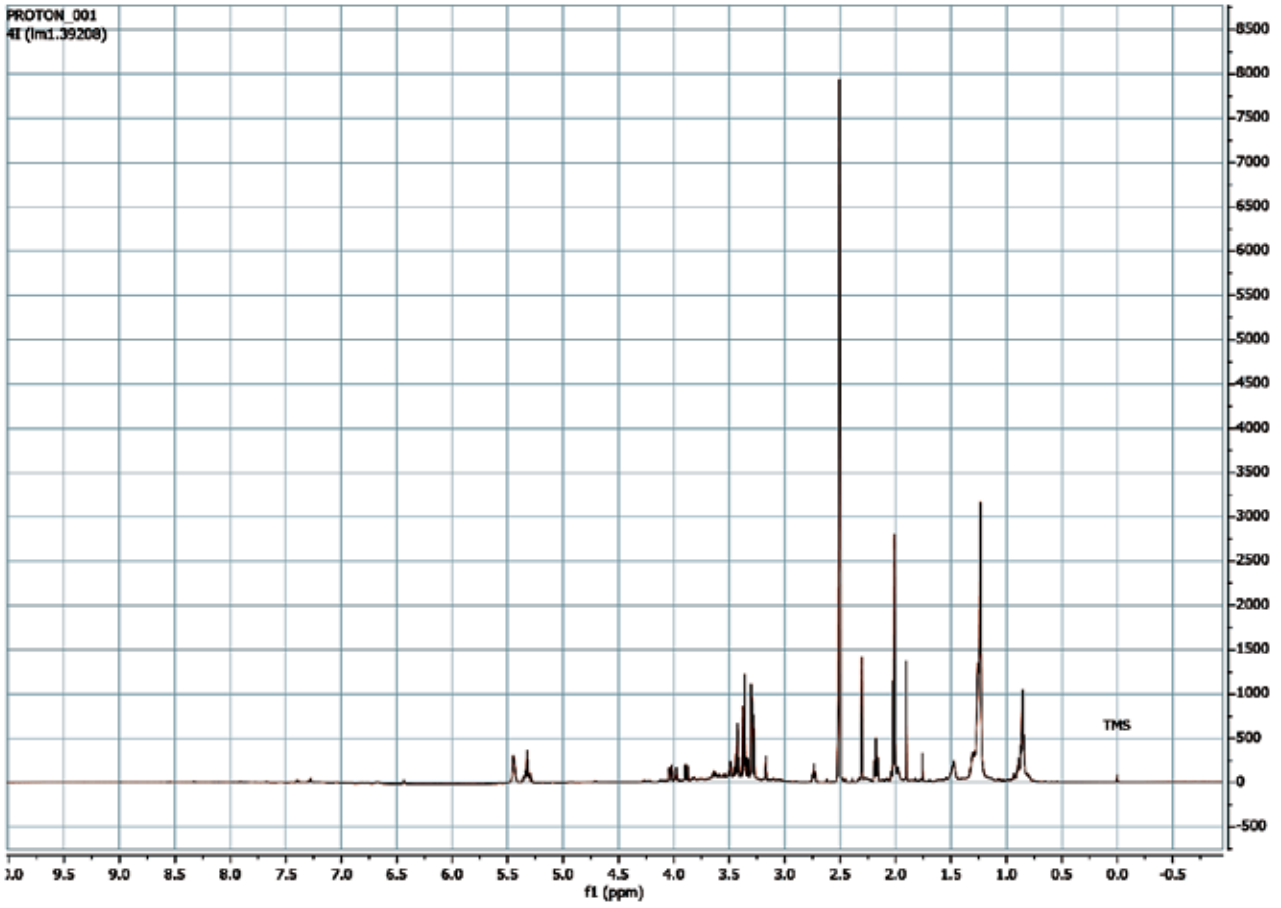

Fig. 3. 1H NMR spectrum of the most active fraction of CS1 extract (measured in DMSO)

The antioxidant activity was determined for each of the fractions in the same concentration range (12.50—0.10 mg.ml^{-1}). The results showed that the fourth fraction of CS1, had the highest polarity and also the highest DPPH˙ radical scavenging activity (IC50DPPH = 7.72 mg.ml^{-1}), while with the CS2 extract, the highest activity was observed with the second fraction obtained by separation (IC50DPPH = 12.55 mg.ml^{-1}).

Fig. 3 shows the 1H NMR proton spectrum of the fraction of the CS1 extract exhibiting the highest polarity. A detailed and demanding analysis of 1H, 13C, COSY, HSQC and HMBC spectra identified the chemical structure of one of the five compounds as cis-oleic acid.

CONCLUSIONS

The results of the antioxidant activity of the total ethanol extracts of samples CS1 and CS2, showed the higher activity of CS1 compared to CS2. This was confirmed also by the values of the mean inhibition concentrations of both extracts. The common characteristic of both samples was that the antioxidant activity of the total extracts was higher than the highest activity of any fraction obtained by column chromatography separation on silica gel. This may be caused by the mutual positive action of all chemical compounds in non-fractionated ethanol extracts. Using NMR analysis, we identified the chemical structure of cis-oleic acid as one of the components of the highest polarity fraction of sample CS1.

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COMPARISON OF MERCURY CONTENT IN FRUIT BODIES OF MUSHROOMS OF THE *GENUS PLEUROTUS* GROWING ON VARIOUS WOOD SUBSTRATES

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ABSTRACT

This study investigated the content of mercury in fruit bodies of mushrooms of the genus *Pleurotus*, namely oyster mushrooms (*Pleurotus ostreatus*) cultivated on wood logs (beech, oak, nut-tree, aspen, and poplar), and Indian oyster (*Pleurotus pulmonarius*) growing naturally on aspen. Wood-decay mushrooms are capable of accumulating in their bodies toxic elements such as mercury, which occurs at increased levels particularly in contaminated environments (e. g. close to waste dumps). Our study showed different levels of Hg in individual fruit bodies of oyster mushrooms. Those growing on beech (0.03017 mg.kg^{-1} DW), oak (0.02934 mg.kg^{-1} DW), nut-tree (0.09899 mg.kg^{-1} DW) and aspen (0.04482 mg.kg^{-1} DW) substrates contained only low levels of Hg, while this element reached considerably higher levels in mushrooms growing on poplar wood (0.84157 mg.kg^{-1} DW). The elements in fruit bodies of mushrooms vary considerably depending on composition and contamination of the respective substrate.

Key words: wood substrate; mercury; *Pleurotus ostreatus*; *Pleurotus pulmonarius*

INTRODUCTION

Oyster mushroom (*Pleurotus ostreatus*) is currently considered one of the most important wood-decay fungi as it is a source of a broad spectrum of biologically active compounds used in the treatment of civilization diseases. Oyster mushrooms are precious, also because of their high nutrition value which is related to the presence of various chemical elements. They are bound to chitin and other polysaccharides of the mushroom cell wall, but their content is highly variable [5]. However, these mushrooms can also accumulate toxic elements such as heavy metals. Mercury is an example of an element which increasingly accumulates more in fruit bodies of mushroom than in the bodies of plants and animals [2, 3, 4]. Mercury intoxication damages first of all the nerve system and kidneys. The typical symptoms of poisoning include nervousness, tremor and visual and memory impairment. Very sensitive are pregnant women and growing children [8].

The aim of the study was to investigate the content of mercury in fruit bodies of oyster mushrooms cultivated on different wood substrates and growing naturally on aspen.

MATERIALS AND METHODS

Materials

In our experiment, we investigated the fruit bodies of oyster mushrooms (*Pleurotus ostreatus*) cultivated on various wood substrates (beech, oak, nut-tree, aspen, and poplar) and Indian oyster (*Pleurotus pulmonarius*) growing naturally on aspen trees. Oyster mushrooms were cultivated by a common method on wood logs which were appropriately prepared and inoculated with oyster mushroom spawn (Fig. 1). The grown mushrooms were collected in two seasons, i. e. in spring and in autumn. The Indian oyster was collected in the summer. All mushrooms were mechanically cleaned, dried at 40 °C (Thermo scientific, Thermo electron led GmbH, Germany), and ground into a powder in a porcelain mortar.

Determination of the content of Hg

The total content of Hg in mushrooms was determined by a single-purposed atomic absorption spectrometer AMA 254 (ALTECH Prague, CR), intended for the direct determination of the total Hg in solid and liquid samples without previous chemical treatment of the samples. The required amount of sample (approx. 100 mg) was weighed in a platinum boat and transferred to a burning chamber of the pre-programmed analyser where it was dried and burned under the flow of oxygen. The released Hg vapours were trapped and concentrated on a gold amalgam at about 120 °C, in order to prevent the condensation of water vapours produced during the burning of the samples. Mercury was then released from the gold amalgam by a brief heating to higher temperatures and the released Hg vapours passed together with the carrier gas, into the measurement and reference cells, where their absorbance was measured at a wavelength of 253.65 nm. The content of Hg was determined by means of an external calibration curve. A low pressure mercury lamp emitting light of the required wavelength was used as a light source and the decrease in light intensity was detected by a semiconductor UV diode. The instrument operates in two ranges, namely 0—30 ng of Hg and 0—500 ng of Hg. The accuracy of the measurements was checked by a certified reference material, TORT-2 Lobster hepatopancreas (National Research Council, Canada), with a certified Hg value of 0.27 ± 0.06 mg.kg^{-1}. The total mercury content in the mushrooms was expressed as mg.kg^{-1} dry weight (DW).

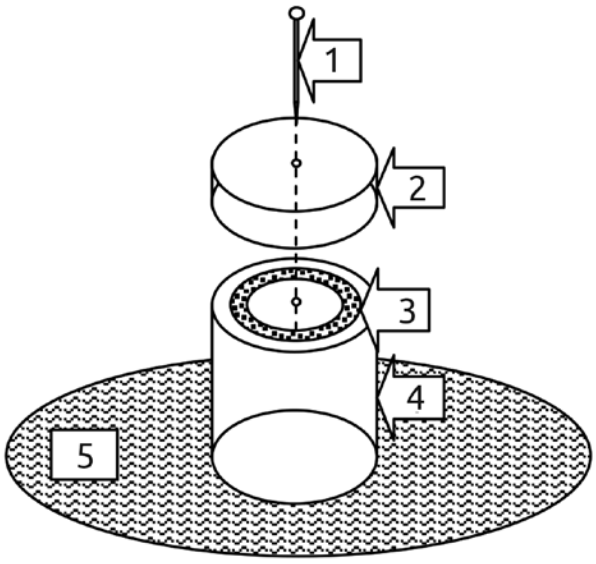

Fig. 1. Preparation of a wood log and inoculation with *Pleurotus ostreatus* spawn
1 — nail; 2 — log cap; 3 — mushroom spawn; 4 — log body; 5 — soil

RESULTS

The results of the analysis of the total mercury in the individual samples of the oyster mushrooms grown on various wood substrates are shown in Table 1 and Fig. 2. A very low content of Hg was detected in mushrooms grown on; beech, oak, and aspen wood, and the levels of Hg in mushrooms harvested from the nut-tree log were also low. However, fruit bodies of mushrooms cultivated on poplar exhibited several-fold higher levels of Hg compared to the other samples.

DISCUSSION

The specific structure of the cell wall of oyster mushroom fruit bodies enables the accumulation of macro- and micro-elements including the toxic ones [5]. Our experiment showed the presence of Hg but the content of this element determined in individual samples differed considerably. The content of Hg in mushrooms cultivated on beech, oak, and aspen substrates, showed low levels of Hg which agreed with the published papers [7]. The mushrooms cultivated on the

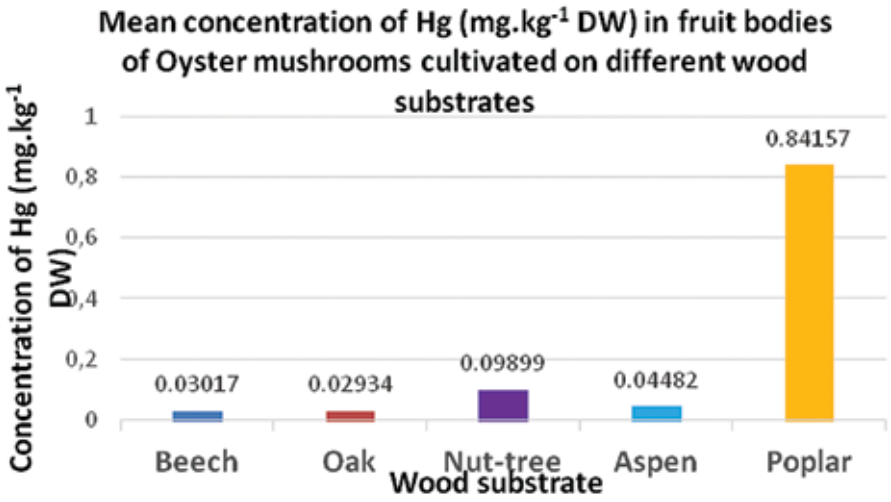

Fig. 2. Mean content of mercury in oyster mushroom samples

Fig. 3. White poplar (Populus alba) from which the log was taken

Fig. 4. Local brook starting at the poplar shown in Fig. 2

Table 1. Levels of Hg detected in samples of oyster mushrooms

Substrate	Content of Hg [mg.kg^{-1} DW]			Mean [mg.kg^{-1} DW]	SD	RSD [%]
	Exp. 1	Exp. 2	Exp. 3			
Beech	0.03014	0.03007	0.03031	0.03017	0.00012	0.41
Oak	0.02821	0.02832	0.0315	0.02934	0.00186	6.36
Nut-tree	0.09465	0.10475	0.09758	0.09899	0.0052	5.25
Poplar	0.79172	0.88324	0.84975	0.84157	0.04631	5.5
Aspen	0.04287	0.04354	0.04805	0.04482	0.00281	6.27
CRM substrate: TORT-2 = 0.24522 mg.kg^{-1} Hg; certified value = **0.27 ± 0.06 mg.kg^{-1} Hg**						

nut-tree log contained double the amount of Hg determined in the previously mentioned samples, but the highest level of Hg was found in mushrooms cultivated on poplar wood.

It is very demanding to assess the safety of these mushrooms, as the world-wide standards specify limits only for methylmercury (the no-observed-adverse-effect level "NOAEL" is 0.24 mg of methylmercury per week) and mushrooms accumulate mainly the inorganic Hg forms [6]. In 2011, the FAO and WHO determined that the provisional tolerable weekly intake of humans for inorganic mercury was 4 µg mercury per kg body weight per week. Thus, after recalculation, person weighing 70 kg should not consume more than 330 g of these mushrooms per week.

The trees, which were used for obtaining the cultivation logs, grew in the same location which suggests that the high level of Hg only in samples grown on poplar may be related to the local contamination of the soil. The content of Hg in fruit bodies of mushrooms depends, besides other factors, also on the content of this metal in the soil; the higher the soil content, the higher the level of Hg in the mushrooms [1]. The high content of Hg in the soil is naturally present only close to its deposits. However, inorganic forms of Hg are released to the environment also from; waste dumps, fungicidal preparations, or from iron smelting and subsequently produce complexes with soil particles. An increased deposition of Hg in the soil was recorded in regions rich in iron ore [8]. The area of poplar from which we obtained the log for cultivation was known in the past century as an area of iron smelting and manufacturing blades for ploughs ("lemeš" in Slovak; there exists a village called Lemešany in this area). A local brook with orange coloured water (probably because of high content of iron) starts close to the mentioned poplar tree (Fig. 3 and 4). In order to confirm this, one should carry out detailed determinations of the composition of the soil, water and wood in this area.

Mushrooms, of the genus *Pleurotus*, accumulated mercury in their fruit bodies, in agreement with observations of other authors. We concluded that the increased content of this heavy metal in oyster mushrooms grown on poplar substrate, might be related to the local craftsmen activities in the past century. Oyster mushroom may thus serve as bioindicators of local environmental contamination. This study also indicates the need for increased caution when selecting a suitable substrate and environment for the cultivation and harvesting of oyster mushrooms, due to the potential accumulation of heavy metals in their fruit bodies.

ACKNOWLEDGEMENT

We wish to express our thanks to Mrs. Anna Valenčíková from the accredited workplace of the State Veterinary and Food Institute in Košice for her kind help in the determination of the mercury in the samples of the oyster mushrooms.

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EVALUATION OF FRAGMENTATION OF DNA AND EXPRESSION OF ACETYLCHOLINE ESTERASE GENE IN CULTURE OF BOVINE LYMPHOCYTES EXPOSED TO THIACLOPRID

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ABSTRACT

The persistence of insecticides in ecosystems can damage the genome integrity of cells and organisms of animals and humans. We investigated the commercial insecticide Calypso 480 SC which contains the active ingredient of thiacloprid. The insecticide was added to the culture of peripheral blood lymphocytes of young cattle. The cultured cells were exposed for 24 and 48 h to various concentrations of the preparation Calypso 480 SC. In the first part of our experiment, we tested the ability of thiacloprid to induce DNA fragmentation which is an important phase of cell apoptosis. DNA fragmentation was observed soon after the action of the lowest concentration of technical thiacloprid at both investigated exposure times (24 and 48 h). In the second part, we used real-time PCR to observe the expression of the bovine acetylcholine esterase (AChE) gene. Increased expression of the bovine AChE gene was observed in the treated lymphocyte cultures of both tested animals.

Key words: bovine lymphocytes; DNA fragmentation; expression of acetylcholine esterase gene; thiacloprid

INTRODUCTION

Neonicotinoid insecticides are used in agriculture and in households to control pests. They have strong neurotoxic effects and act as agonists of nicotine acetylcholine receptor (nAChR) of insects. Their mechanism of effect on nAChRs of mammals has not yet been explained. According to information provided by Tomizawa and Casida [8], neonicotinoids make up about one fourth of the world market in pesticides and their use in some countries continues to increase [3]. Neonicotinoids are chemical compounds with systemic effects which enables their distribution into pollen grains and nectar, and thus exert negative effects on the reproduction of pollinators, such as bumblebees and honeybees [5]. Recently, there was observed a more rapid decline in insectivorous birds in locations contaminated with neonicotinoids [4]. Reduced growth and reproductive abilities have been observed in fish and reptiles [2]. The risk related to the application of neonicotinoid pesticides to large areas of cultivated soil is based on their relatively high solubility in water and their penetration to water sources and the soil.

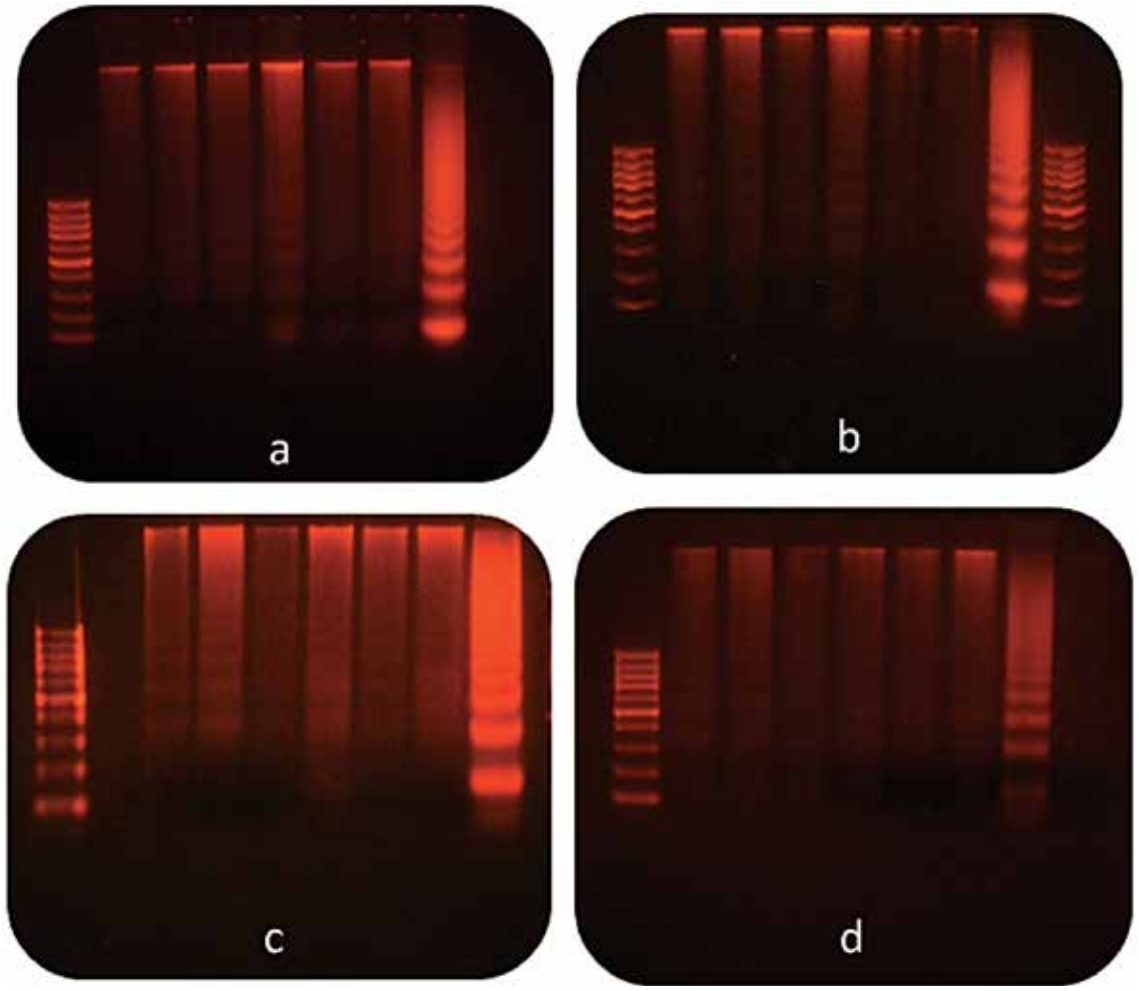

Fig. 1. Fragmentation of DNA from cultivated bovine lymphocytes
Lanes from the left: 100bp DNA ladder; DMSO; thiacloprid 30 $\mu g.ml^{-1}$; 60; 120; 240; 480 $\mu g.ml^{-1}$; positive control: cells U 937 affected by camptothecin 4 $\mu g.ml^{-1}$; a) animal No. 1, 24 h; b) animal No. 1, 48 h; c) animal No. 2, 24h; d) animal No. 2, 48 h.

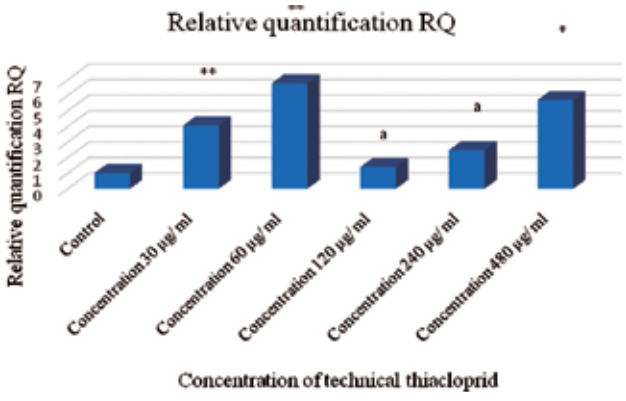

Fig. 2. Changes in the expression of AChE gene in cultures of bovine lymphocytes after 24 hour exposure to technical thiacloprid

Our study focused on the neonicotinoid insecticide thiacloprid, the active ingredient of a commercial preparation called Calypso 480SC, which is relatively frequently used in practice. We investigated the effects of thiacloprid on DNA fragmentation of cultivated bovine lymphocytes and on the expression of the bovine acetylcholine esterase (AChE) gene.

MATERIALS AND METHODS

Our experiments were carried out using the thiacloprid-based insecticide Calypso 480 SC (N-{3-[(6-chloro-3-pyridinyl)methyl]-1,3-thiazolan-2-yliden}cyanamide; 480 $g.l^{-1}$, (Bayer AG, Germany). Blood obtained from two

clinically healthy bulls was cultivated in 4 ml of cultivation medium RPMI 1640 (Sigma, St. Louis, MO, USA), supplemented with; 1ml of bovine foetal serum (BOFES, Sigma, St. Louis, MO, USA), antibiotics (penicillin 250 U.ml^{-1} and streptomycin 250 µg. ml^{-1}), and phytohaemagglutinin (PHA, 180 µg. ml^{-1},Welcome, Dartford, Germany). Twenty four or forty eight hours before the end of the total time of the cultivation of the lymphocytes (72 h), the tested insecticide in concentrations of: 30 µg.ml^{-1}, 60 µg.ml^{-1}, 120 µg.ml^{-1}, 240 µg.ml^{-1}, and 480 µg.ml^{-1}, was added to the cell cultures. The DNA was isolated by means of an Apoptotic DNA Ladder kit (Roche Diagnostics GmbH, Mannheim, Germany). The extracted DNA was analysed by electrophoresis in 1 % agarose gel (1 h 30 min; 75 V; 1 x TAE buffer). Electrophoretic gels were stained with GelRedTM (Biotinum) and the results were visualised by a Gel-documentation system D1-HD (Major Science).

The total RNA was isolated, employing a kit Aurum-TM Total RNA Mini Kit (BioRad, USA). Concentrations of RNA were determined spectrophotometrically (Nano-Drop 1000 Spectrophotometer, Thermo Scientific, USA). The cDNA was prepared by transmission of RNA by means of iScriptTM cDNA Synthesis Kit (BioRad, USA). Primers with fluorescence labelled probes for the detection of BoAChE, were prepared by Generi Biotech (Czech Republic). The gene for polymerase II (POLR2A) was used as a reference gene. Real-time PCR was conducted using a thermocycler CFX96 Touch™ Real-Time PCRSystem (BioRad, USA). The initial denaturation at 95°C/3 min was followed by 40 cycles of alternation of denaturation at 95 °C/10 s and hybridization and extension at 60 °C/20 s.

To evaluate the relative quantification (RQ), we used the ddCt, RQ = 2 – (ΔΔCt) method by Livak et al. [6]. Statistical evaluation of results was carried out by the Student *t*-test.

RESULTS AND DISCUSSION

After a 24 h exposure to thiacloprid, DNA fragmentation could be observed in the agarose gel at all tested insecticide concentrations (30—480 µg.ml^{-1}). Similar results were obtained after a 48 h exposure, as the samples after electrophoretic separation showed positive fragmentation even at the lowest pesticide concentration (Fig. 1). In the second part of the study, we investigated the influence of technical thiacloprid on the expression of bovine AChE gene in lymphocyte cultures from two clinically healthy young bulls using real-time PCR. Significantly, increased expression of the bovine AChE gene was detected at the lowest pesticide concentrations (30 and 60 µg.ml^{-1}; 4—6.7-fold increase in expression of bovine AChE gene; $P < 0.01$), and at the highest tested concentration (480 µg.ml^{-1}), a 5.7-fold increase was observed ($P < 0.05$) (Fig. 2).

Olgun et al. [7] observed an induction of production of DNA fragments in mice thymocytes after exposure to a mixture of pesticides and concluded that the cytotoxicity of pesticides caused an induction of apoptosis. Induction of apoptosis is associated with changes in the expression of AChE gene, which was recorded in our study mainly at lower pesticide concentrations. Higher concentrations of thiacloprid (120 and 240 µg.ml^{-1}) most likely caused such damage to DNA, that it resulted in the necrosis of some subpopulations of lymphocytes. Our assumption is supported by Galdíková et al. [1], who observed a significant increase in the frequency of breaks in bovine chromosomes at the mentioned concentrations. The increased expression of the AChE gene may be related to apoptosis induced by the insecticides which was documented by the presence of the DNA fragmentation. Our observations of bovine cells agree with the current knowledge about apoptotic cells of various human primocultures and cell lines, in which the increased expression of AChE gene and increased activity of AChE enzyme was detected [9].

CONCLUSION

The observation of DNA fragmentation and changes in the expression of AChE gene in cultivated bovine lymphocytes, indicate that the cytotoxic effects of the insecticide preparation CALYPSO 480 SC consists mostly in the induction of apoptosis.

ACKNOWLEDGEMENT

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INTERCONNECTION OF CANIS THERAPY AND ART THERAPY

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ABSTRACT

The essential element of the care of mentally or physically disabled clients is the integration of psychotherapy into their everyday life. A range of important therapies is based on the all-embracing development of an individual. This study investigated the influence of a combination of canis therapy and art therapy activities on mentally and physically handicapped individuals using observations and in-depth interviews oriented on; subjective feelings, personal experiences, views, knowledge and the skills of the therapists who were given eight basic questions. The individuals were two canis therapists and two art therapists, practicing their profession in various establishments in the Slovak Republic and the Czech Republic. The interviews demonstrated that although all respondents observed short-term and long-term effects of these therapies, only one of them used them in parallel. The study also suggested some interconnecting activities which can be used in various institutions intended for different clients.

Key words: art therapy; canine-assisted therapy; canis therapy; handicap; integration; psychotherapy;

INTRODUCTION

The stimulus to initiate this study, was the potential interconnection of two closely associated psychotherapeutic methods, canine-assisted therapy (canis therapy) and art therapy. Canis therapy is a supportive therapy method, based on the favourable effects of the interaction of humans and dogs, particularly on the physical, psychological and social facets of the human clients [1]. It has been used in establishments engaged in the care and education of; children (kindergartens, schools, and foster homes), health care institutions, retirement homes, and special institutions for psychologically disturbed children and adults [2]. It is a complementary psychotherapeutic method used in various situations in which other methods appeared unsuccessful or could not be used because of some specific conditions.

Art therapy is the intentional, professional and systematic use of creative arts oriented toward helping people to change their; behaviour, thinking, emotions and personal structures toward a more socially and individually acceptable direction [3]. It is effective, not only as a receptive medium, but first of all, by the process of creation. It is used in situations involving disturbances or limitations of verbal manifestation. It is advantageous because of the possibility of its interconnection with other methods; such as imagination, relaxation, music therapy, body therapy and also canis therapy [4].

The aim of this study was to investigate the existence of an interconnection between canis therapy and art therapy in four selected institutions in Slovakia and the Czech Republic. The investigation involved seeking the opinions of therapists about the successfulness of these associated methods and their observed short-term and long-term effects and, also on the basis of these observations, to suggest some helpful activities.

MATERIALS AND METHODS

The techniques used in this qualitative research included; observations, in-depth interviews and analysis of the results of the respective activities, i.e. exploration techniques based on direct contact with the respondents. The development of the research plan was preceded by participation of the author in several canis therapy and art therapy sessions as an indifferent observer, in order to better understand the personal experiences of the therapists. The preparation stage included, also test interviews which were followed by the development of the system of questions and their sequence (from introductory, up to the most relevant and specific).

The standardized interviews involved open questions and enabled the quantification of coincident responses. All respondents were interviewed by the same researcher who recorded their responses, thus preserving their consistency and authenticity. The interview itself was preceded by; introductions, explanation of the research aims, and a short debate to evoke a friendly atmosphere. The length of each interview was 20—30 minutes.

To obtain responses to the following 2 basic questions, the therapists were asked 8 specific sub-questions:

1. How did the canis therapy or art therapy affect your clients?
2. Do you use canis therapy and art therapy in parallel?

Specific questions:

- How long have you practiced canis therapy/art therapy?
- Is the therapy you practice individual or in groups? Does it always have the same course?
- Do you observe a beforehand prepared programme during the therapy?
- Do you observe any short-term psychological or physical effects of the therapy on your clients?
- Do you observe any long-term effects?
- How do you perceive the direct influence of therapy during the session?
- How long does it takes for the effects to disappear?
- Do you use, in your practice, canis therapy and art therapy activities in parallel?

If yes, give an example. If not; why not?

Respondents

Two criteria were used for selecting the respondents for this study: a) age; b) practicing regularly for a minimum of one year with an individual or group therapy unit in a relevant institution.

The 4 respondents selected were active therapists, 26—51 years old, two females (canis-therapists) and two males (art therapists), working in different establishments with different types of clients:

Respondent 1: Maják n. o., social service home for deaf-blind young people, Zdoba, SR; (Fig. 1).

Respondent 2: Hospital in Homolce, Department for seniors with physical disabilities, Prague, CR; (Fig. 2).

Respondent 3: Elementary School of Art (ZUŠ), visual art branch, Košice, Bernolákova street, (mentally disabled children integrated into a regular classroom); (Fig. 3).

Respondent 4: Primary school Gaštanová, cynological interest-group (school children grades 1—5, without disability), Žilina, SR; (Fig. 4).

The clients in these establishments differed by; age, type and character of handicap, and the method and length of the application of, canis therapy or art therapy.

Following the 4 interviews, the observations and responses were grouped and analysed.

RESULTS AND DISCUSSION

Tables 1 to 4 present information obtained by interviews according to categories that resulted from the analysis of the responses.

Table 1. Forms and types of therapy

Respondent	Form	Type
1	Canis therapy	Group
2	Canis therapy	Individual
3	Art therapy	Individual /group
4	Art therapy	Individual

Table 2. Short-term effects of canis therapy or art therapy observed by respondents/therapists

Respondent	Short-term effects observed by therapists
1	• Increased power of concentration • Positive motivation to work • Improved frame of mind • Empathy for animals and classmates
2	• Warming of body at changing position • Willingness to collaborate after termination of session • Improved socializing • Alleviation of feelings of loneliness
3	• Increased self-confidence • Spontaneity • Calming down • Concentration
4	• Motivation for carrying out physical activity • Willingness to work as a member of team • Improved frame of mind • Improved non-verbal communication

Table 3. Long-term effects of canis therapy or art therapy observed by respondents/therapists

Respondent	Long-term effects observed by therapists
1	• Improved fine and gross motor skills (observable at writing) • Improved physical condition (muscle building) • Stirring up interest in nature and animals • Obtaining specialised cynological knowledge • Improved communication
2	• Elimination of blocks • Increased self-confidence • Releasing muscle spasms • Stirring up interest in what is happening in their surroundings • Improved psychological health affecting the physical one
3	• Integration into the collective of healthy children • Creating a team • Improved fine motor skills • Better spatial imagination • Self-reliance
4	• Clients learned to use new senses and communicate through artistic product • Improved fine motor skills • Improved communication with surroundings • Increased self-confidence

Table 4. Interconnection of canis therapy and art therapy

Respondent	Response to question: Do you use in your practice canis therapy and art therapy activities in parallel?
1	Yes, I use them in parallel. Children enjoy working with colours and it is not difficult to involve dogs in such activities.
2	It is not possible with seniors with physical disability. But for children in a camp I plan to use in parallel ergotherapy, canis therapy and art therapy and develop a common project.
3	No. I wish this was possible, however, the internal regulations of the school do not allow such possibility.
4	No. Some clients did not respond positively to the presence of animals so we abandoned such idea.

Our investigations showed that only one respondent (No. 3) practiced equally both individual and group therapy.

Each respondent reported more than one positive effect of therapy. Canis therapy affected in the short-term, mostly the psychological health and their personal experiences (improved frame of mind, empathy, willingness to collaborate). The long-term effects involved more of the physical aspects (improvement of condition, release of spasms, and improvement of fine motoric skills). The short-term effects of art therapy described by therapists included, overall relaxation, and improved concentration. The long-term effects observed by all respondents involved; creating a team spirit, improved fine motor skills and communication. Our results agree with those reported by other authors of similar orientations [1], [5].

Fig. 1. Maják, n.o., social service home for deaf-blind young people, Zdoba, SR, (Resp. 1)

Fig. 2. Hospital Na Homolce, Department for seniors with physical disabilities, Prague, CR, (Resp. 2)

Fig. 3. Elementary School of Art (ZUŠ), visual art branch, Košice, Bernolákova street (mentally disabled children integrated into a regular classroom), (Resp. 3).

Fig. 5. Interconnection of canis therapy and art therapy — suggested activity

Fig. 4. Primary school Gaštanová, cynological interest-group (school children grades 1—5, without disability), Žilina, SR, (Resp. 4)

The second basic question was oriented on interconnection of canis therapy and art therapy and whether the respondents use both these forms in parallel. In the case of a positive response, we then asked the respondent to identify the favourite activity common to both of them. In the case of a negative response, we asked for the reason. Here the responses of individual respondents differed. The first one mentioned, that she uses both forms equally and in parallel, and the example was on the sidewalk using coloured chalk for the tracing of a dog's body. The second respondent practices only canis therapy. The main reason is that the physical condition of the clients who are confined to a bed precludes art therapy. However, she is willing to use both forms in parallel with different clients. The third respondent does not practice both therapies in parallel but has personal experience with both of them. However, keeping dogs in the institution where he applies the therapy is not allowed. He decided to consider integrated therapy outside of the establishment. The last respondent had no personal experience with canis therapy and practices only art therapy. He obtained information from the personnel of the establishment that, in the past some clients disliked the immediate presence of animals and because of that, this form of therapy was abandoned.

Canis therapy and art therapy contribute to a better quality of life for handicapped people and despite its positive effects that can be expected from their interconnection, this is usually not practiced although it is financially undemanding (Fig. 5).

Up to this day, the Slovak legislation has not defined canine-assisted therapy and art therapy as therapy forms. Because of that, there are no unified rules for their implementation, requirements on the education of persons providing this therapy, or for the preparation and testing of animals involved in these therapies. This affects negatively the quality of providing the services.

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