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NEUROPROTECTIVE EFFECT OF *GREWIA CARPINIFOLIA* EXTRACT AGAINST VANADIUM INDUCED BEHAVIOURAL IMPAIRMENT

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ABSTRACT

Vanadium (V), a heavy metal, has been reported to induce central nervous system toxicity leading to various behavioural impairments. It is characterized by the production of reactive oxygen. The present study was designed to test the possibility of *Grewia carpinifolia* ethanolic extract in preventing behavioural alterations following acute vanadium toxicity in mice. Twenty five Swiss albino mice (25—27 g) were completely randomized into 5 groups (A—E) of 5 animals each. Group A received distilled water and served as a control; group B, received vitamin E (500 mg.kg^{-1} b.w. every 72 hours), a known antioxidant orally, along with a daily dose of sodium metavanadate intraperitoneally (i.p.) for 7 days; group C and group D received *Grewia carpinifolia* leaf extract at 100 and 200 mg.kg^{-1} b.w orally respectively, along with the sodium metavanadate i.p. for 7 days; while group E received sodium metavanadate i.p. only for 7 days. The behavioural and motor functions were analysed by the open field, negative geotaxis, and hanging wire tests; the daily body and brain weights were recorded. *Grewia carpinifolia* ethanolic extracts significantly reduced the number of grooming, stretched attend posture, and freezing time that were significantly increased in the vanadium only group and also enhanced the vestibular functions. In addition, the latent time spent on the hanging wire in groups simultaneously administered with the extract and V compared favourably ($P > 0.05$) with the control groups but a decrease in latent time was observed in the V only group. The results suggest that acute V toxicity results in various behavioural deficits and support a possible role of *Grewia carpinifolia* as a protective agent against acute vanadium-toxicity with a better result at 200 mg.kg^{-1} b.w.

Key words: behaviour; *Grewia carpinifolia*; mice; vanadium

INTRODUCTION

Vanadium is a trace element that is widely distributed in nature. Power- and heat-producing industrial plants us-

ing fossil fuels (petroleum, coal, oil) cause the most widespread discharge of vanadium into the environment. The incidence of exposure to toxic levels of vanadium to industrial workers has been of increasing concern [21, 24]. Environmental exposure occurs *via* inhalation in the surrounding area of metallurgical plants or through the consumption of contaminated foods [4, 24, 25], and recently from massive oil burning, as seen in Arabian Gulf [6], the Niger-Delta region of Nigeria [20], and the Gulf of Mexico. The mangrove forest of the Niger-Delta of Nigeria, covering about 70 000 km^2 of wetlands (the largest in Africa and the third largest in the world) with a population of about 20 million has been the centre of constant exploration for oil by many international oil companies [44]. These exploratory activities often lead to gas flaring and oil spillage impacting negatively on the aquatic and terrestrial habitats, as well as animal and human health [22].

Vanadium compounds have been reported to cause toxic effects by most routes of exposure in most species [14]. The disposition of vanadium in specific tissues may be involved in the pathogenesis of certain neurological disorders and cardiovascular diseases [46]. Its capacity to affect the activities of various other intracellular enzyme systems and modify physiological processes damaging cell membrane *via* the production of free radical has also been documented [6]. The central nervous system (CNS); rich in polyunsaturated fatty acid side chains, with high oxygen tension but poor in antioxidant capacity, is very vulnerable to free radical damage [1] by vanadium leading to: tremors, CNS depression and various behavioural alterations [44]. Several investigators have demonstrated that antioxidants such as α-tocopherol and ascorbic acid protect the brain against vanadium-induced (ree radical injury [13, 33]. Consequently, some medicinal plants have been reported to contain some phytochemicals, mostly polyphenols and flavonoids, which exhibit high antioxidant activity [16]. Furthermore, natural sources of antioxidants have been severally studied in a bid to discover potentially safer, effective, and cheaper antioxidants [5, 28, 36]. Therefore, there is a need for the study of plants that may offer some protection against the effects of vanadium in a country such as Nigeria which is known for oil spillage and gas flaring where about 61% of its citizen are impoverished and cannot readily afford conventional drugs [31].

Most species in the genus, *Grewia* have been reported to have antioxidant properties and are used in the treatment of various disorders in man and other animals [17]. Triterpenoids, steroids, glycosides, flavones, lignans, phenolics, alkaloids, lactones, anthocyanins, flavones, and organic acids have been isolated from various species of this genus [27, 34].

To this end, this study was designed to investigate the hypothesis that a nutritional strategy like co-administration of ethanolic extract of *G. carpinifolia* leaves could ameliorate vanadium-induced neurotoxicity in mice.

MATERIALS AND METHODS

Experimental animals

Twenty five male mice weighing between 25—27 g were randomly divided into five groups (A—E) of five animals per group. They were obtained and kept in the experimental animal house of the Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Ibadan. The animals were 5 weeks old and housed under standard conditions of temperature, (25 ± 2 °C) and light, (approximately 12/12 h light-dark cycle), fed on standard diet (Animalcare® Feeds Ltd., Nigeria) with fresh water *ad libitum*. The cages were cleaned of waste daily. All the animals were acclimatized to the laboratory conditions for two weeks before the commencement of the experiments. The study was approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/025).

Plant material and extraction

Fresh leaves of *Grewia carpinifolia* were collected from the Botanical Garden of the University of Ibadan. It was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN) where herbarium specimen (voucher number FHI 109693) was deposited.

The plant sample (5 g) was homogenized in 80 % aqueous ethanol at room temperature and centrifuged at 10,000 rpm for 15 min and the supernatant was preserved. The residue was re-extracted twice with 80 % ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature.

Experimental design

The experiment to determine the ameliorative effect of *Grewia carpinifolia* on white laboratory mice following

acute vanadium intoxication was designed to span 8 days. Group A received distilled water throughout the experimental period and served as a control; group B, the standard group received vitamin E (500 mg.kg^{-1}) every 72 h orally along with a daily dose of sodium metavanadate (Sigma-Aldrich, St. Louis, USA) at 3 mg.kg^{-1} [29] intraperitoneally (i. p.) for 7 days consecutively; group C and group D received *Grewia carpinifolia* leaf extract at a single daily dose of 100 and 200 mg.kg^{-1} orally respectively, along with sodium metavanadate at 3 mg.kg^{-1} i. p. for 7 days consecutively; and group E received only sodium metavanadate at 3 mg.kg^{-1} i. p. for 7 days consecutively. The present study was conducted in line with the European laws on the protection of animals (86/609/EEC). The protocols for the experiments were approved by the institutional animal care and ethics committee.

Open field test

Locomotor and exploratory activities were measured by an open-field task box (Coulbourn Instruments L. L. C., PA, USA). Each animal was placed individually at the centre of the apparatus and observed for 5 minutes to record the movement time, length and number of line crossing and time spent (exploratory activity) at the centre of the open field box by TruScan software v 2.07 (Coulbourn Instruments L. L. C., PA, USA)

The following observations as previously described by Brown et al., [10] were also recorded:

(A) Line crossing: number of times a mouse crossed from one square to another with at least its two front paws.

(B) Rearing: number of times mouse stood on its hind legs.

(C) Grooming: sets of heterogeneous components comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking while stationary.

(D) Stretched attend posture: duration of time the mouse stood still with forward elongation of the head and shoulders.

(E) Time spent at the centre, duration of time the mouse spent at the centre square.

(F) Freezing: duration with which the mouse was completely stationary.

The open field box was cleaned with methylated spirit before placing the subsequent animals in it in order to avoid possible bias effects due to odour clues left by a previous mouse.

Fore limb support (hanging wire) test

This test is based on the latency of a mouse to fall off a metal wire upon exhaustion based on the method described by Van Putten et al. [45]. A 2 mm thick metallic wire secured to two vertical stands was used. The wire was tightly attached to the frame to avoid vibration or unwanted displacement of the wire during the measurements. Each mouse was placed on the wire with its fore limb and monitored for a maximum period of 120 seconds. The period of time it took the animal to stay on the wire before falling was taken and recorded [9, 11]. The animals that did not fall off the hanging wire during the test period of 120 seconds were given a maximum score [30].

Negative geotaxis test

Each animal was placed in the middle of a board, 300 inclined to the surface plane, in a head-down position and the latency to turn and orient its position; to be facing up the slope, is recorded [8].

Two trials were performed for each mouse in both the hanging wire and negative geotaxis tests. The second trial was done 3 to 4 hours after the completion of the first trial; hence all mice were well rested before the second trial.

Body weight

Rats in all the groups were weighed daily throughout the experiment.

Relative brain weight (RBW)

Rats were anesthetized with ketamine following which the frontal, parietal and temporal bones were gently removed to expose the brain which was carefully removed, weighed and observed macroscopically [47]. The relative brain weight of each rat was calculated as follows:

$$\text{RBW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}}$$

Statistical analysis

The results were analysed using the statistical package GraphPad prism version 5.01 (San Diego, USA). These data were subjected to one-way ANOVA and subsequently to the Bonferroni post-test to perform multiple comparisons in order to assess the statistical significance of differences between all possible pairs of groups. Repeated measures

on the same animals were analysed using the Wilcoxon matched-pair, signed-rank test [41]. Differences were regarded as statistically significant when $P < 0.05$.

RESULTS

Open field test

The mean values for observations of the Open field test in the experimental animals are presented (Table 1). A statistically significant ($P < 0.05$) decrease was observed in the number of line crossing in the group administered with 100 $mg.kg^{-1}$ of the extract (group C) and the vanadium group (E) when compared with the control and other test groups. There was also a significant increase ($P < 0.05$) in the number of rearing in the group administered with only vanadium (E); however, there was no difference in these parameters in the other test groups when compared with the control. The time spent at the centre of the open field after seven days co-administration of ethanolic extract of *Grewia carpinifolia* at 200 $mg.kg^{-1}$ p.o. with vanadium (group D) was not significantly different from those of the control and the standard group administered with vitamin E; conversely a significant increase in time spent at the centre of the box was recorded in the vanadium only group (E) when compared with the other groups.

Hanging wire test

The extract treated groups (C and D) spent more time on the hanging wire before falling off, although this was of no significant difference ($P > 0.05$) when compared to the control group; however, vanadium at 3 $mg.kg^{-1}$ resulted in a statistically significant ($P < 0.05$) decrease in the time spent on the hanging wire test (Figure 1). Two trials were performed for each mouse. The second trial was done after all the animals had successfully completed the first trial; hence all mice were well rested before the second trial.

Negative geotaxis test

The mean time for negative geotaxis of the vanadium only group (E) was statistically ($P < 0.05$) higher than those of the extract at tested doses and the standard drug (Figure 2).

Body weight

An increase in daily body was observed in all the groups from day 1 to day 5. There was a decrease in body weight in groups B–E on day 6, after which daily weight gain resumed in groups B–D on day 7 and 8; however the daily body weight continued to decrease in group E after day 6.

Relative brain weight

There was no significant difference ($P > 0.05$) in the relative brain weight of the animal across all the groups (Table 2).

DISCUSSION

Vanadium (V) is a pro-oxidant and indirectly results in the production of free radicals leading to oxidative damage [6, 12] and the possible role of vanadium in behavioural changes has been studied [32]. Vanadium (V) crosses the blood brain barrier and possibly alters the biochemistry of the brain of treated animals [18]. The central nervous system (CNS) myelin could be a preferential target of vanadium mediated lipid peroxidation in rats and mice since the brain has a high metabolic activity as well as a high concentration of myelin [26]. This may consequently increase the susceptibility of the nervous tissue to peroxidative damage [15, 39] by V which causes behavioural alterations.

Behavioural studies can be used in risk assessment following neurotoxicity as it represent the net output of the sensory, motor and cognitive functions occurring in the nervous system [33].

The Open field test is one of the most commonly used tests in animal behavioural studies [40]. In the present study, the number of rearing and grooming was significantly increased after vanadium exposure in the Open field test which may indicate an increase in anxiety in the vanadium only treated when compared to the control animals as well as the extract treated groups. The locomotion was also reduced in the vanadium treated group as indicated by an increase in time spent at the centre of the open field maze, as well as a reduction in the number of new square crossing. The extract at 200 $mg.kg^{-1}$ conversely had values comparable with the control and standard group; this may be linked to the ability of the extract at this dosage to reduce or eliminate anxiety-like behaviours in vanadium exposed mice.

Stretched attend posture (SAP) is an essential component of risk-assessment defensive behaviour in rodents [7]. The mice exposed to vanadium in this study, unlike

Table 1. The mean values for observations of the Open field test
(mean ± SD)

Observation	Group A (Control)	Group B (V + vit. E)	Group C (V + *G. carpinifolia* at 100 mg.kg^{-1})	Group D (V + *G. carpinifolia* at 200 mg.kg^{-1})	Group E (V only)
Line crossing	55.64 ± 27.29	59.00 ± 2.53	24.00 ± 16.15*	59.00 ± 5.93	18.60 ± 4.67*
Rearing	17.67 ± 13.50	13.00 ± 1.41	3.25 ± 1.89*	10.00 ± 1.82	73.25 ± 7.23*
Grooming [sec]	21.33 ± 11.85	16.00 ± 11.31	12.00 ± 4.69	21.65 ± 4.72	14.40 ± 7.33
Stretched attend posture [sec]	34.36 ± 4.09	38.60 ± 5.02	36.0 8± 7.32	35.21 ± 2.58	62.09 ± 10.36*
Time spent at the centre [sec]	2.54 ± 0.03	2.86 ± 0.83	3.81 ± 0.51	3.00 ± 0.12	22.64 ± 10.40*
Freezing [sec]	110.08 ± 9.06	103 ± 7.43	98.21 ± 7.65	112 ± 14.56	135 ± 13.48

SD — standard deviation; number of animals in the group n = 5
* —statistically different from the control at P < 0.05

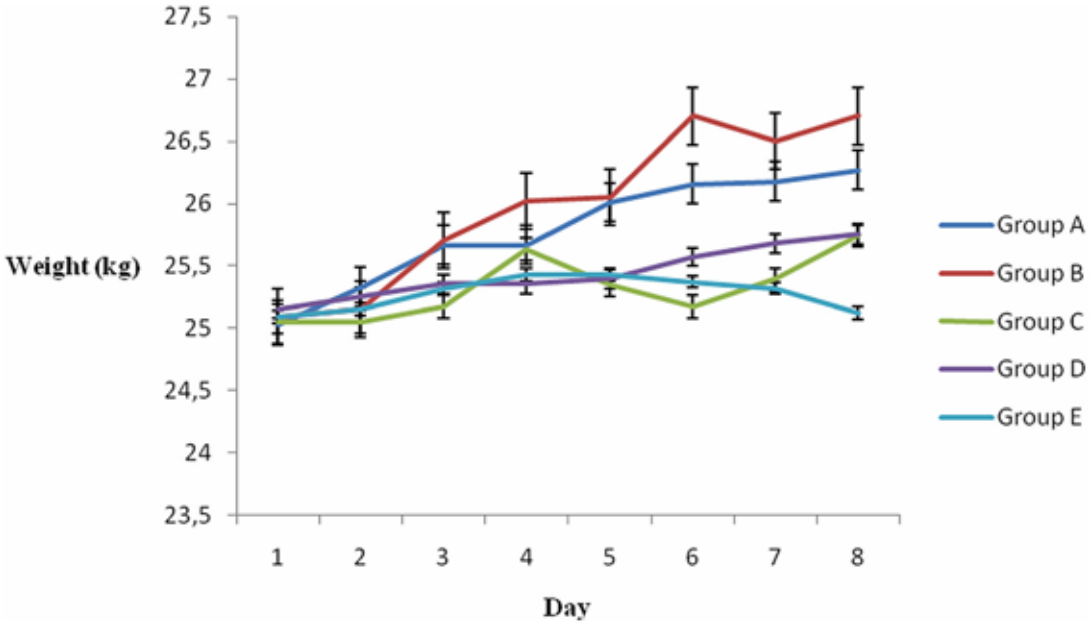

Fig. 1. Time spent on the hanging wire
* — statistically different from the control at P < 0.05

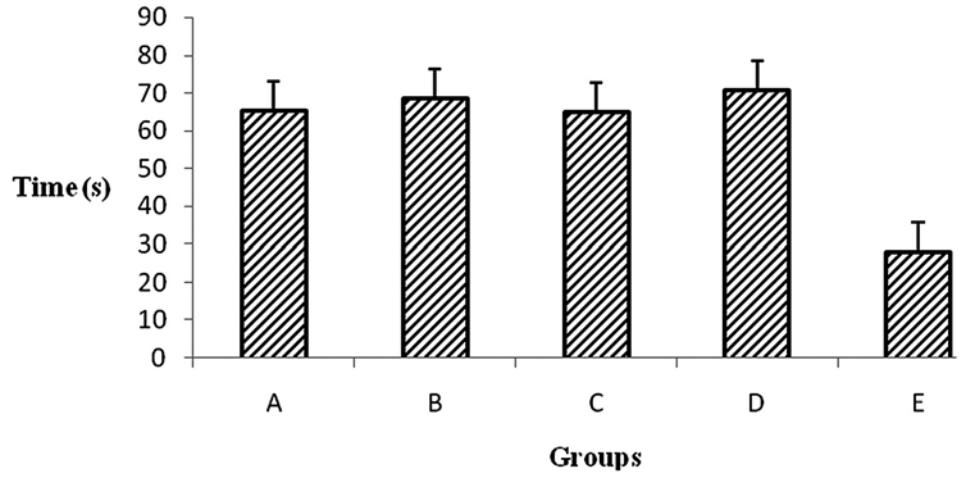

Fig. 2. Mean values for negative geotaxis of experimental animals

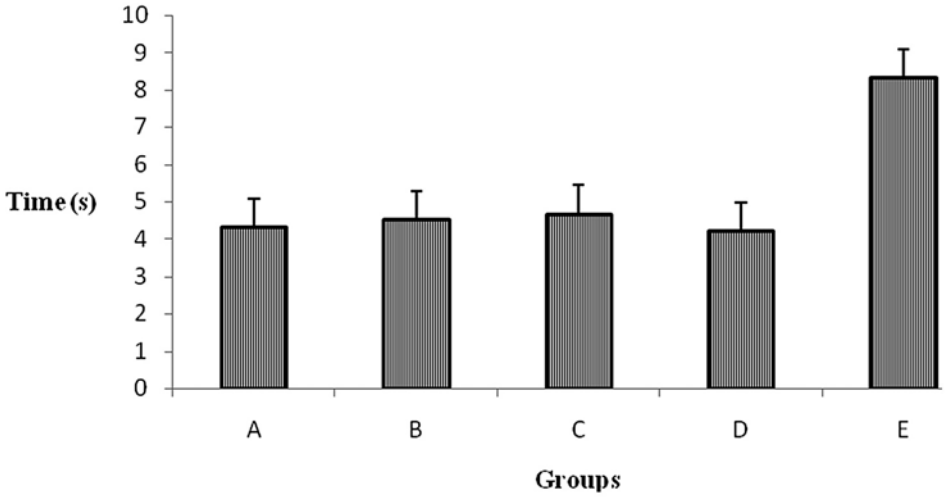

Fig. 3. Mean values for daily body weight

Table 2. Mean values for relative brain weight

Group	Relative Brain Weight ($\times 10^{-2}$)
A	5.34 ± 0.17
B	5.02 ± 0.21
C	5.20 ± 0.22
D	5.45 ± 0.10
E	5.14 ± 0.23

the control and test groups, recorded a significant increase in stretched attend posture. This increase in stretched attend posture (SAP) may suggest anxiety of the mice, which has been indicated to result in low motivation to explore novelty which makes an animal take the stretch-attend posture. This is in concordance with results obtained by Mustapha [29], Soazo and Garcia [42] following vanadium intoxication. Furthermore, this hesitance by the vanadium treated group to move may be attributed to vanadium-induced muscular weakness [32]. In general, observations in the open field test were similar in the *G. car-*

pinifolia extract treated and control groups in this study. Although, Pfau and Skog [35] had documented that the genus Grewia possess some anxiolytic activity, the mechanism by which the extract of *G. carpinifolia* reduced anxiety is still not certain.

In this study, muscular strength was found to be significantly decreased after vanadium treatment as observed in the hanging wire test. This finding highlights the fact that heavy metals have been implicated in muscles and joints pain [3] and further supports that V intoxication results in muscular weakness [32]. The present study may point to the fact that high levels of vanadium may interfere with motor functions leading to decreased motor activities, as well as grip strength in mice. However, the findings of this study also showed that concurrent administration of *G. carpinifolia* extract to V exposed animals proved beneficial as the animals performed better on the hanging wire test, as evidenced by an increase in the suspension time. The hanging wire test can be used to assess global sub-acute muscle function and coordination over time in mice [23] and the ability of the mice to produce sustained tension in the limb musculature. Thus, *G. carpinifolia* extract may have ameliorative effect on muscular weakness caused by V intoxication by improved muscle function and coordination in mice.

The negative geotaxis is a reflex test which reflects vestibular function, motor development and activity [2]. Vanadium has been reported to induce vestibular damage [29], and generally, mice with vestibular dysfunction become disoriented and unable to generally explore a novel setting [37]. This could explain the increased time in the negative geotaxis shown by the mice given vanadium only. Nevertheless, the similarities in the lower latent time of the extract treated group at 200 $mg.kg^{-1}$ and those of the control and standard group, further supports the neuroprotective activity of the plant extract at this dosage. This result of the negative geotaxis further substantiates the findings in the Open field test.

In this study, the daily body weights of the vanadium only exposed mice decreased continuously after the sixth day following the vanadium administration. The observed insignificant decrease (when compared with other test groups and control) in daily body weight observed in the present study is in consonance with the findings by Garcia et al., [14, 15] in acute vanadium toxicity in rats. Vanadium toxicity have been previously reported to induce anorexia [19] leading to the reduction in daily body weights which becomes significant following chronic exposure as reported by Sánchez et al. [38] and Todorich et al. [43]. The insignificant difference in relative brain weight in this study is in line with that observed by Garcia et al., [14] but contrary to that of Altamirano et al. [4] who had described a significant decrease in brain weight following vanadium toxicity, the variance in these results may be ascribed to the fact that six [6] weeks old mice were used in this study, while in their studies the mice were exposed at post-natal day one [1]. It seems that vanadium has more effect on brain weight when exposed to neonates undergoing a high degree of cellular proliferation.

Grewia carpinifolia extract contains numerous phytochemical constituents including: tannins, phlobatinins, saponin, flavonoids, terpenoids, cardiac glycosides, coumarin, alkaloids and anthraquinone [17]. Many of these compounds have proven their potential as antioxidants in various oxidative stress models as scavengers of free radicals as reported in prior studies [36]. The observed beneficial effects of *G. carpinifolia* extract in vanadium-induced changes in behaviour may thus be attributed to these diversified chemical components.

CONCLUSIONS

This study has shown that *G. carpinifolia* extract administered in combination with vanadium ameliorates vanadium induced behavioural impairments. The extract showed better protective activity at 200 $mg.kg^{-1}$. Therefore, the plant should be given more emphasis as a candidate in developing a modern drug to minimise vanadium induced toxicity. Further study is on-going to isolate and characterize the bioactive principle(s) of the extract with the aim of determining its exact mechanism of action.

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EFFECTS OF MANURE BEDDING ON THE RATE OF CLAW DISEASES IN DAIRY COWS

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ABSTRACT

This study was conducted to determine the prevalence of claw diseases in dairy cows housed on two different bedding systems: deep straw bedding and unsterilized recycled manure solids. On the farm (farm A) with the straw bedding, 403 and on the farm (farm B) with manure solid bedding, 226 dairy cows were examined. The prevalence of cows with one or more claw lesions on the farm with straw bedding and with manure solids were 37 % and 69 %, respectively (P < 0.001). In both bedding systems, two claw lesions dominated: digital dermatitis and inflammatory disorders of corium (white line diseases, toe ulcer, and sole ulcer). The prevalence of the digital dermatitis housed in deep-bedded freestalls and in freestalls with manure solids were 17.8 % and 53.1 % (P < 0.001), respectively. The prevalence of the white line diseases and toe ulcer were 12.1 % and 15.7 % on farm A and 3.88 % and 2.59 % on farm B, respectively (P < 0.001). There was no significant difference in the prevalence of the sole ulcer between farms. These data indicate a relatively high prevalence of claw lesions on the studied dairy farms. Moreover, a long-term contact of claws with manure (boxes and corridors) on the farm with unsterilized recycled manure solids seems to be associated with a higher prevalence of digital dermatitis.

Key words: claw diseases; dairy cows; lameness; manure bedding

INTRODUCTION

Claw diseases, especially injuries and infections of the feet, constitute one of the most serious and painful, yet least well-managed categories of diseases for the modern high yielding dairy cow. This problem has been recognised and studied for many years, but is not getting any better. Approximately 70 % of intensively managed dairy cattle in North America were affected by claw diseases [6], and the recently reported lameness prevalence in the U. K. dairy herds ranged from 0—80 % [1].

A high proportion of cows becomes lame within the first two months of their first lactation and regularly re-

lapse. This indicates quite clearly that conventional, accepted practices for housing, feeding and managing of the modern Holstein-Friesian dairy cow lead to injury or otherwise affect the feet to a degree where 50 % of the animals will experience the chronic pain of lameness in any one lactation [30].

The obvious consequences of lameness are: less time spent eating, less movement to bunks, subsequent weight and production losses, and failure to show heat. Extreme lameness with weight loss causes a negative energy balance and can cause anoestrus [16]. Assessing the effect of such diseases on milk yield is a difficult task [10]. Milk production before disease incidence can confound the effect of such disease on overall milk yields. Milk yield was higher before than after a lameness occurrence; high milk production was associated with lameness [13]. Lameness prevention remains a significant priority for the dairy industry as consumer demand drives changes in housing and management to promote improved wellbeing, and farmers strive to improve productivity. Providing a clean, dry, and comfortable surface for cows to rest on is important for the welfare of dairy cows, as they spend approximately 12 h per day resting [15]. The lameness prevalence appears to be greater in freestall facilities compared with other management systems such as tie-stall housing [3, 23]. Leg lesions were observed less frequently in cows housed in deep-bedded sand stalls than cows on mattresses [11] and severe lesions were less prevalent in sand beds than on mattresses [28]. The exposure to concrete walking surfaces in alleys and other changes such as the regrouping of cattle around the time of calving are potential differences typical of freestall design and management that may be important factors elevating lameness risk [5]. Increased costs and reduced availability of other common bedding sources has prompted many dairy producers to search for more feasible alternatives such as sand or recycled manure solids. Although sand can be considered the ideal bedding source for dairy cows, not all producers are willing and able to convert to sand bedding, as it presents several challenges related to manure management [17].

The aim of this study was to compare the prevalence of claw diseases in dairy cows housed on two different bedding systems: straw bedding and recycled manure solids.

MATERIALS AND METHODS

This study was conducted on two dairy farms. All the animals were housed in a free stall system. On the farm with the deep straw bedding (Farm A), 403 and on the farm with manure solid bedding (Farm B), 226 Holstein-Frisian dairy cows were examined. The dairy cows on the farm A with deep straw bedding (group size of 60 cows per pen, approximately) had lying areas with 80 cm of straw available. The farm B with manure solids was equipped with cubicles with concrete basement floor. Recycled manure solids were obtained from the mechanical separation of raw manure and used for cubicle bedding without any chemical and thermal processing. The depth of this bedding varied from 20—30 cm.

The average yearly milk yield on the Farm A and B was 7,400 and 8,200 kg, respectively. The dairy cows were fed by total mixed ration (TMR) on both farms with maize and alfalfa silage as the main components. Claw trimming was performed twice a year by external professional claw trimmers. No claw bathing was performed on either farms.

All the dairy cows were examined and treated in the trimming crush for claw lesions by one person. The clinical examination was focused on the following claw lesions [29]: digital dermatitis, heal horn erosions, white line diseases, toe ulcer, sole ulcer (pododermatitis circumscripta specifica), interdigital fibroma, sole haemorrhage, and chronic laminitis (horizontal horn fissures and concave wall). The cases of digital dermatitis were recorded for each leg and other lesions for each claw (eight claws per cow).

The statistical analysis was performed by running a chi-squared test using the statistical software StatSoft, version 8.0. P-values ≤ 0.05 were considered significant.

RESULTS

The prevalence of cows with one or more claw lesions on the farm with straw bedding (A) and with manure solids (B) were 37 % and 69%, respectively ($P < 0.001$). The dairy cows of farm B had more than one lesion per head, demonstrating a higher prevalence of claw diseases in the herd with manure solids (Table 1).

The results from the chi-squared test describing individual claw lesions on deep straw bedding versus manure solids are shown in Table 2.

Table 1. Prevalence of claw lesions in dairy cows on different beddings

Variable	Number of dairy cows	Cows with lesions	Lesion count	Lesion count per cow	Lesion count per cow with lesions
Deep straw bedding	403	151 (37 %)[a]	223	0.55	1.48
Manure solids	226	156 (69 %)	309	1.37	1.98

[a] — $P < 0.001$

Table 2. Prevalence of individual claw diseases in dairy cows on different beddings

Variable	Deep straw bedding	Manure solids
Number of dairy cows	403	226
Lesion count (100 %)	223	309
Digital dermatitis	17.5 %	53.1 %a
Heel erosion	21.5 %	18.8 %
White line diseases	12.1 %	3.88 %a
Toe ulcer	15.7 %	2.59 %a
Sole ulcer	9.43 %	3.23 %
Interdigital fibroma	17.9 %	12.3 %
Sole haemorrhage	3.59 %	5.83 %
Chronic laminitis	2.24 %	0.32 %

[a] — $P < 0.001$

In both bedding systems, two types of claw lesions dominated: digital dermatitis and inflammatory disorders of the corium (white line diseases, toe ulcer, and sole ulcer). The prevalence of the contagious digital dermatitis on farm A and B were 17.8 % and 53.1 % ($P < 0.001$), respectively. The prevalence of the white line diseases and toe ulcer was 12.1 % and 15.7 % on farm A and 3.88 % and 2.59 % on farm B, respectively ($P < 0.001$). There was no significant difference in the prevalence of the sole ulcer between the farms.

No acute laminitis could be observed in the examined dairy cows. In addition, several cases of sole haemorrhage and chronic laminitis were found on both farms (Table 2).

DISCUSSION

Bovine lameness can be based from 85 % on claw lesions associated with painful inflammatory processes. The remaining 15 % of lameness cases are due to other locomotory disorders, including: diseases of joints, tendons, tendon sheets, muscles, and bones or animals suffering from neurological disorders [29].

Cows housed in the deep straw-bedded freestalls (n = 403) had a lower prevalence of claw lesions (37 %) than cows housed in the freestalls with manure solids (69 %; n = 226). This difference was based on a high occurrence

of digital dermatitis on the farm with manure solids. Digital dermatitis represents a lesion that is frequently affecting more than one leg. However, some forms of digital dermatitis like M3 and M4 [8, 24] are not associated with painful conditions. Digital dermatitis prevalence was clearly on the farm with manure solids in the present study. We can speculate that the mixture of straw and faeces can represent in the case of deep straw bedding, a lower risk of skin infection with causative agents. Bovine digital dermatitis is a common, worldwide, painful, infectious disease of the feet of intensively managed cattle [21]. The cause of digital dermatitis is multifactorial with an essential spirochaetal bacterial component. Several cultural, phenotypic and molecular studies have demonstrated that the spirochetes belong to a diverse phylogenetic group of *Treponema* spp. [20]. Acute digital dermatitis lesions were reproduced experimentally in Holstein heifers, thus, a systematic method to determine the efficacy of interventions aimed at the control of acute digital dermatitis is now available [12]. A new hypothesis about the participation of *Dichelobacter nodosus* in the development of skin lesions typical for digital dermatitis has been demonstrated recently [22]. The authors hypothesise that external noxious stimuli allow D. nodosus to break down the epidermal barrier creating a suitable environment for the secondary invaders, *Treponema* species, which gradually take over the infection site. In agreement with the prevalence of the claw diseases in our study, a recent observation has revealed that the two most frequent claw diseases in dairy cows were digital dermatitis and septic corium inflammation [19].

Significant effects of stall surface on lameness prevalence have been reported [4, 9]. In both studies, the lameness prevalence was compared between herds with deep-bedded sand and mattress-based freestalls. A lower lameness prevalence could be observed in the sand stalls in comparison with the non-sand ones [4]. Similarly, high producing Holstein cows had a lameness prevalence of 17.1 % in herds with sand-based freestalls compared with 27.9 % in herds with mattresses [9]. It is interesting to note that the claw lesion prevalence for deep straw beds in the current study was much lower than the lesion prevalence observed with manure solids bedding. Differences in lameness prevalence likely occur between deep-bedded and mattress based stalls due to greater resting comfort in deep-bedded stalls. When provided the choice between deep beds with either sand or sawdust bedding and mattresses with 2 to 3 kg of bedding, cows showed a preference for deep beds [27]. Several studies have shown cows prefer stalls with greater surface cushion and spend more time lying down and less time standing when stall surfaces provide a greater degree of comfort [15, 26]. The use of mattresses as a stall surface has been implicated as a risk factor for lameness in dairy cows [7]. Deep-bedded freestalls likely provide greater comfort than mattresses with small amounts of bedding.

The higher prevalence of the white line diseases and toe ulcers in dairy cows on the farm A with deep straw bedding might be related to the impropriate walking surfaces of farm alleys. Risk factors for increased lameness were: the presence of damaged concrete in yards, cows pushing each other or turning sharply near the parlour entrance or exit, cattle grazing a pasture that is also grazed by sheep, the use of automatic scrapers, and delayed treatment of lame cows [2].

There are several preventive measures against high lameness prevalence, including: feeding, welfare, hoof trimming, and foot baths. Studies have shown that long intervals between hoof trimmings, or a lack of routine hoof trimming, is associated with an increased lameness [18, 25]. Professional trimming was found to be more effective on farms with no nutritional disorders and where refurbishment works were carried out. The greatest decrease in the prevalence of claw lesions was observed on farms which provided: professional trimming, effective footbathing, improved walking and resting surfaces, and which treated severely lame cows between regular trimmings [14]. However, claw trimming remains the most effective method available to facilitate producers to prevent claw disorders from evolving from the subclinical to the clinical stage.

These data of the present study indicate a relatively high prevalence of claw lesions on the studied dairy farms. In addition, a long-term contact of claws with manure (boxes and corridors) on the farm with unsterilized recycled manure solids seems to be associated with a higher prevalence of digital dermatitis. Generally, there was no overall effect of the manure solids on claw diseases rate in dairy cows.

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PARASITOFAUNA OF BROWN BEAR (*URSUS ARCTOS*) IN THE PROTECTED LANDSCAPE AREA CHKO — POĽANA

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ABSTRACT

During the years 2015—2016 we obtained 15 samples of faeces of brown bears (*Ursus arctos*) and 2 samples of gastrointestinal (GI) tracts of young female brown bears for helminthological examinations. The samples of faeces were collected from various sites in the protected landscape area CHKO-Poľana, and the gastrointestinal tracts originated from bears hunted down in the same area within permitted regulation of bear population for 2015. Of the 17 samples collected from the CHKO-Poľana area, 13 were positive for the presence of parasites (76.47 %). Parasitological examinations revealed the presence of 5 species of endoparasites: *Eimeria*, *Cryptosporidium*, *Sarcocystis*, *Baylisascaris* and *Ancylostoma*. Roundworms *Baylisascaris transfuga* (46.15 %) and *Ancylostoma* spp. (30.77 %) were the dominant species. Observation of the seasonal dynamics showed the highest prevalence of parasites during autumn and winter.

Key words: *Ancylostoma*; *Baylisascaris transfuga*; brown bear (*Ursus arctos*); *Cryptosporidium*; *Eimeria*; *Sarcocystis*

INTRODUCTION

The brown bear (*Ursus arctos*) is the most numerous large beast living in the Slovak territory. At the end of the 1920s, they were almost eradicated as their number decreased down to about 15—75 individuals. After the year 1932, when the State Protection of Nature of the Slovak Republic (SR) enacted a nationwide protection of this animal, the population of brown bears increased more than 40-fold. Between 2013—2015 the State Protection of Nature of SR set up a selection procedure for the implementation of a complex study of assessing of the population of brown bears (*Ursus arctos*) based on a non-invasive

method of determination of DNA in samples of excrements and the population of brown bears in Slovakia was assessed to be 1256 ± 233 individuals. This animal is most numerous in the mountain chains Veľká (Greater) Fatra and Malá (Lesser) Fatra, the Low Tatras, the High Tatras and Poľana [12]. In 1981, Poľana was declared a Protected Landscape Area (CHKO) for the purpose of protecting plant and animal communities and non-living nature. It is located in central Slovakia where it spreads over 20,360 hectares [14].

The majority of information about endoparasites in bears comes from the North America and Canadian territories where there are several bear species (grizzly, Baribal, and other). Only sporadic information is available about parasitofauna of the brown bears in Slovakia. This study conducted during 2002—2003 showed that in the areas of Western, Low and Belianske Tatry the eggs of *Baylisascaris transfuga* and *Toxascaris transfuga* were found at the highest prevalence in brown bears [6]. The examination of samples of bear faeces collected in 2008—2009 in the area of National park Poloniny confirmed the highest prevalence of the species *Baylisascaris transfuga* [1, 10]. The study conducted in 2010—2011 recorded 55.6 % prevalence of *Cryptosporidium* spp. in samples of brown bear faeces collected in central and eastern Slovakia [13]. This protozoan opportunistic pathogen with zoonotic potential is the causative agent of enteritis and diarrhoea in weakened individuals.

A special group of parasitoses are helminthoses, the most important of which are roundworms of the genus *Baylisascaris*, as well as species of the genus *Ancylostoma*. Larvae of these helminths cause larva migrans syndrome in humans, occurring in various forms (nervous, ocular, visceral). The infectious larva migrates in organs and tissues of humans which results in a wide scale of symptoms and raises serious risk to human health.

The aim of this study was to investigate the prevalence of endoparasites in brown bears in the Protected landscape area CHKO-Poľana in relation to their potential risk to human health.

MATERIALS AND METHODS

From spring to winter in the years 2015–2016 we collected 15 samples of faeces of brown bear in the territory Protected Landscape Area (CHKO) Poľana. We collected samples from different sites in order to decrease the possibility of obtaining several samples from the same animal. We also obtained 2 gastrointestinal tracts from young (4—5 years) female bears hunted down within permitted regulation of brown bears in the hunting grounds of the Protected hunting area (CHPO) Poľana used by the state enterprise Woodland of SR (LESY SR). They were subjected to complete helminthological examination.

The diagnostic methods employed in our study included the flotation method for the diagnosis of protozoan cysts and helminth eggs with the use of flotation solutions according to Breza and Faust [9]. To diagnose cryptosporidian oocysts in the faeces, we prepared smears and stained them by the Kinyoun method [4]. The samples were examined also by the ELISA method using the commercial kit *Cryptosporidium* ELISA (f. Diagnostic Automation, INC, Calabasas, CA). This ELISA is an *in vitro* immunoassay for the qualitative determination of *Cryptosporidium* antigen in faeces.

RESULTS

Of the total number of 17 samples (15 samples of faeces, 2 GI tracts) 13 were positive (76.47 %) for the presence of parasites. By means of the flotation method, we detected in one sample the presence of oocysts of the genus *Eimeria* (7.69 %), in 2 samples *Sarcocystis* spp. (15.38 %) and in 4 samples (30.77 %) eggs of the helminth *Ancylostoma* spp. The highest prevalence was observed for the species *Baylisascaris transfuga* (46.15 %) which was confirmed by total helminthological examination of the GI tract of two young female bears where we detected presence of both adult and juvenile forms of roundworms. The ELISA meth-

Table 1. Species composition and prevalence of endoparasites found in samples from brown bears in the Protected Landscape Area (CHKO) Poľana

Genus/Species	Prevalence
Cryptosporidium	15.38 %
Eimeria	7.69 %
Sarcocystis	15.38 %
Ancylostoma	30.77 %
Baylisascaris transfuga	46.15 %

Table 2. Prevalence of endoparasites in brown bear according to seasons

Season	Prevalence
Spring	33.33 %
Summer	66.67 %
Autumn	85.71 %
Winter	100 %

od confirmed the cryptosporidium antigen in two samples (15.38 %) (Table 1).

The highest prevalence of endoparasites was recorded during autumn and early winter months (October—December). In autumn the prevalence was 85.71 % while in early winter it reached 100 %. In the spring, the prevalence was low (33.33 %) and in the summer it increased 2-fold (66.67 %) (Table 2).

DISCUSSION

Detailed surveys of parasitofauna of brown bear has been conducted in various areas of North America and Canada. The most frequently found species were roundworms *Baylisascaris transfuga*, *Trichinella spiralis*, *Uncinaria* spp., and tapeworms of genera *Diphylobotrium*, *Taenia*. Other endoparasites such as; *Echinostoma revolutum*, *Dirofilaria ursi*, and ectoparasites *Dermacentor andersoni* and *Arctopsylla* spp. occurred only sporadically [15]. Between 2011—2013 in Canada, in the territories of Alberta and British Columbia, 7 various endoparasites were found in the American black bear Baribal (*Ursus americanus*) and grizzly bear (*Ursus arctos horribilis*): *Baylisascaris transfuga*, *Dirofilaria ursi*, *Uncinaria rauschi*, *Uncinaria yukonensis*, *Taenia arctos* and tapeworms with a zoonotic character, such as, *Diphyllobothrium dendriticum* and *Diphyllobotrium nihonkaiense* [1].

It has been suggested that the maximum size of the brown bear population acceptable for the entire total Slovak territory is about 700 bears which would correspond to 1,150 hectares of wooded area per one animal. This comes to about 23 % of the Slovak territory where these animals are regularly found. The increased pressure of human activities on the natural environment, such as: tourism, picking forest fruits, hunting, timber harvesting, new settlements and constant construction activities, results in the displacement of bears to populated areas and raises the risk of their contact with humans. Changed living conditions of wildlife and the considerable increase in the population of the brown bears in the recent years are factors supporting the transmission of propagative stages of protozoa and helminths which have the potential to produce diseases in humans, the so-called parasito-zoonoses [6, 10, 11].

There is available only sporadic information about the parasitofauna of brown bears in the Slovak territory. Investigations of endoparasites in brown bears were carried out by Mituch in 1972 [11] who performed helminthological dissections of 24 bears and recorded the occurrence of five different species of helminths: *Taenia hydatigena*, *Toxascaris transfuga*, *Trichinella spiralis*, *Thomix aerophilus* and *Aelustrongylus abstrusus*. Goldová et al. detected the presence of 6 parasite species: *Baylisascaris transfuga*, *Ancylostoma* spp., *Toxascaris* spp., *Cryptosporidium* spp., *Taenia* spp. and *Capilaria* spp. [6]. During 2008—2009 coprological examinations showed the presence of 4 parasite species with the prevalence reaching 72.34 %. The dominant species was *Baylisascaris transfuga* [10].

The majority of authors investigating the seasonal dynamics of parasites in bears reported that the highest prevalence of endoparasites was recorded in autumn and the lowest in spring. Several explanations were suggested, why bears hibernate only for short time or not at all during the winter season: global warming and relatively warm winter months; disturbance of bears by people; either tourists or hunters; or hunting parties during the major pre-denning period [2].

Our observations of the seasonal dynamics of endoparasites in brown bears showed that their prevalence was the highest during autumn (85.71 %) and early winter months (100 %). The majority of authors investigating seasonal dynamics of parasites in bears reported that the parasites were most prevalent in autumn and the prevalence in spring was the lowest. In agreement with our results, Major et al. [10] in their coprological study in 2008—2009 also observed the highest prevalence of endoparasites in autumn and winter and the lowest one in spring. Study in grizzly bears (*Ursus arctos horribilis*) in Canada also showed a higher prevalence of gastrointestinal parasites in autumn than in spring and the presumed reason for such dynamics was the elimination of adult helminths from their bod-

ies before denning and subsequent re-infection in spring, as bears limit uptake of food shortly before their "winter sleep" and evacuate their bowels [5]. Choquete et al. [8] similarly presumed that endoparasites were eliminated before denning. However, Frechette and Rau and Finnegan speculated that adult parasites die during winter months denning due to the lack of carbohydrates and are subsequently decomposed and absorbed, or eliminated in the first spring faeces [2, 3]. We contemplated that the high prevalence of endoparasites in winter months observed in our study was related to a relatively warm 2015/2016 winter which resulted in a short period of denning or its complete absence and bears consumed food also during this period. During late summer and autumn, when bears form fat reserves important for winter denning, they enter the phase known as hyperphagia when the intensity of feed intake increases by 2—3 fold compared to the normal activity during spring and summer and as a result of this, their body weight may increase by 30—35 % [7]. This biological peculiarity of brown bears may be the reason behind the recorded higher prevalence of endoparasites in this animal during late autumn and the winter months.

CONCLUSIONS

All species of endoparasites identified in the samples of bear faeces or their gastrointestinal tract present danger to human health as they are classified as parasito-zoonoses, i.e. diseases that can naturally be transmitted to humans. *Baylisascaris transfuga* and *Ancylostoma* spp. are considered the potential causes of visceral, nervous or ocular form of larva migrans in mammals including man. In humans, particularly in immunodeficient patients, coccidia of *Cryptosporidium* spp., may cause serious infections with pronounced clinical symptoms, such as watery diarrhoea associated with colic and abdominal pain, nausea, weight loss, and even dehydration. The enormous increase in the bear population in Slovakia in the past several years; regular occurrence of bears close to populated areas; repeated encounters of these animals with people; increased pressure of human activities on natural environment; insufficient denning during winter months; and the possibility of transmission of infectious stages of protozoa and helminths, raise considerable risk to the health of the human population.

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A RARE CASE OF ATYPICAL RENAL ARTERIES ARRANGEMENT WITH ECTOPIC KIDNEYS IN A GUINEA PIG

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ABSTRACT

We recorded a very rare case of atypical renal arteries arrangement in a guinea pig using the corrosion technique in the study of the arterial system. The right renal artery originated from the ventral wall of the abdominal aorta at the level of the caudal aspect of the 5th lumbar vertebra. The left renal artery originated from the left common iliac artery approximately 12 mm caudally to the aortic bifurcation. The right kidney was located ventral to the aortic bifurcation and the left kidney inside the pelvic cavity between the common iliac arteries. According to the vascular pattern, we determined that the ectopic kidneys in this guinea pig were unusual. This is the first case describing bilateral ectopic kidneys in a guinea pig.

Key words: abdominal aorta; guinea pig; iliac artery; kidney; renal artery

INTRODUCTION

The general arrangement of renal arteries in the guinea pig is that they originate off of the lateral walls of the abdominal aorta caudal to the origin of the posterior mesenteric artery [10]. The literature describes several variations in the origin and arrangement of the guinea pig renal arteries [12]. In humans, they are found also as branches originating from various branches of abdominal aorta [3] or from the thoracic aorta [6]. The anomalous arrangement and origin of renal arteries is very often associated with unilateral or bilateral kidney ectopy.

Although a simple ectopic kidney is seldom responsible for symptoms, the association with malrotation of the renal pelvis with a calculus may increase the risk of hematuria, hydronephrosis, and stone formation with colicky pain [8]. Other signs and symptoms of ectopic kidneys include: incontinence, a palpable abdominal mass, urinary tract infection, renovascular hypertension secondary to an anomalous blood supply, and dystocia from a pelvic kidney [7]. An ectopic kidney is often associated with other abnormalities such as: agenesis of the opposite kidney, vascular

malformation, and genital anomalies. Ectopic kidneys and an anomalous origin of the renal arteries were described in: the rat [5], dogs [4], a cat [2], a swine [11] and a bovine [9].

The purpose of this study is to describe a rare case of guinea pig ectopic kidneys using the corrosion technique of arterial system.

MATERIALS AND METHODS

In a routine study of the guinea pig arterial system, we found in one male an atypical position of the kidneys. The study of the arterial system in 26 adult English self guinea pigs (*Cavia porcellus*, L. 1758), aged 220 days was performed using the corrosion technique. We investigated guinea pigs of both sexes (female n = 13; male n = 13), in a weight range of 0.8–1 kg, in an accredited experimental laboratory at the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15–20 °C, relative humidity 45 %, 12 h light period). A granular feed mixture (FANTASIA, Tatrapet, Liptovsky Mikulas, Slovak Republic) and drinking water were available to all animals ad libitum. The animals were sacrificed by intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}). Immediately after euthanasia, the vascular network was perfused with a physiological solution. During the manual injection through the ascending aorta, the right atrium of the heart was opened in order to lower the pressure in the vessels in order to ensure an optimal injection distribution. Spofacryl (SpofaDental, Czech Republic) using a volume of 20 ml was used as a casting medium. After polymerization of the medium, the maceration was carried out in 2–4 % KOH solution for a period of 2 days at 60–70 °C. This study was carried out under the authority of decision No. 2647/07-221/5.

RESULTS

The right renal artery originated from the ventral wall of the abdominal aorta at the level of the caudal aspect of the 5th lumbar vertebra. The origin was located 4 mm cranial to the aortic bifurcation and 7 mm caudally to the origin of the caudal mesenteric artery. Furthermore, the right renal artery turned ventrally and caudally (Fig.1). During its course, in the place of contact of the right renal artery with the right kidney, it gave off two small interlobar arteries entering the kidney parenchyma. The main continuation entered the ventral directed renal hilus. The right kidney was located ventrally to the aortic bifurcation at the level from the caudal half of the 4th lumbar vertebra to the cranial half of the 6th lumbar vertebra (Fig. 2).

Fig. 1. Origin of the right renal artery. 1 — abdominal aorta; 2 — right renal artery; 3 — caudal mesenteric artery 4 — left common iliac artery; 5 — right common iliac artery; 6 — median sacral artery. Left lateral view. Magn. ×3.2

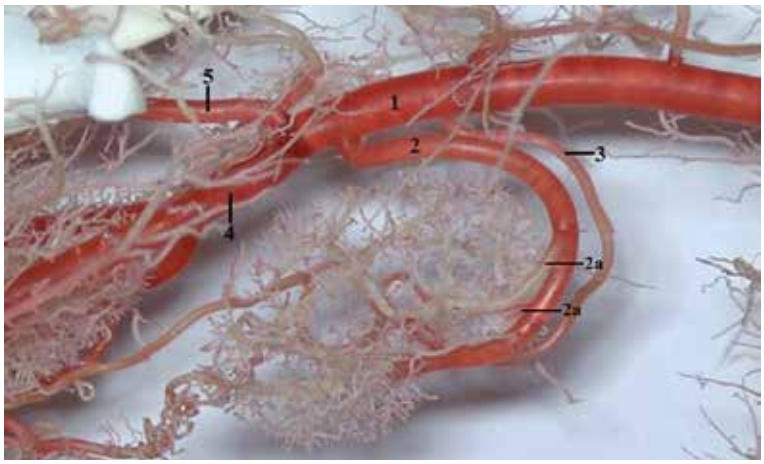

Fig. 2. Origin of the right renal artery. 1 — abdominal aorta; 2 — right renal artery; 2a — interlobar arteries 3 — caudal mesenteric artery; 4 — right common iliac artery; 5 — median sacral artery. Right lateral view. Magn. ×3.2

Fig. 3. Origin of the left renal artery. 1 — right common iliac artery; 2 — left common iliac artery; 3 — left renal artery 4 — caudal mesenteric artery; 5 — left internal iliac artery. Ventral view. Magn. ×3.2

The left renal artery originated from the left common iliac artery approximately 12 mm caudally to the aortic bifurcation and 8 mm cranially to the origin of the internal iliac artery. After running in a ventromedial direction, the left renal artery entered the left kidney through the craniolaterally positioned renal hilus. The position of the left kidney was inside the pelvic cavity between bilateral common iliac arteries (Fig. 3).

The cranial abdominal, adrenal and phrenic arteries were independent branches arising from the cranial part of the abdominal aorta.

DISCUSSION

Popesko et al. [10] described the origin of renal arteries in the guinea pig from the abdominal aorta by means of the cranial and caudal right renal artery, and cranial and caudal left renal arteries. The origin of the cranial renal arteries was found at the level of the 1st lumbar vertebra, and of the caudal renal arteries at the level between the 1st and 2nd lumbar vertebra. The cranial abdominal arteries originated independently from the abdominal aorta cranial to the cranial renal artery.

In about 20 % of cases, both renal arteries were single, the right renal artery was double in over 50 % of the cases, and the left renal artery was double in over 60 % of the cases. Single or double renal arteries were divided into two branches before entering the kidney. The largest branch arising from the renal artery was the cranial abdominal artery. It branched from the cranial renal artery in cases of double renal arteries or just proximally to the division into branches in cases of single renal arteries [12].

An ectopic kidney usually does not show specific clinical signs. Unilateral ectopic kidneys are more common in several species of domestic mammals. In a cat, was described an ectopic right kidney while the left kidney was found in the correct place [2]. Ectopic kidneys were also described in dogs. In three dogs, was found in one case, bilateral ectopic kidneys and in two cases only a left ectopic kidney [4].

An ectopic kidney can cause poor outflow and is more susceptible to calculi, hydronephrosis and infection. Hydronephrosis is the most common finding in humans with an ectopic kidney [2]. There is also an increased incidence of other extrarenal malformations in humans, including skeletal anomalies (particularly vertebral), imperforate anus, cryptorchidism, other genitourinary and cardiovascular anomalies, and chromosomal defects [1].

CONCLUSIONS

The diagnosis of an ectopic kidney could be included in the differential diagnoses of abdominal or retroperitoneal masses. This is of particular importance, since ectopic kidneys are more susceptible to diseases than normal ones. Although a clinically silent ectopic kidney is identified, careful monitoring of the patient may be needed in order to detect possible urologic problems.

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CONTENT OF CHEMICAL ELEMENTS IN WOOD-DESTROYING FUNGI

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ABSTRACT

The aim of this study was to examine the content of chemical elements in the dried fruiting bodies of edible wood decaying fungi such as Honey mushrooms (*Armillaria mellea*), Shiitakes (*Lentinus edodes*) and Oyster mushrooms (*Pleurotus ostreatus*). Powdered samples of fungi were mineralized in a microwave digestion. Twenty-one (21) chemical elements were detected in the plasma of the device ICP-MS AGILENT 7500c by accredited methods with the aid of calibration curves. The content of individual elements varied within a considerable range. The highest contents of K, Mn, Cu and Cd were found in the fruiting bodies of Honey mushrooms (*Armillaria mellea*). Shiitakes (*Lentinus edodes*) had the highest content of B and Mo. Significant differences were found in the content of elements in the Oyster mushrooms (*Pleurotus ostreatus*) from Slovakia, Hungary and China. The highest content of Al was found in the Oyster mushrooms (*Pleurotus ostreatus*) from Hungary. The Chinese oysters had a maximum contents of Ca, Mg, Co, Pb, As and U. The Oyster mushrooms (*Pleurotus ostreatus*) from Lemešany (Slovakia) had the highest contents of Na, Zn, Fe, Se, Ag, Hg and Cr. The difference of chemical element content could be influenced by the genotype of the fungus and by the composition of substrate on which mushroom grow up.

Key words: *Armillaria mellea*; chemical elements; ICP-MS; *Lentinus edodes*; *Pleurotus ostreatus*

INTRODUCTION

Fungi and mushrooms play an important role in ecosystems. They participate in the process of decomposition of live and dead matter, which often causes, particularly in the forest sector, considerable damage. During their short life, mushrooms in their bodies accumulate important substances for human nutrition. Cultivated mushrooms were found to be a good source of: vitamins [24, 27], dietary fibres, polysaccharides, α- and β-glucans which have immunostimulating, immunomodulating and anti-cancer properties [29, 30]. Mushrooms contain β-carotene, lyco-

pene, tocopherols [24], phenolics, and flavonoids with antioxidant effects [6]. They contain a number of biologically active substances that function as enzymes, protease inhibitors, lectins and so on [15]. Edible wild grown mushrooms are relatively rich in essential trace elements such as potassium, phosphorus, selenium, iron, zinc, manganese, and copper [3], but also absorb toxic elements such as mercury, cadmium, lead, silver and arsenic from the substrate, which are hazardous in the diet [5, 8, 9, 10, 11]. Many studies have drawn attention to the occurrence and concentration of toxic elements found in the fruiting body of mushrooms. In order to obtain valuable biologically active substances from fungi, researchers have developed new technological methods of cultivating fungi on substrates such as straw, cereals and other agricultural raw materials [17], or fungi are grown as a tissue cultures with a strictly defined composition of the substrate [16].

The aim of this study was to examine the content of chemical elements in the dried fruiting bodies of edible wood decaying fungi such as Honey mushrooms (*Armillaria mellea*), Shiitakes (*Lentinus edodes*) and Oyster mushrooms (*Pleurotus ostreatus*) grown in various substrates in different countries.

MATERIALS AND METHODS

As experimental materials, we used homogeneous powder of the dried fruiting bodies of Honey mushrooms (*Armillaria mellea*) originating from Slovakia — mushrooms were picked up in the forest; Shiitakes (*Lentinus edodes*) originating from China — mushrooms were bought at the store and Oyster mushroom (*Pleurotus ostreatus*) originating from Hungary and China (mushrooms were also bought at the store) and originating from Slovakia (small village Lemešany) — mushrooms were cultivated on wooden blocks.

Powdered samples of fungi were mineralized in a microwave digestion with 6 cm^3 of concentrated HNO_3 and 1 cm^3 of 30 % H_2O_2. The mineralized samples were analysed in the plasma of the device ICP-MS AGILENT 7500c (Agilent, USA) by accredited methods used at the State Veterinary and Food Institute in Košice. The contents of 21 chemical elements were detected by means of the aid of the calibration curves (Table 1). The reagents used were of the highest quality (Suprapur and p. a.). Mercury was determined using a special device (AMA 254) for the determination of Hg directly in the powdered material without mineralization. The results are reported as the average of three measurements in $mg.kg^{-1}$ DW with the corresponding SD.

RESULTS AND DISCUSSION

Mushrooms have been part of a normal human diet for thousands of years. Analysed edible wood decaying fungi such as Honey mushrooms (*Armillaria mellea*), Shiitakes (*Lentinus edodes*) and Oyster mushrooms (*Pleurotus ostreatus*) are valued due to their aroma and taste, as well as their proteins, dietary fibre, low fat content, vitamins, essential trace elements and a number of other biologically active substances. Fresh and preserved fruiting bodies of them can be culinary — processed in different ways. For many years, edible wood decaying fungi were widely used in traditional medicine. In recent years, much attention has focused on various immunological and anti-cancer properties of certain mushrooms that offer potentially important health benefits, including antioxidants, anti-hypertensives, cholesterol-lowering properties, and liver protection, as well as anti-inflammatory, anti-diabetic, anti-viral and anti-microbial properties [5]; [13]. These properties have attracted the interest of many pharmaceutical companies as a rich source of innovative biomedical molecules.

Mushrooms have the ability to accumulate not only toxic but also the essential chemical elements from the environment in which they grow. Therefore, mushrooms can be used as an indicator of pollution in particular in the areas of mining and metallurgical metal processing [8]; [9]; [10]; [11]. Cultivated mushrooms are characterized by a lower content of toxic elements [8].

The mean content of chemical elements is presented in the Table. 1. The results are expressed in $mg.kg^{-1}$ dry weight (DW).

The results of this study demonstrated that the concentrations of chemical elements in the various kinds of wood decaying fungi were different. The highest concentration of boron was measured in fruiting bodies of Shiitakes *(Lentinus edodes*) originating from China. The lowest content of boron was found in the fruiting bodies of Oyster mushroom (*Pleurotus ostreatus*) originating from Hungary. Kaya and Bag [12] found in Honey mushrooms (*Armillaria mellea*) a boron value of 0.243 $mg.kg^{-1}$

and in Rogers mushrooms (*Lentinus tigrinus*) the value of 1.132 mg.kg^{-1}. The boron content in the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) was in the range from ND (not detected) to 2.630 mg.kg^{-1}. In comparison with these results, the boron content in our samples of the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) varied from 0.323 to 0.563 mg.kg^{-1}. Vetter [25] determined higher boron content (5—15 mg.kg^{-1}) in *Marasmius wynnei*. Konuk [14] in compliance with our results have published 0.50 µg.g^{-1} boron content in Honey mushrooms (*Armillaria mellea*).

Extremely high levels of aluminium were found in Oyster mushrooms (*Pleurotus ostreatus*) originating from Hungary (14 527 mg.kg^{-1}). This is probably a consequence of the high aluminium content in the soil. Hungary is rich in bauxite, which is an aluminium ore. The lowest aluminium content was measured in the fruiting bodies of Shiitakes (*Lentinus edodes*) originating from China (69.67 mg.kg^{-1}) (Table 1).

Oyster mushrooms (*Pleurotus ostreatus*) from different countries showed widely different results for the aluminium content. Kaya and Bag [12] reported aluminium content in 24 different mushroom species ranging from 18.71 to 1 486 mg.kg^{-1} DW. Other scientists have mentioned the aluminium content in different mushroom species in the range from 8.5 to 365 µg.g^{-1} or 68 to 420 µg.g^{-1} (cited in Kaya and Bag [12]). Our results, except for the extreme value of aluminium found in Oyster mushrooms (*Pleurotus ostreatus*) originating from Hungary, are in accordance with the values of aluminium published in the literature for different varieties of mushrooms.

The high aluminium content presents a risk to human health. The WHO recommends an acceptable daily intake of aluminium of 60 mg per day.

The sodium content was found in a range of 4.684 and 51.49 mg.kg^{-1}. The highest value of sodium was found in Oyster mushrooms (*Pleurotus ostreatus*) grown on a wooden block (Table 1). The farmer used sodium chloride to protect Oyster mushrooms against snails and this probably caused an increased sodium content in fruiting bodies.

Ayaz et al. [1] reported a high content of sodium 363.57 ± 13.40 mg.kg^{-1} DW in Honey mushrooms (*Armillaria mellea*). Muszyńska et al. [19] reported 100 ± 67.00 mg.kg^{-1} of sodium in fruiting bodies DW of Honey mushrooms (*Armillaria mellea*). In comparison with the published values, our results of sodium content in mushrooms were lower.

Mushrooms are an important source of potassium. For edible wild grown mushrooms Kalač [10, 11] determined that the potassium content was in a range of 20 000 to 40 000 mg.kg^{-1}. Zhang et al. [31] reported the potassium content in a range of 29 000 to 45 000 mg.kg^{-1}. These values of the potassium content are extremely high in comparison with our results. Our maximum value of potassium content was 21 009 mg.kg^{-1}. Other values of potassium content were lower and ranged from 10 588 to 15 684 mg.kg^{-1} (Table 1).

The calcium content of 34.77 mg.kg^{-1} was found in Honey mushrooms (*Armillaria mellea*) and of 126.32 mg.kg^{-1} in Oyster mushrooms (*Pleurotus ostreatus*) originating from China (Table1).

Kalač [10, 11] determined that the calcium content was in a range from 100 to 500 mg.kg^{-1} DW. Zhang et al. [31] reported that the calcium content was in a range from 35 to190 mg.kg^{-1} DW. Our results of the calcium content in mushrooms corresponded with these published results.

The usual contents of magnesium in wild growing mushrooms are in a range from 650 to 1300 mg.kg^{-1} DW. Kalač [10]; [11] determined that the magnesium content was in a range from 800 to 1800 mg.kg^{-1} DW. Our results of the magnesium content (924.1 to 1028.1 mg.kg^{-1} DW) in mushrooms were in a range of these published results (Table 1).

The highest manganese (13.49 mg.kg^{-1} DW) and copper (8.729 mg.kg^{-1} DW) contents were in Honey mushrooms (*Armillaria mellea*) originating from Slovakia. The highest zinc (34.17 mg.kg^{-1} DW) and iron (97.03 mg.kg^{-1} DW) contents were in Oyster mushrooms (*Pleurotus ostreatus*), also originating from Slovakia (Table 1).

Kalač [10] determined the usual range of manganese content (5–60 mg.kg^{-1} DW), copper content (10—70 mg.kg^{-1} DW), zinc content (30—150 mg.kg^{-1} DW) and iron content (30—150 mg.kg^{-1} DW). Zhang et al. [31] determined that the manganese, copper, zinc and iron contents were in the range of 6.4—43; 14—39; 140—260; and 24 200 mg.kg^{-1} DW, respectively. Radulesku et al. [20] determined that the manganese, copper, zinc and iron contents in the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) were in the range of 12.40—11.50; 12.50—10.20; 41.30—37.90; and 387.00—284.0 mg.kg^{-1} DW, respectively. The same authors determined that the manganese, copper, zinc and iron contents in the fruiting bodies of Honey mushrooms (*Armillaria mellea*) were in the range 10.91—7.85; 10.43—6.43; 158.2—80.5; and 745.0—432.0 mg.kg^{-1} DW, respec-

Table 1. Mean content of chemical elements in the dried fruiting bodies (DW) of wood decaying fungi (*Armillaria mellea, Lentinus edodes- Shiitake* and *Pleurotus ostreatus*) originating from Slovakia, China and Hungary

Chemical element	*Armillaria mellea* Slovakia		*Lentinus edodes* China		*Pleurotus ostreatus* Hungary		*Pleurotus ostreatus* China		*Pleurotus ostreatus* Slovakia	
	mg.kg^{-1}	SD	mg.kg^{-1}	SD	mg.kg^{-1}	SD	mg.kg^{-1}	SD	mg.kg^{-1}	SD
Boron (B^{11})	0.615	0.031	1.078	0.012	0.323	0.007	0.563	0.011	0.426	0.002
Sodium (Na^{23})	4.684	0.036	11.05	0.075	12.59	0.011	30.07	0.007	51.49	0.005
Potassium (K^{39})	21 009	0.019	11 281	0.071	15 684	0.077	13 171	0.014	10 588	0.011
Calcium (Ca^{43})	34.77	0.013	35.56	0.010	73.27	0.015	126.32	0.005	58.88	0.024
Magnesium (Mg^{24})	942.8	0.032	961.9	0.031	924.1	0.012	1 028.1	0.018	956.1	0.021
Aluminium (Al^{27})	145.2	0.013	69.67	0,005	14 527	0.119	925.49	0.291	226.3	0.112
Manganese (Mn^{55})	13.49	0.011	10.95	0.002	4.735	0.003	7.852	0.011	9.594	0.004
Copper (Cu^{63})	8.729	0.005	3.542	0.005	3.428	0.001	5.517	0.003	4.653	0.007
Zinc (Zn^{66})	23.7	0,008	22.65	0,012	27.01	0,021	30.01	0.008	34.17	0.014
Iron (Fe^{57})	39.39	0.007	24.16	0.003	61.83	0.037	64.64	0.014	97.03	0.021
Cobalt (Co^{59})	0.022	0.001	0.019	0.001	0.054	0.003	0.163	0.002	0.041	0.001
Molybdenum (Mo^{95})	0.002	0.001	0.006	0.001	0.002	0.001	0.002	0.001	0.003	0.001
Selenium (Se^{82})	1.199	0.007	1.169	0.012	1.436	0.031	1.179	0.011	3.335	0.021
Silver (Ag^{107})	0.748	0.014	0.102	0.003	0.052	0.001	0.274	0.001	2.503	0.037
Mercury ($(Hg)^{202}$AMA	0.074	0.018	0.011	0.006	0.021	0.007	0.097	0.003	0.799	0.012
Cadmium (Cd^{111})	4.182	0.006	1.519	0.021	0.158	0.010	0.375	0.007	0.588	0.004
Lead (Pb^{208})	0.681	0.007	0.631	0.005	1.026	0.002	1.978	0.011	0.703	0.001
Arsenic (As^{75})	0.094	0.001	0.349	0.001	3.312	0.004	13.23	0.002	0.449	0.002
Chrome (Cr^{53})	0.322	0.002	0.225	0.001	0.222	0.001	0.555	0.001	0.562	0.001
Nickel (Ni^{60})	0.245	0.012	0.153	0.007	0.658	0.001	0.291	0.001	0.357	0.004
Uranium (U^{238})	0.022	0.007	0.019	0.003	0.059	0.012	0.073	0.002	0.026	0.002

tively. In comparison with these published values, our results were lower. Much higher values of these elements in the fruiting bodies of Honey mushrooms (*Armillaria mellea*) and Oyster mushrooms (*Pleurotus ostreatus*) were determined by Kaya and Bag [12] and Ayaz et al. [1].

Many Slovaks like to pick up wild mushrooms in the forest and consume it by different ways. Moreover, everyone can buy both cultivated and wild mushrooms in open markets or stores. The consumption of wild and cultivated mushrooms continues to increase in many countries. This can increase transport of toxic chemical elements into humans and cause health risks to consumers.

It is necessary to know how much of mercury, cadmium, lead, chrome, arsenic and nickel are found in the fruiting bodies of wild and cultivated mushrooms. Certain countries have established statutory limits for metals in edible mushrooms. In the Slovak Republic, the limits of 0.75 mg.kg^{-1} and 1.0 mg.kg^{-1} FW (fresh weight) have been established for mercury and cadmium in wild-growing mushrooms; whereas 0.2 mg.kg^{-1} FW have been established for cadmium in cultivated mushrooms. Limits of 1.0 mg.kg^{-1} FW have been established for lead in wild-growing mushrooms and 0.3 mg.kg^{-1} FW in cultivated mushrooms. The maximum level for arsenic is 0.5 mg.kg^{-1} FW and for nickel is 0.5 mg.kg^{-1} FW in other foods (Slovak Codex Alimentarius, Chemical contaminants) [22]. Slovak Codex Alimentarius does not have any limits for chromium in mushrooms.

The maximum level for certain contaminants in foodstuffs established by the Commission of the European Communities (Commission Regulation EC No 466/2001) [28] is set at about 0.2 and 0.3 mg.kg^{-1} fresh weight for cadmium and lead, respectively, in cultivated fungi. Assuming that the dry matter content of mushrooms is 10 %, these same limits for dry material will be ten times higher and approach 2.0 and 3.0 mg.kg^{-1} dry weight for cadmium and lead, respectively [21].

The mercury content in our samples of mushrooms was found in the range from 0.011 mg.kg^{-1} DW in Shiitakes (*Lentinus edodes*) fruiting bodies originating from China to 0.799 mg.kg^{-1} DW (which is equivalent 0.0799 mg.kg^{-1} FW) in fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) originating from Slovakia (Table 1). The mercury content in our samples of mushrooms did not exceed the hygiene limit for fresh mushrooms.

The cadmium content in our samples of mushrooms was found in the range from 0.158 to 4.182 mg.kg^{-1} DW. The maximum value of cadmium was accumulated in Honey mushrooms (*Armillaria mellea*) originating from Slovakia. The cadmium content of 4.182 mg.kg^{-1} DW (0.418 mg.kg^{-1} FW) in this mushroom did not exceed the hygiene limit of 1.0 mg.kg^{-1}for fresh mushrooms (Table 1).

The lead content in our samples of mushrooms was found in the range from 0.631 mg.kg^{-1} DW in Shiitakes (*Lentinus edodes*) fruiting bodies originating from China to 1.978 mg.kg^{-1} DW in fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*), also originating from China (Table 1). Both cultivated mushrooms did not exceed the hygienic limit of 0.3 mg.kg^{-1} for fresh cultivated mushrooms.

In Honey mushrooms (*Armillaria mellea*) fruiting bodies we found the arsenic content to be 0.094 mg.kg^{-1} DW and in the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) originating from China the arsenic content was 13.23 mg.kg^{-1} DW, which exceeded the hygiene limit of 0.5 mg.kg^{-1} FW for foods.

The oyster mushrooms (*Pleurotus ostreatus*) originating from China were probably cultivated on rice straw which is usually treated with some arsenic detergents. Arsenic accumulates in rice straw and then passes from the rice straw substrate into the fruiting bodies of Oyster mushroom (*Pleurotus ostreatus*).

The chromium content in our samples of mushrooms was found in the range from 0.222 to 0.562 mg.kg^{-1} DW (Table 1). Nearly the same chromium contents were found in the fruiting bodies of Shiitakes (*Lentinus edodes*) originating from China and Oyster mushrooms (*Pleurotus ostreatus*) originating from Hungary.

The nickel content was found in the range from 0.153 to 0.658 mg.kg^{-1} DW. The lowest value was determined in the fruiting bodies of Shiitakes (*Lentinus edodes*) originating from China and the highest nickel content was found in the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) originating from Hungary (Table 1). These values did not exceed the hygienic limit for nickel of 0.5 mg.kg^{-1} FW in other foods.

Relatively low levels of molybdenum (0.002—0.006 mg.kg^{-1} DW), cobalt (0.019—0.163 mg.kg^{-1} DW), and uranium (0.019—0.073 mg.kg^{-1} DW) were determined in the samples of mushrooms (Table 1).

The concentrations of the accumulated chemical elements reported by some scientists vary considerably for the same mushroom species. Kalač and Svoboda [8] stated for the fruiting bodies of wild growing mushrooms,

a mercury content ranging from < 0.5 to 10 mg.kg^{-1} DW. In the Honey mushrooms (*Armillaria mellea*) fruiting bodies, they found the mercury content lower than 0.5 mg.kg^{-1} DW, and cadmium content was in the range from 2.5 to 5.0 mg.kg^{-1} DW. Others scientists found cadmium content in the fruiting bodies of wild growing mushrooms in the range 5—50 mg.kg^{-1} DW. Wild growing mushrooms are good cadmium accumulators [11, 4]. The lead content was found in the range from 1.0 to 10 mg.kg^{-1} DW. In the Honey mushrooms (*Armillaria mellea*) fruiting bodies these authors found the lead content to vary from 1 to 2 mg.kg^{-1} DW. In many species of mushrooms the arsenic content was lower than 0.05 mg.kg^{-1} DW [26].

The study of the accumulated metal elements in cultured Champignon mushrooms (*Agaricus bisporus*) fruiting bodies showed arsenic content in the range from 0.54 to 22.8 mg.kg^{-1} DW when substrate contained arsenic content 3.8 to 1000 mg.kg^{-1} DW [23]. Kalač and Svoboda [8] and Kalač [10,11] published the chrome content in mushrooms growing in uncontaminated areas in the range from 0.1 to 2.0 mg.kg^{-1} DW and the nickel content in the range from 0.4 to 2.0 mg.kg^{-1} DW.

In our study, the highest selenium content (3.335 mg.kg^{-1} DW) and highest silver content (2.503 mg.kg^{-1} DW) were found in the fruiting bodies of Oyster mushrooms (*Pleurotus ostreatus*) originating from Slovakia (Table 1). In the other four samples of mushrooms, the mean selenium content was 1.246 mg.kg^{-1} DW. Our results of selenium content are in the range of the selenium content published by Kalač [10] (1—5 mg.kg^{-1} DW). Various species of mushrooms may accumulate different amount of selenium. In extract from the fruiting bodies of the Honey mushrooms (*Armillaria mellea*), the selenium content was 2.359 mg.kg^{-1} and in extract from fruiting bodies of the family *Boletaceae* (*Boletus edulis*), the selenium content reached 17.0 mg.kg^{-1} [18].

As it is evident from our results, edible wood decaying fungi have the ability to take up some amount of silver from the substrate. The highest silver content of 2.503 mg.kg^{-1} was found in the fruiting bodies of the Oyster mushrooms (*Pleurotus ostreatus*) originating from Slovakia (Table 1). A considerably lower silver content of 0.748 mg.kg^{-1} was found in the fruiting bodies of the Honey mushrooms (*Armillaria mellea*). The ability of edible wood decaying fungi to accumulate silver from the substrate decreased in the following order: Oyster mushrooms (*Pleurotus ostreatus*) originating from Slovakia > Honey mushrooms (*Armillaria mellea*) originating from Slovakia > Oyster mushroom (*Pleurotus ostreatus*) originating from China > Shiitakes (*Lentinus edodes*) originating from China > and Oyster mushrooms (*Pleurotus ostratus*) originating from Hungary.

Some species of mushrooms may accumulate high amounts of silver from the soil contaminated with silver [2]. This provides the opportunity to obtain silver in other way than mining.

CONCLUSIONS

In general, fungi play an important role in the biosphere of soils and are intimately involved in the cycling of elements in both organic and inorganic substrates [7].

Wild growing mushrooms are also known as an unfailing source of biologically active substances used for many years in herbal medicine which is now the inspiration for finding and developing modern drugs. Mushrooms are consumed as a delicacy, and particularly for their specific aroma, taste and texture can be culinary processed in different ways.

However, it is necessary to remember that edible mushrooms can accumulate high amounts of toxic elements (mercury, cadmium, lead or arsenic). This can cause serious health problems especially in mining and metallurgical processing areas where the mushrooms grow in an environment highly contaminated by pollutants. The contamination of cultivated mushrooms with toxic elements is lower than contamination of edible wild growing mushrooms. But it also depends on the quality and composition of the substrate. If the substrate is contaminated with toxic heavy metals there is a risk of their accumulation in the fruiting bodies of mushrooms.

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ANTIOXIDANT ACTIVITY OF HONEY MUSHROOMS (*ARMILLARIA MELLEA*)

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ABSTRACT

Mushrooms *Basidiomycota* have long been appreciated for their taste, flavour, desirable aroma, texture, nutraceutical and medicinal attributes. Honey mushrooms (*Armillaria mellea*) are edible mushroom generally used in traditional oriental medicine. The aim of this study was to examine extracts from the fruiting bodies of these mushrooms. The content of the components with antioxidant properties, such as total phenols, total flavonoids, β-carotene, lycopene and β-glucans were determined by spectrophotometric methods. The results obtained showed that the water extracts contained higher levels of total phenols and total flavonoids (367.1 and 548.5 mg.dm^{-3} gallic acid equivalent — GAE, respectively) in comparison with methanol extracts (108.2 and 113.4 mg.dm^{-3} GAE, respectively). Very low contents of β-carotene and lycopene were determined in the methanol extract (0.756 mg.g^{-1} dry weight and 0.05 mg.g^{-1} dry weight, respectively). Methanol extracts from the fruiting bodies of Honey mushrooms (*Armillaria mellea*) inhibited the uptake activity of 2,2-diphenylpicrylhydrazyl (DPPH) free radicals by 45 %. The IC50 (mg of compound, that inhibit 50 % of DPPH radicals) of methanol extract was below 10 mg.cm^{-3} (6.448 mg.cm^{-3}), suggesting a high antioxidant potential of fruiting bodies of the Honey mushrooms *Armillaria mellea*.

Key words: antioxidant property; *Armillaria mellea*; Honey mushrooms; polyphenols; total flavonoids

INTRODUCTION

Honey mushrooms (*Armillaria mellea*) are an autumn mushroom growing in our latitudes. They grow in tufts on the living and dead trees, causing significant damage in forestry. They are an excellent edible mushroom which can be culinary processed in different ways. They can be dried or pickled. Some people are allergic to Honey mushrooms (*Armillaria mellea*) and for some consumers *Armillaria mellea* are difficult to digest or slightly toxic. They are collected as young fruiting bodies. The stems are often ligneous, therefore, they are not consumed. These mushrooms

contain minerals, healthy fibre, vitamins and are low in fat. They have been traditionally used in alternative medicine in many countries, mainly in Asia, for their antimicrobial and anti-carcinogenic effects [8] and the content of health-promoting substances such as: polysaccharides, sterols, sphingolipids, fatty acids, sesquiterpenoids, indole compounds, peptides, and for their enzymes involved in the immunostimulatory and immunomodulatory responses [9, 13]. They participate in the protection of the brain [20] and bone marrow cells [4]. Honey mushrooms (*Armillaria mellea*) contain large amounts of biologically active substances. From arylesters of sesquiterpenes, there are included: melleolid, armillarin, armillaridin, arnamial, armillaric acid (exhibit antimicrobial activity against gram-positive bacteria), as well as judeol, melleolid, 4-o-methylmelleolid and meleollids B–D, K, L and M [12], [16]. Aerobic organisms need oxygen for their life cycle. In cells, the oxidation-reduction reactions result in the formation of: reactive oxygen species, free oxygen radicals, such as superoxide $O_2^{\bullet -}$, hydroxyl radical HO$^\bullet$, peroxide radical ROO$^\bullet$, alkoxy radical RO$^\bullet$, hydroperoxy radical $HO_2^\bullet$, hydrogen peroxide H_2O_2, nitric oxide NO and hypochlorous acid (HOCl). Reactive oxygen species are generally considered to be by-products of damaged cells. It is now known that these oxygen species are the essential mediators of cellular signalling and regulation [14]. A disturbance of the balance between the need for oxygen and an excess of free radicals in cells leads to oxidative stress and violation of cell membranes, which may cause damage to the body. Uncontrolled production of reactive oxygen species is the trigger for many diseases, such as: cancer, atherosclerosis, liver damage, degenerative processes associated with lipid peroxidation of cell walls and inhibition of protein synthesis, and many other diseases [5, 6, 18]. Almost all organisms are protected against reactive oxygen species by enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase. To reduce the oxidative damage to an organism caused by oxygen free radicals, synthetic antioxidants are now used such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tercbutylhydroquinone (TBHQ) [2, 18]. Currently, there is a search for the source of natural antioxidants which are less toxic than the synthetic antioxidants. One of the sources of antioxidants is the edible fungi *Basidiomycota*.

The aim of this study was to determine, in the fruiting bodies of Honey mushrooms (*Armillaria mellea*), the content of some substances which are supposed to have antioxidant properties.

MATERIALS AND METHODS

For our analysis were used 1 kg of freshly harvested Honey mushroom (*Armillaria mellea*) fruiting bodies with a moisture of 87.24 %, collected in the autumn of 2014 in an area of Bankov near Košice, in the Slovak Republic. The mushrooms were dried at 60 °C to a constant weight and then homogenized into a fine powder.

The analysis was carried out using different extracts. Water and methanol extracts were prepared by the extraction of 100 mg samples in 2 cm^3 of water or methanol for 24 hours with occasional vigorous stirring at 8 °C in a refrigerator. After returning to room temperature, the extraction mixtures were filtered through filter paper (Whatman No. 4) and the filtrates were used for the determinations of the total phenolic compounds and total flavonoids and the uptake activity of metanolic extracts of fungi. For the determination of lycopene and β-carotene, we prepared the extract from 100 mg of dried powdered Honey mushroom fruiting bodies using a solvent mixture of acetone and n-hexane at a ratio of 4 : 6 (v/v) [19].

The total phenolic compounds were determined by a micromethod, using Folin-Ciocalteu reagent (Sigma, USA) according to the method described by Waterhouse [21]. The determination of the total flavonoids was carried out according to the method published by Konczak [10]. The determination of the uptake activity of methanolic extracts of the fungi was carried out based on the methodology described by Tsai et al. [17]. Beta-glucans were isolated according to the patent No. 285 062 of 2006 [19]. The content of Beta-glucans (lycopene and β-carotene) was determined by a spectrophotometric method of Nagata and Yamashita [7] and Dasgupta [1].

All chemicals and water were of an analytical grade and p. a. purity. For the determination of the total phenolic compounds, the calibration of signals was performed using gallic acid (Fisher Scientific, UK). Ascorbic acid (LACH NER Ltd., Czech Republic) was used for the determination of the inhibitory activity of methanolic extracts of mushroom against 2,2-diphenylpicrylhydrazyl (DPPH) radicals (Sigma, USA). Spectrophotometric measurements were performed by a UV VIS spectrophotometer (Biochrom Li-

Table 1. Mean content of the total phenolic compounds, total flavonoids, β-carotene and lycopene in the extracts of Armillaria mellea

Armillaria mellea	mg.dm^{-3} GAE	SD	mg.g^{-1} GAE DW	µg.mg^{-1} GAE DME
Total phenolic compounds				
Methanol extract	108.2	0.005	2.2	6.1
Water extract	367.1	0.007	7.3	ND
Total flavonoids				
Methanol extract	113.4	0.007	2.3	6.4
Water extract	548.5	0.012	11.0	ND
	mg.100 cm^{-3}		**mg.g^{-1} DW**	
B-glucans acetone + n-hexane 4:6 (v/v)				
β-carotene	0.0076	0.0001	0.756	
lycopene	0.0005	0.0001	0.05	

SD — standard deviation; ND — not detected;
GAE — gallic acid equivalent; DW — dry weight; DME – dry methanol extract

Table 2. Uptake activity of Armillaria mellea methanol extract against DPPH free radicals

Measured quantities	% of inhibition	mg.dm^{-3} AAE	mg.g^{-1} AAE DW	µg.mg^{-1} AAE DME	IC50 mg.cm^{-3}
Methanol extract	45	53.21	1.06	3.02	6.448

AAE — ascorbic acid equivalent; DW — dry weight; DME — dry methanol extract
IC50 = mg of compound, that inhibit 50 % of DPPH radicals

bra S12, England). The wavelengths used for measurements are stated in the cited methods.

Results are reported as means of three measurements with the corresponding SD.

RESULTS AND DISCUSSION

It was found that 1 ml of methanol extract contained 17.6 mg of methanol extractable compounds, which is equivalent to 352 mg of methanol extractable compounds in 1 g of dried fungi. The mean contents ± SD of the total phenols (TP), total flavonoids (TF), β-carotene and lycopene are listed in Table 1. The antioxidant activity of methanol extracts of Honey mushrooms (*Armillaria mellea*) against DPPH free radicals is presented in Table 2.

Honey mushrooms (*Armillaria mellea*) contain phenolic compounds and flavonoids. From Table 1 it is evident, that the water is a better extracting agent for these compounds. In comparison with methanol, 3.4-times higher amounts of total phenolic compounds were extracted by water. Water extracted 4.8-times higher amounts of total

flavonoids than methanol. Similar results were obtained by Lung and Chang [5], who extracted almost identical compounds from Honey mushroom (*Armillaria mellea*) tissue cultures by hot water and methanol. The yield of extraction by hot water was considerably higher than that by methanol. The antioxidant activity of extracts correlated with the amount of the respective antioxidants in the extracts [3, 6]. Phenolic acids, lignans and flavonoids with -OH and -COOH functional groups are better extracted by polar solvents [22]. When comparing the content of the total phenolic compounds and flavonoids in methanol extracts converted to gallic acid equivalent (GAE), we obtained almost identical results for 1 g of dry mushroom (2.2 and 2.3 GAE.g-1 DW) and for 1 mg of dry methanol extract (6.1 and 6.4 μg.mg-1GAE DME). These results indicate that the contents of total flavonoids and phenolic compounds extracted by methanol were quite similar.

The water extracts showed greater differences, so we can conclude that the other investigated substances are extracted only by water and not by methanol. The samples of Honey mushrooms (*Armillaria mellea*) showed a low content of β-carotene, which also contributed to the antioxidant activity of fungi. The lycopene exhibited an even lower content (50 mg.g^{-1} DM). From 100 g samples, we isolated only 1.13 g of β-glucans, which was a low yield compared to 7.6 g per 100 g of edible portion as stated by Muszyńska [13]. It may be caused by differences in laboratory isolation compared to the conditions specified in Patent No. 285 062.2006 [19]. β-glucans and chitins affect the immune system, reduce blood pressure and blood glucose, and have an antibacterial, antiviral and anti-inflammatory effect [15]. An important indicator of antioxidant activity was an uptake activity of DPPH free radicals. In our experiments the methanol extract of Honey mushrooms (*Armillaria mellea*) showed 45 % inhibition of DPPH free radicals, equivalent to 53 mg of ascorbic acid per 1 dm^{-3} solution and 1.06 mg of ascorbic acid per 1 g of dry Honey mushrooms (*Armillaria mellea*) and it is equivalent to 3.02 μg of ascorbic acid per 1 mg of methanol extractable substances. Lung and Chang [5] detected 83.2 % inhibition of DPPH radicals in a methanol extract of Honey mushroom (*Armillaria mellea*) tissue culture using a concentration of 10 mg of methanol extract per 1 ml of solution. The methanol extracts of different species of fungi have different inhibitory activity against DPPH radicals as declared by Mau [11], who determined for methanol extracts of mycelia, 10 mg.ml^{-1} an inhibitory activity equal to 78.8 % for Parasol mushrooms (*Termitomyces albuminosus*), 79.4 % for Maitake mushrooms (*Grifola frondasa*) and 94.1 % for Morel mushrooms (*Morchella esculenta*).

An important indicator of the antioxidant activity was the IC50 value with the unit mg.cm^{-3}, which determines how many mg of extractable compounds in 1 ml solution inhibit 50 % of the DPPH radicals. It was stated that extracts possessed good antioxidant properties when their IC50 value was below 10 mg.cm^{-3} [11]. The IC50 equal to 6.448 mg.cm^{-3}, which was established in our study for the methanol extract of Honey mushrooms (*Armillaria mellea*) was in compliance with this requirement which indicates that Honey mushrooms (*Armillaria mellea*) exhibits good antioxidant properties.

CONCLUSIONS

The present study characterizes the fruiting bodies of Honey mushrooms (*Armillaria mellea*) collected in the Slovak Republic in terms of the content of total phenols, total flavonoids, β-carotene, lycopene and β-glucans; the compounds which are believed to have antioxidant properties. The results obtained by analysing methanol and water extracts confirmed the presence of these substances in the fruiting bodies of Honey mushrooms (*Armillaria mellea*). The methanol extracts showed inhibitory activity against DPPH free radicals. The IC50 of methanol extract was below 10 mg.cm^{-3}, confirming a high antioxidant potential of Honey mushroom (*Armillaria mellea*) fruiting bodies. This knowledge makes these mushrooms a functional food with the possibility of using their antioxidant potential in the pharmaceutical industry.

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THE INFLUENCE OF PROBIOTIC LACTOBACILLI AND FLAXSEED ON THE HEALTH OF WEANED PIGLETS AND METABOLISM OF POLYUNSATURATED FATTY ACIDS (PUFAs)

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ABSTRACT

This study investigated for 14 days post-weaning, the influence of dietary supplementation of synbiotics in the form of probiotic cheeses containing cultures of *L. plantarum* and *L. fermentum* and crushed flaxseed (source of ω-3 polyunsaturated fatty acids — PUFAs and fibre) on 36 commercial piglets originating from an infected herd (*Coronavirus* and *E. coli*) during the critical period of weaning. We focused on the health and metabolism of PUFAs in this critical period of a piglet's life. The dietary supplementation positively affected: the overall health state of weaners, reduced diarrhoea by 29 % by 14 days post-weaning and significantly increased the counts of lactic acid bacteria, bifidobacteria and the production of volatile fatty acids. The PUFA concentrations in the *m. biceps femoris* of the piglets were analysed by gas chromatography. High levels of ω-3 alpha-linolenic acid (ALA) in flaxseed increased significantly the level of ALA, eicosapentaenic acid (EPA) and docosahexaenic acid (DHA) in the pig muscles on days 7 and 14 post-weaning. The levels of ω-6 linolenic acid (LA) were less affected by the diet, but were increased on day 14 post-weaning, while the conversion products of LA, and arachidonic acid (AA), were decreased on days 7 and 14. The increased level of dietary ALA favoured the activity of Δ-6-desaturase for the conversion of ALA to EPA and DHA, at the expense of AA synthesis from LA. The ability of synbiotics to incorporate high levels of DHA in the pig muscles appear prospective for improving the nutritional properties of pork and reducing the occurrence of civilization diseases in consumers of this product of animal origin.

Key words: flaxseed; health; polyunsaturated fatty acids (PUFAs); probiotics; weaned piglet

INTRODUCTION

Weaning of piglets is associated with abrupt dietary changes resulting in morphological and functional changes in their intestinal ecosystem. This is one of the reasons why weaners are particularly susceptible for developing

digestive diseases [33, 37]. Biological barriers in the digestive system provide basic primary protection to organisms against negative influences and here probiotics offer a prospective alternative to antibiotics. The effect of probiotics can be supported by prebiotics (potentiated probiotics, also referred to as synbiotics) [4]. These natural bioregulators participate in: the maintenance of the balance of intestinal microflora, prevent colonisation of pathogenic microorganisms [5], optimise digestive processes [9] and, at the same time, stimulate the immune system [19].

Flaxseed can be used as a prebiotic substrate of natural origin. Flaxseed hulls contain soluble viscous polysaccharides which serve as a specific substrate for probiotic bacteria [35]. Flaxseed is also a rich source of polyunsaturated fatty acids (PUFAs), the structural components of cell membranes. Changes in the composition of fatty acids (FAs) in cellular membranes and intestinal mucosa may affect the mechanisms and adherence sites of intestinal bacteria and, subsequently, cause changes in populations of the bacteria in the intestine [33]. Through such mechanisms, the dietary PUFAs may positively affect the health of weaners and support their rapid adjustment to the character of post-weaning diet [21]. The high content of essential ω-3 alpha-linolenic acid (ALA) in flaxseed may positively affect the serum levels of lipoproteins and triacylglycerols [31] and affect the animal products nutritionally by additional ω-3 PUFAs produced at biosynthesis in animal tissues [18].

Food research is a field very much oriented on the search for terrestrial sources of ω-3 PUFAs which could supplement human diet deficient in this type of fatty acids. Animal products could be used as a suitable source, particularly of long-chain fatty acids synthesised in the body of mammals, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). High proportion (50–61 %) of essential ω-3 ALA in flaxseed oil can therefore be used very effectively to increase the content of ω-3 PUFAs in animal products, particularly those of monogastric animals in which the FAs taken up in feed are absorbed in the small intestine in an unchanged form and are subsequently incorporated into tissues. Contrary to ruminants, in monogastric animals FAs are not subjected to biohydrogenation before they enter the small intestine. In the process of biohydrogenation in ruminants, which takes place under the action of micro-organisms in the rumen, FAs present in the feed are converted to unsaturated and saturated ones. For this reason, only smaller proportion of dietary PUFAs are incorporated in phospholipids and triacylglycerols of body tissues [18, 40]. In pigs, there is a close correlation between the amount of FAs in the diet and their tissues and the FA profile may be then easier affected by the diet [40]. Under our conditions, flaxseed appears to be a very suitable option for increasing the content of ω-3 PUFAs in animal tissues and subsequently in animal products, particularly due to the high content of essential ALA in flaxseed, the precursor of EPA and DHA synthesis in animal bodies.

With regard to the ever increasing occurrence of "civilization diseases", development of resistance to antibiotics and increasing interest of the public in using ecological methods in food industry, agriculture and medicine in the field of prevention and treatment of diseases, researchers should focus their attention on suitable biologically active additives of natural origin that are beneficial to health and do not present risk to the food chain.

For the above reasons, the aim of our study was to investigate the influence of feed supplementation with synbiotics, probiotic cheeses containing cultures of *L. plantarum* and *L. fermentum* and crushed flaxseed (source of ω-3 PUFAs and fibre), to commercial piglets originating from infected herd during the critical post-weaning period with a focus on their health and metabolism of PUFAs.

MATERIALS AND METHODS

Animals and diets

This study was carried out on thirty-six 28-day old piglets of Slovak white × Landrace cross-breed, originating from a herd (KOAN s.r.o., Krásnovce, Slovak Republic) where the *Coronavirus* and enterotoxigenic *E. coli* (ETEC) infections were confirmed. The piglets were transported to the experimental housing facility at the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine and Pharmacy (UVMP) in Košice, the Slovak Republic, where the dietary supplementation experiments were performed. The experimental design is illustrated in Fig. 1. The experiments were approved by the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2519/10–221). The animals were handled in accordance with the guidelines established by the relevant commission. They were housed at an ambient temperature of 20—22 °C in stainless steel cages where ¼ of the floor was slotted and ¾ were covered with an insulating rubber layer.

The animals were divided into two groups: control (C; n = 18, control cheese) and experimental group (LFA; n = 18, probiotic cheeses with addition of Lactobacilli + crushed flax-seed as a source of PUFAs). Throughout the study, the animals were fed commercial mixed feed for early weaned piglets OŠ-02 (Spišské Vlachy, SR) and had ad libitum access to water. The mixed feed was supplemented (LFA group) with crushed flax-seed (cultivar Flanders, Agritec, Czech Republic) at a concentration of 10 % (continuously added to rations). In the period starting 10 days before weaning and lasting up to 14 days post-weaning, the piglets in the LFA group were supplied probiotic cheeses (4 g per head per day of each cheese), and crushed flax-seed. Piglets in the C group were supplied control cheese (8 g/per head per day). Both types of cheeses were sprinkled on the surface of the feed.

Cheddar cheese chemical composition: proteins 23.8 %, sugars 2.8 %, lipids 30.1 %, and metabolisable energy 1.62 MJ.kg^{-1}. This cheese was used as a vehicle for probiotic strains. Each of two cheeses contained one strain at 1×10^9 CFU.g^{-1} of cheese (referred to as probiotic cheeses). The probiotic bacteria were added to the cheese milk together with 2 % starter culture (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*) during the typical Cheddar cheese production.

The cheese used as the control was also Cheddar cheese, but without probiotic strains (referred to as control cheese).

Probiotic bacteria

The Lactobacillus probiotic strains were isolated in the laboratory of the Institute of Microbiology and Gnotobiology, UVMP in Košice, the Slovak Republic. The *Lactobacillus plantarum* Biocenol™ LP96 (CCM 7512) strain was isolated from the gut of healthy suckling piglets. This strain was characterized by: strong adherence to the epithelial cells of porcine intestine; inhibitory activity against *Escherichia coli* O8:K88ab:H9 under *in vitro* conditions; and production of hydrogen peroxide [23]. The *Lactobacillus fermentum* Biocenol™ LF99 (CCM 7514) was isolated from the gastrointestinal tract of adult chickens. The strain was characterized by: growth in the presence of bile acids and gastric juice; sensitivity to antibiotics; inhibitory activity against *Salmonella enterica* serovar *Enteritidis* and *Salmonella enterica* serovar *Düsseldorf* [24].

Clinical observations and muscle sampling

During the experimental period, all piglets underwent clinical observations. The health data were recorded twice daily (at 08.00 a. m. and 03.00 p. m.), namely: body temperature, consistency of faeces and moisture of faeces. Samples of faeces were assessed visually using a scale from 1 to 5 (1 — solid faeces; 2 — paste; 3 — sparse; 4 — hydrous; 5 — faeces with blood or mucus admixture). The moisture of faeces was determined by drying a sample of faeces at 80 °C to a constant weight.

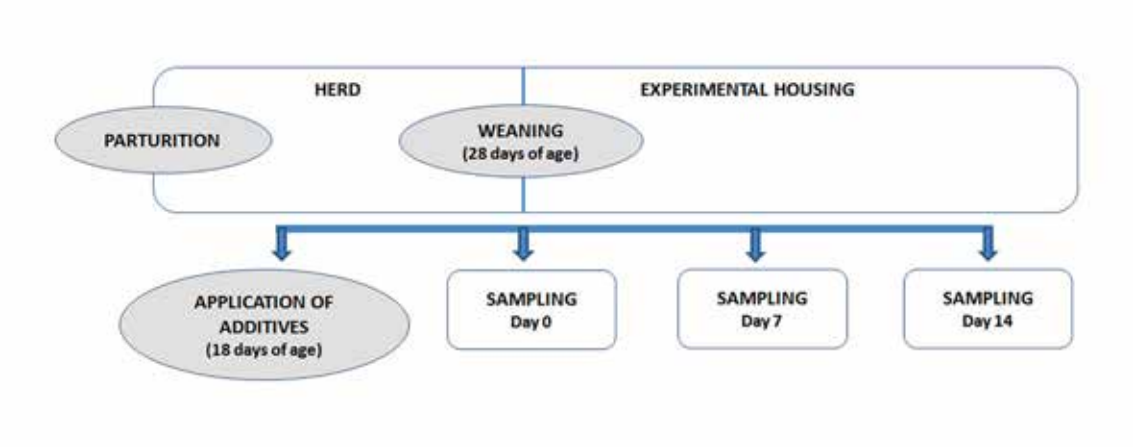

Fig. 1. Schematic representation of experimental design

Note: Day 0 is the day of piglet weaning, days 7 and 14 are the days of supplementation of the rations after weaning. Supplementation to experimental animals started 10 days before weaning (at 18 days of the piglets' age) and lasted up to 14 days after weaning. The muscle samples were collected from the m. biceps femoris of piglets after feeding with or without synbiotic additives on these days.

Piglets from both groups were humanely euthanized by intracardiac administration of T61 a. u. v. (Intervet International B.V. Boxmeer, the Netherlands) at a dose of 1 ml. kg^{-1}.$head^{-1}$ on days 0 (day of weaning; n = 6), 7 (n = 6) and 14 (n = 6) post-weaning and skeletal muscle samples for the analysis of PUFAs were taken from the musculus biceps femoris (sample = 2 g; see Fig. 1). All samples were stored at –70 °C until analysis.

Gas chromatography

The extraction of fatty acids was performed by modification of the method of Folch [8] using dichloromethane instead of chloroform [6] according to Tvrzická et al. [34]. A Clarity Chromatography Station data integration system was used to integrate the peak areas. The FA concentration (c) in pig serum was calculated as follows:

$$c\,(\mathrm{mg.g^{-1}}) = \frac{\text{peak area of a given FA} \times c \text{ of internal standard } (\mathrm{mg.ml^{-1}})}{\text{peak area of internal standard or sample (ml)}}$$

Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). Significant differences between the groups were determined using *t*-test, and the differences between days within groups by using Repeated Measures Anova (GraphPad Prism 5.0 for Windows, GraphPad Software, San Diego, CA, USA).

RESULTS

Health conditions

Fortification of feed with probiotic cheeses and flaxseed positively affected overall health state of weaned pigs from the problematic herd (body temperature, consistency and water content of excrements) and reduced the prevalence of post-weaning diarrhoea by 29.2 %. The faecal score of these weaners was significantly lower and their body weight higher already after 14 days of feed supplementation.

Intestinal metabolism

The synergistic effect of additives on caecal microbial activity of weaners was reflected in: a significant increase in lactic acid bacteria (LAB) and bifidobacteria, increased production of volatile fatty acids (VFA) (acetic, propionic and butyric acids) and concurrently decreased counts of coliform bacteria and haemolytic *E. coli*. Supplementation of additives to weaners positively affected the population of LAB in the jejunum and supported the production of lactic acid (unpublished data).

PUFAs metabolism

The levels of ω-3 PUFAs in the muscle of piglets are summarized in the Fig. 2. High levels of ω-3 PUFA ALA in the diet significantly affected the level of this acid in muscles of experimental animals. In the group of animals fed rations fortified with lactobacilli and flaxseed an increase in ALA on days 7 and 14 post-weaning (both $P < 0.001$) was recorded in comparison with control pigs fed unfortified feed. Synthesis of EPA and DHA in the body of animals copied the levels of essential ALA. In the fortified group, we recorded a significant increase in the level of EPA on days 7 and 14 post-weaning ($P < 0.001$). In comparison with the control group, the animals fed supplemented rations exhibited significantly increased biosynthesis of EPA on days 7 ($P < 0.01$) and 14 ($P < 0.001$) post-weaning. Conversion of EPA to DHA in the fortified group showed a similar increasing tendency and DHA levels were significantly higher after 14 post-weaning days and also in comparison with the control animals ($P < 0.001$). Fortification of feed resulted in an increased synthesis of EPA ($P < 0.001$) and subsequently increased conversion of EPA to DHA ($P < 0.001$) compared to day 0 and the control group.

The levels of ω-6 linolenic acid (LA) were less affected by the diet (see Table). In the fortified group they were significantly increased on day 14 compared to day 0 ($P < 0.01$) and also in comparison with the control ($P < 0.001$). At the same time, supplementation of the diet suppressed the conversion of LA to arachidonic acid (AA) on days 7 and 14 ($P < 0.05$) and in comparison with the control ($P < 0.001$). The increased intake of ω-3 ALA in the experimental diet in the LFA group enhanced the activity of Δ-6-desaturase in favour of ω-3 EPA and DHA synthesis on days 7 and 14 in comparison to day 0 ($P < 0.001$) and on days 7 ($P < 0.01$) and 14 ($P < 0.001$) in comparison to the control.

The levels of ω-6 fatty acids in the m. biceps femoris of piglets after feeding supplemented (LFA) and non-supplemented (C) diet for 14 days after weaning are presented in Table1.

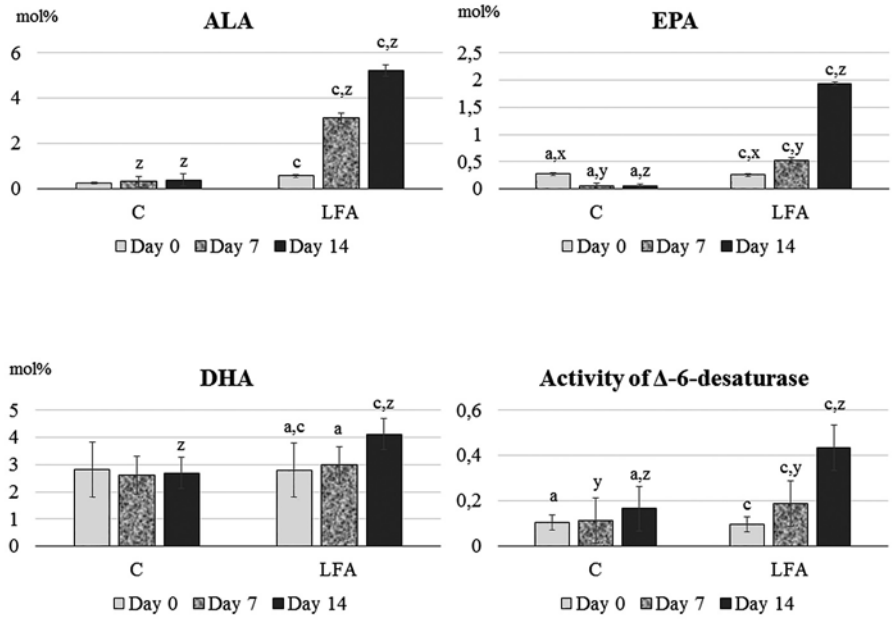

Fig. 2. Effects of feeding PUFAs and flaxseed to weaned piglets (LFA) at 14 days post-weaning on the metabolism of ω-3 PUFAs and activity of Δ-6-desaturase in the m. biceps femoris of piglets in comparison with the control group (C)

ALA — alpha-linolenic acid; EPA — eicosapentaenic acid; DHA — docosahexaenoic acid; Activity of Δ-6-desaturase is the ratio of eicosapentaenic to arachidonic acid. Data are the means (mol %) ± SEM (Repeated measures ANOVA). $^{a, b, c, x, y, z}$ — Mean values with same superscript letters differ significantly ($^{a, x}$ — $P < 0.05$; $^{b, y}$ — $P < 0.01$; $^{c, z}$ — $P < 0.001$). Superscript letters $^{a, b, c}$ — show differences on days 7 and 14 post-weaning compared to day 0; $^{x, y, z}$ — show differences between the groups.

Table 1. The concentration of ω-6 FAs in the *m. biceps femoris* of piglets after feeding supplemented (LFA) and non-supplemented (C) rations for 14 days after weaning

Fatty acid [mol %]	C			LFA		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
LA	19.27 ± 1.02^{x}	19.98 ±1.06	21.3 ± 2.06^{z}	20.99 ± 3.1^{b,x}	23.54 ± 2.44^{a}	27.86 ± 1.99^{a,b,z}
GLA	0.33 ± 0.01^{y}	0.39 ± 0.09	0.27 ± 0.01^{y}	0.41 ± 0.03^{y}	0.39 ± 0.05	0.21 ± 0.02^{y}
DGLA	0.28 ± 0.01^{b,z}	0.21 ± 0.03^{b,z}	0.22 ± 0.04	0.39 ± 0.02^{z}	0.34 ± 0.03^{z}	0.2 ± 0.01
AA	11.01 ± 1.1^{a,z}	10.83 ± 2.21^{a,z}	9.28 ± 0.98^{a,z}	4.8 ± 1.1^{a,z}	5.1 ± 0.95^{a,z}	4.61 ± 0.89^{a,z}

LA — linoleic acid; GLA — gamma-linolenic acid; DGLA — dihommo-gamma-linolenic acid; AA — arachidonic acid; data are means (mol %) ± SEM (Repeated measures ANOVA). $^{a, b, x, y, z}$ — Mean values in rows with same superscript letters differ significantly ($^{a, x}$ — $P < 0.05$; $^{b, y}$ — $P < 0.01$; ^{z} — $P < 0.001$). Superscript letters $^{a, b}$ — show differences on days 7 and 14 compared to day 0; $^{x, y, z}$ — show differences between the groups.

DISCUSSION

Fortification of feed with synbiotics (*L. plantarum, L. fermentum* and flaxseed) improved: the overall health of weaned piglets from a problematic herd, consistency and water content of excrements and increased their weight gains, similar to the results obtained in weaners infected with enterotoxigenic *E. coli* (ETEC) and fed diet fortified with probiotic strain of Bacillus amyloliquefaciens [14] and in gnotobiotic (germ-free) piglets supplemented with synbiotics (*L. Plantarum* and flax oil) [25]. Stimulation of the LAB population in the jejunum and ileum and increased concentration of lactic acid (final fermentation product of these bacteria) can positively affect the intestinal microenvironment of weanlings, particularly in terms of its antimicrobial effects [3]. Volatile fatty acids play an important role in the protection against intestinal infections; they prevent the development and absorption of toxic products of metabolism, participate in preservation of mucosal integrity and support growth of intestinal epithelial cells [39].

High level of essential ω-3 PUFA ALA after 17 days of supplementation significantly increased ALA levels in skeletal muscles. On the basis of increased amount of substrate, conversion of ALA to long-chain ω-3 PUFAs increased in the body and resulted in their increased concentration in muscle tissue. Our results agree with those obtained in studies conducted in pigs [2, 11, 13, 29], chickens [41] and sheep [36]. Similar to our study, increased proportions of ω-3 PUFAs were observed in skeletal muscles and adipose tissue, but also in the tissues of heart, liver and brain after increased intake of ALA by feed [13, 26, 32]. However, they recorded an increased levels of ALA, eventually also EPA and docosapentaenoic acid (DPA), but the content of DHA (the acid important for the organism) was comparable with the control. A significant increase in DHA after the application of fish oil and fish meal was observed by other authors [13, 17]. This increase in DHA was caused by a direct incorporation of DHA present in fish additives into tissues and not by the synthesis of DHA in the body which negatively affected organoleptic properties of products of animal origin. For this reason, Haak et al. [13] presented an opinion that the synthesis of DHA in the body is most likely not affected by the intake of its precursor in the diet, but is regulated strictly metabolically where desaturation and elongation of ω-3 PUFA is blocked at the level of DPA synthesis. On the other hand, in agreement with our results, Perini et al. [26] applied flax diet to mice and observed the highest level of ALA in the liver and consequently a significantly increased conversion of ω-3 ALA to EPA and DHA with the highest levels on day 56 of application, accompanied by a significant decrease in the conversion of ω-6 LA to AA and almost a 3-fold decrease in the ω-6:ω-3 PUFA ratio. Similarly Kašteľ et al. [15, 16] conducted a study with supplementation of flaxseed and lactobacilli to gnotobiotic pigs and piglets, the optimum model for digestive physiology and lipid metabolism, and observed significantly increased levels of ω-3 PUFAs EPA and DHA and decreased level of ω-6 AA in the blood serum of these animals. Omega-6 AA is the major precursor of eicosanoids at cleavage of phospholipids by means of phospholipase A2 which serves as a substrate for enzymes of cyclooxygenase (COX) and lipoxygenase (LOX) pathways resulting in the production of pro-inflammatory cytokines. On the contrary, ω-3 PUFAs produce eicosanoids with anti-inflammatory properties [27]. The mechanisms by which ω-3 PUFAs induce their immunosuppressive effect is the inhibition of AA metabolism, therefore the production of pro-inflammatory eicosanoids, inflammatory cytokines, and concentration of lipids and lipoproteins in the blood [42], and significant decrease in the activity of genes is responsible for inflammatory reaction [38].

After continuous fortification of feed with synbiotics in our study, we observed decreased conversions of LA to GLA (gama-linolenic acid) and AA, despite significantly increased level of LA. The results of our study confirmed that after increased absorption of the ω-3 PUFA-fortified feed received by animals, they showed: predominant conversion of ω-3 PUFA ALA to EPA and DHA, increased activity of Δ-6-desaturase in favour of ω-3 PUFAs synthesis and reduced synthesis of ω-6 PUFAs GLA and AA. Mammals lack the enzymes for the synthesis of highly non-saturated fatty acids such as AA and DHA from acetyl-CoA, and synthesises them from precursors, essential PUFAs [22], with the amount of substrate available to the common enzyme Δ-6-desaturase which plays the key role. An increased absorption of ALA (ω-3) is therefore the cause of the predominant biosynthesis of EPA to DHA at the expense of AA (ω-6) synthesis [12, 26]. After supplementation of a diet with flaxseed, a 2.4-fold increase in DHA was observed by Shapira et al. [28] in hens. Significantly higher concentrations of all ω-3 PUFAs, including DHA, and a subsequent decrease in ω-6:ω-3 PUFA ratio was recorded by Kouba

et al. [17] in rabbits and by Enser et al. [7] in sows and boars. Our results agree with those of these authors and confirm the claims of other authors [10, 12]. The increased absorption of essential ALA in the body stimulates more intensive conversion of ALA to EPA and DHA, which can be used in practice by the food industry already after short-term (21—28 days) application of flaxseed [17]. The results obtained in one research paper proved that nutritional shortcomings during prenatal development of the foetus result in permanent changes in the physiology of progeny, including the metabolism of lipids and increased the risk of chronic diseases in adults [30]. Recent studies indicate that the relevant mechanisms are modulated also by DHA, the main ω-3 FA with potential impact on growth and development of children [20], and by general changes in the content of ω-3 FAs in the nutrition of the mother which subsequently affects the metabolism of lipids in the foetus [1]. Supplementation of animal feed with flaxseed or flax oil is a suitable way of satisfying the requirements of consumers of animal products by nutritionally beneficial components. However, the absence of the effect of feed fortified with flaxseed on the level of DHA in pig tissues has not yet been fully explained. It could be caused by a low ability of the pig to synthesise DHA from EPA or by rapid utilization of DHA in the tissues of these animals. These assumptions should be subjected to further investigation. The ability to increase the level of DHA in pork would bring considerable nutritional benefits. The relevant research should focus on defining the optimum amount and period of ω-3 PUFAs supplementation, in order to decrease the risk of increased lipid oxidation and the related adverse effects on quality of animal products [18].

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THE USE OF ULTRASONOGRAPHY IN DIAGNOSTIC IMAGING OF REPTILES

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ABSTRACT

The aim of this study was to obtain new knowledge and show the possibility of the use of ultrasonographic (USG) examinations in reptilian medicine. As reptiles are patients brought to veterinary clinic in smaller numbers, we focused on the indications and limits of this type of examination in individual groups of reptiles. In the period of 2014—2015 we examined by ultrasound, 28 reptiles with the aim to diagnose gravidity, reproductive problems and to determine their gender. At the same time we examined the internal organs and their availability for potential USG examinations. We also investigated: the issues related to fixation and positioning of the patients; selection of suitable examination probes; and the ways of their application to a suitable body area. The experimental part of our study was focused on the examination of the reproductive apparatus of reptiles. We monitored: individual phases of gravidity in selected reptilian species; evaluated development of follicles in ovaries; and the development of eggs after successful mating up to their laying. We described the pathological states of reproductive organs and the suitability of this examination for the determination of gender.

Key words: diagnostic imaging; lizard; reptile; snake; tortoise; turtle; ultrasonography

INTRODUCTION

Ultrasonography (USG) is an important diagnostic tool in any veterinary practice with many potential uses in reptilian medicine. In general, this examination is considered safe for both the patient and the examination personnel. It is a non-invasive method for the evaluation of anatomical size, position and structure of the internal organs. Neither the patient nor the assisting personnel are exposed to ionizing radiation [5].

The organs best suitable for examination by USG include: the heart, liver, gallbladder, urinary bladder, large intestine and gonads. In some animals, USG enables the examination of: the stomach, small intestine, spleen, pancreas and kidneys [6].

The examination by ultrasound allows one to evaluate: inflammatory changes, tumours, developmental irregularities, presence of foreign bodies, degeneration of tissues, and to confirm gravidity and carry out other investigations [3].

USG is a suitable method for the observation of reproductive functionality, reproductive cycle and diseases of reproductive organs. Other indications include the determination of gender, evaluation of reproductive potential or assessment of therapeutic intervention. The determination of gender on the basis of external morphology is frequently quite demanding. Ultrasonographic examination appears to be a useful method for the confirmation of gender in reptilian species [4].

With regard to the age of the patient and stage of the reproductive cycle, reptilian testicles are ovoid structures of varying size from hardly visible up to distinctive. They are especially prominent in Green iguana and Bearded dragon during the reproduction season. They have distinct granular homogenous echotexture, are isoechogenic, even somewhat more hyperechogenic than the liver [2].

The ovaries are visible depending on their size and phase of the reproductive cycle. At the beginning of this cycle the ovarian follicles are anechogenic. Their size varies from 1 mm up to several mm. Before ovulation, they grow, appear as large circular structures and their echogenicity increases. In the late pre-ovulation phase almost the entire abdominal cavity is filled up with round follicles. After ovulation, the eggs grow and the wall turns hyperechogenic. Depending on the species, the wall may calcify or not at all. Monitoring of the inside of the egg may be based on the degree of calcification. At the beginning of gravidity, we can distinguish in the internal content, the anechogenic egg white layer and hyperechogenic yolk. Sometimes a very little embryo can also be visible. In viviparous reptiles one can observe the development of the embryo, developmental phase, embryonal activity and the heartbeat [2].

The aim of this study was to obtain new knowledge and show new possibilities of the use of (USG) examination in reptilian medicine with a focus on the reproductive cycle in selected reptilian species.

MATERIALS AND METHODS

Examinations were carried out employing a USG instrument Aloka prosound. We used either linear or convex probe. The linear probe of frequency 10—15 MHz was preferred for examination of snakes and lizards, and tortoises and turtles were examined by means of a convex probe of frequency 3.5—7 MHz.

The following reptiles and organs were examined by USG:

- **Tortoises and turtles** (n = 7):
  Hermann's tortoise (*Testudo hermani*): 1 male — heart,
  Pond slider (*Trachemys scripta scripta*): 3 females — heart, liver, reproductive apparatus,
  Red-eared slider (*Trachemys scripta elegans*): 3 females — reproductive apparatus.
- **Lizards** (n = 16):
  Bearded dragon (*Pogona vitticeps*): 1 male + 4 females — heart, liver reproductive organs,
  Leopard gecko (*Eublepharis macularius*): 5 females – reproductive apparatus,
  Gila monster (*Heloderma suspectum*): 1 male + 1 female — liver, heart, gallbladder, reproductive organs,
  Veiled chameleon (*Chameleon calyptratus*): 4 females — reproductive organs.
- **Snakes** (n = 5):
  Indochinese spitting cobra (*Naja siamensis*): 1 female — reproductive apparatus,
  Ocellated carpet viper (*Echis ocellatus*): 1 female — reproductive apparatus,
  Prairie rattlesnake (*Crotalus viridis*): 1 male + 1 female — hemipenises in male; reproductive apparatus in female,
  Ornate flying snake (*Chrysopelea ornata*): 1 female — reproductive apparatus, liver.

RESULTS

Our study included observations of the course of gravidity in four Leopard gecko females (*Eublepharis macularius*). Examinations of females were carried out in regular intervals. First we observed the presence of round follicles and after fertilization, the formation and growth of eggs until laying.

The first examination on March 11th showed the presence of round follicles about 5 mm in diameter. By April 1st the follicles reached a mean size of 8.6 × 9.2 mm.

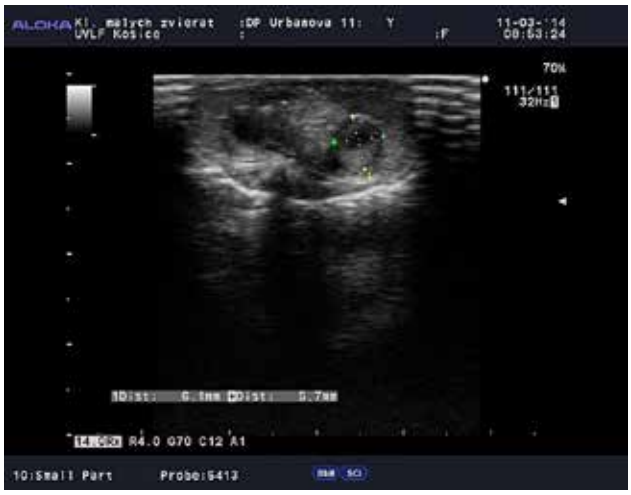

Fig. 1. First examination on March 11th

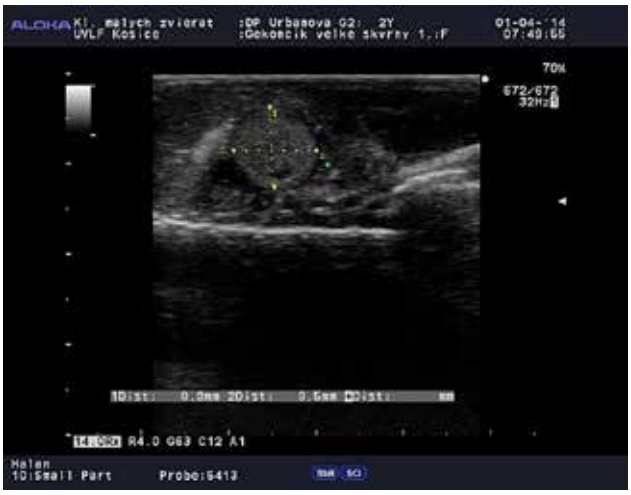

Fig. 2. Examination on April 1st

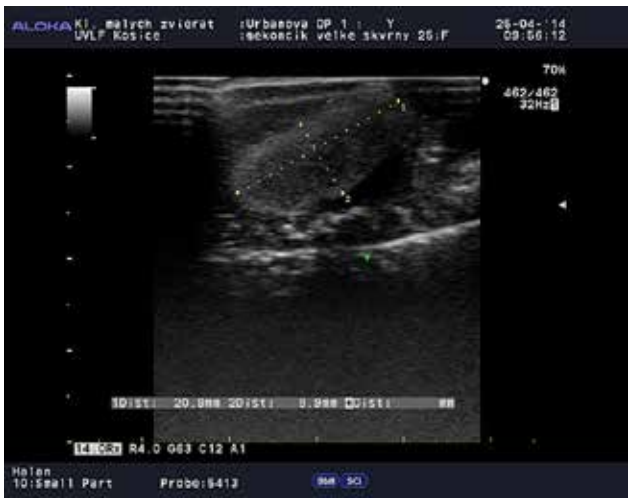

Fig. 3. Examination on April 25th

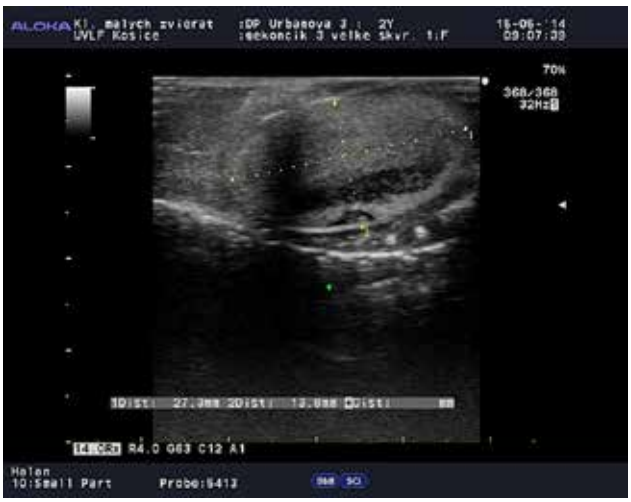

Fig. 4. Examination on May 15th

Table 1. Mean size of follicles and eggs of Leopard geckoes at individual examinations

Date	Mean size [mm]
March 11	5 x 5
April 1	8.6 x 9.2
April 25	9.3 x 18
May 15	13.7 x 28.4

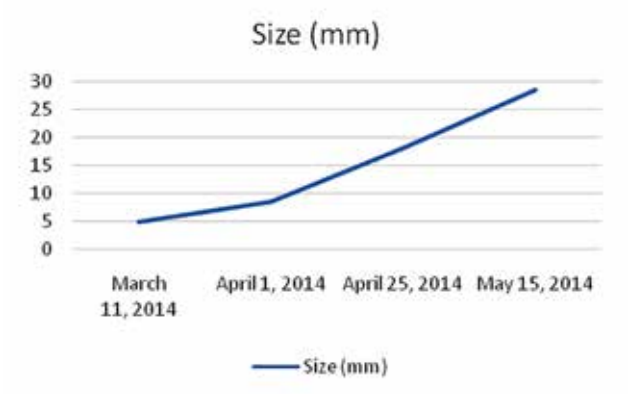

Fig. 5. Development of follicles and eggs in Leopard gecko females

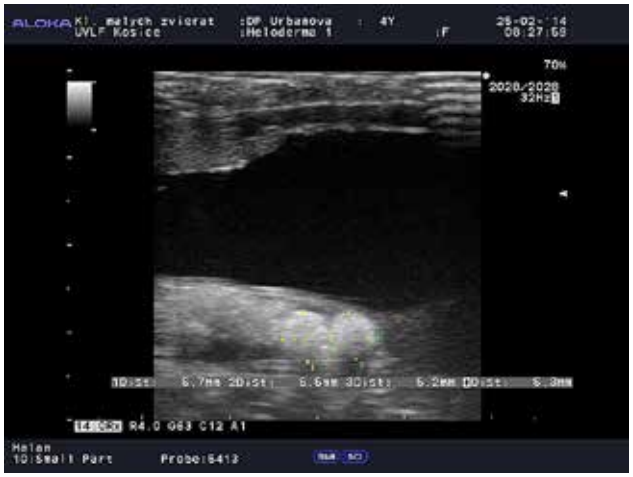

Fig. 6. Examination of Gila monster female on February 25

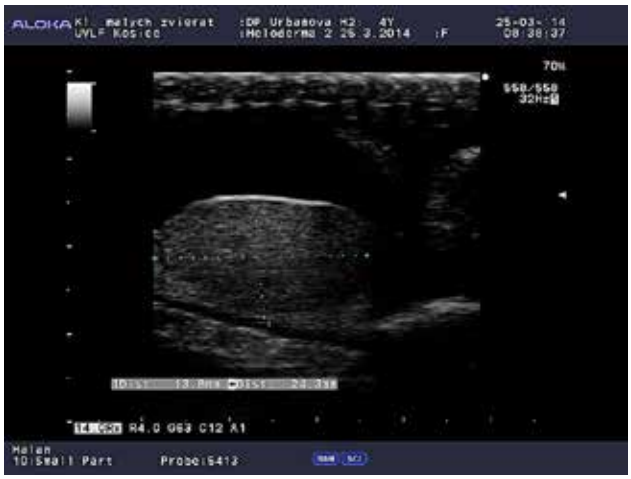

Fig. 7. Examination of Gila monster on March 25

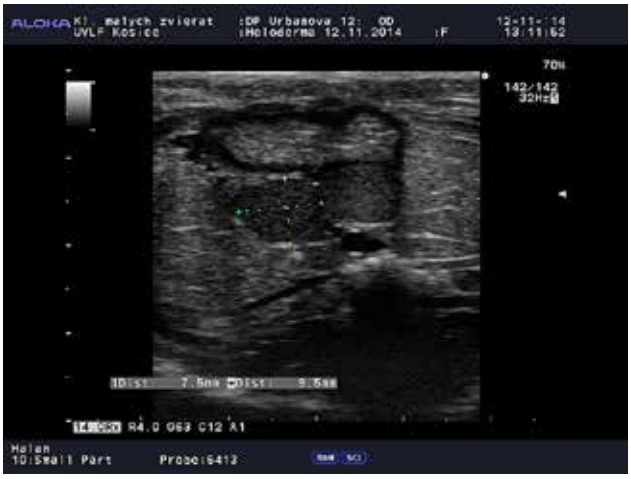

Fig. 8. Examination of Gila monster on November 12

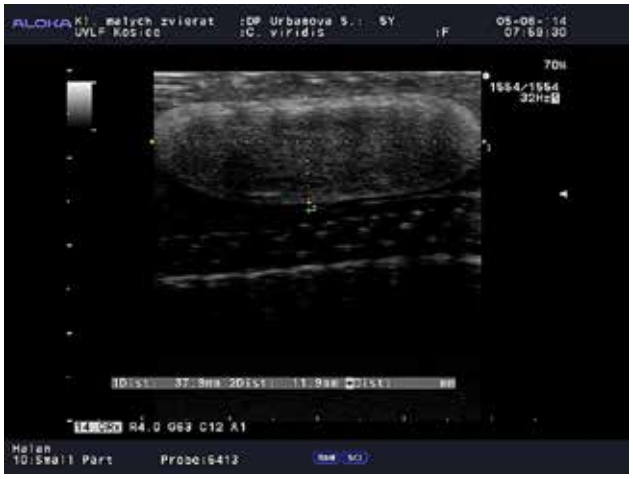

Fig. 9. Examination of Prairie rattlesnake female on August 5

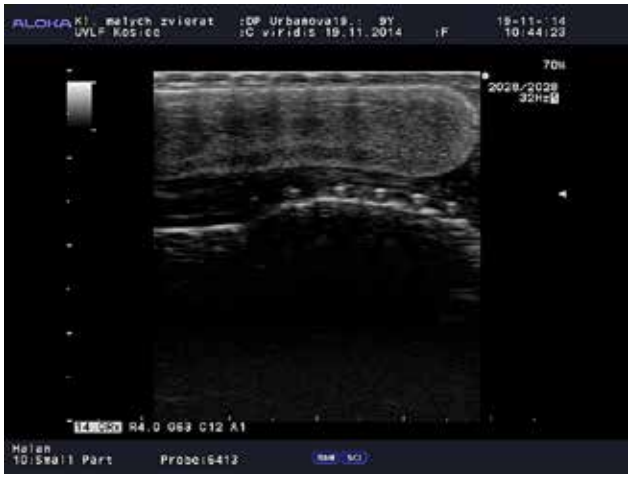

Fig. 10. Examination on August 26

Fig. 11. Examination on September 23

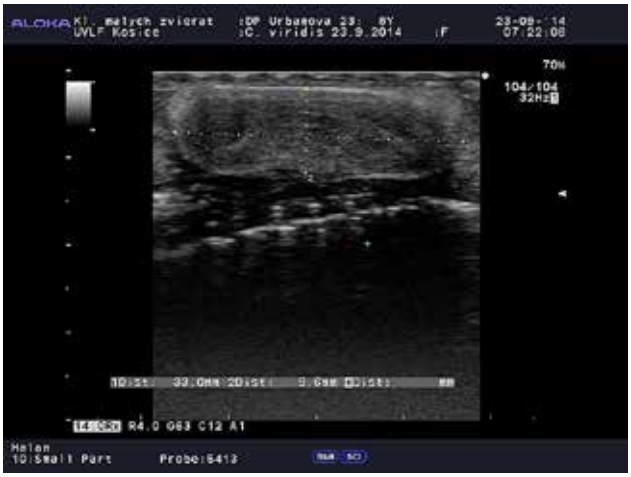

Fig. 12. Examination on November 19

The following examination on April 25th showed the presence of oval-shaped eggs covered in a hyperechogenic surface envelope which filled up most of abdominal cavity. Their mean size was 9.3 × 18 mm. By May 15th, the eggs grew to the mean size of 13.7 × 28.4 mm. The eggs of this size could be observed by a naked eye through abdominal wall. Three days later the eggs were laid.

We also observed the reproductive cycle of a young Gila monster (*Heloderma suspectum*) female at first mating which resulted in unsuccessful gravidity. The first examination of this female on February 25th showed the presence of round follicles with a diameter of 5.5 mm. One month later, oval-shaped eggs started to develop and reached the size of 13.8 × 24.3 mm. Afterword, their development evidently stopped and the eggs were absorbed. The examination on November 12 showed again the presence of round follicles 8.5 mm in diameter.

We examined one viviparous Prairie rattlesnake (*Crotalus viridis*) female for the presence and growth of eggs and the gradual development of embryos. During the first examination on August 5th, we observed eggs of homogeneous structure of size 37.9 × 11.9 mm. Three weeks later the egg size was 36.4 × 9.8mm and there were no signs of the development of the foetuses. At the following examination (September 23rd), there were still present eggs of homogenous structure of size 33 × 9.6 mm, but the developing foetuses were absent which indicated abnormal gravidity. Large homogeneous eggs were still present on November 19th, confirming unsuccessful fertilization. Several days later, the unfertile eggs were disposed of by the female.

In addition to the examination of the reproductive apparatus of the females, we examined in individual reptile species also, heart, liver, gallbladder and reproductive organs of males.

During ultrasonographic examination the heart served as a starting point which allowed us to orientate oneself to the internal structures. The heart of reptiles is three chambered, composed of two atria and one ventricle. During examination, one can visualize the entire cardiac cycle. Systole represents the time during which the ventricle contracts and ejects blood into the circulation. Diastole represents the period of time when all heart cavities are filled up gradually. The flow of blood can be observed by Doppler imaging.

The liver of reptiles appears ultrasonographically as homogenous hyperechogenic tissue. It is located largely in the cranial part of abdominal cavity. During examination we were able to visualise anechogenic vessels the presence of which can be confirmed by Doppler. In the tortoises, the cervicobracheal region is used for the examination of the liver.

The gallbladder of reptiles is localised in the abdominal cavity caudally of the liver, and in snakes, at relatively a long distance from the caudal tip of the liver. In snakes the gallbladder was not well developed. During the examination of the gallbladder, we were able to observe a thin hyperechogenic wall with anechogenic content. Of pathological states, it is possible to discern urinary stones and thickened bladder wall due to irritation or inflammatory processes.

In male reptiles one can observe the testes in the sea-

sonal reproductive cycle. One of the characteristic features of snakes and lizards are hemipenises, the paired structures found behind the cloaca. For their imaging, the probe should be applied behind the cloaca and gradually moved along to the ventral basis of the tail. They appear as heterogenous structures surrounded by muscles.

DISCUSSION

Recently, the interest in rearing terrarium animals has increased. Thus, veterinarians face more frequently issues related to reptilian reproduction. Ultrasonography appears to be a useful diagnostic tool also in these animals.

Girling and Raiti described many indications for which USG examination can be considered as useful. They include: determination of gender and the stage of the reproductive cycle, confirmation of gravidity, examination of soft tissues, intraocular structures, vascularization of tissue, and detection of pathological changes and tumours [1].

However, there are some limits which can complicate this examination. The problems may be related to very small size of some individuals, the period of shedding, manipulation, fixation and the way of application of the probe [6].

In our study we used USG imaging to examine: the heart, liver gallbladder and reproductive organs in males and females of different reptile species. One part of our study focused on the examination of the reproductive apparatus of female reptiles.

Successful imaging of ovaries is dependent on their size and the reproductive phase. In the period of sexual activity they are discernible on the basis of the presence of round follicles. At the onset of gravidity, oval-shaped eggs are formed in the oviduct and their surface gradually calcifies which is manifested by a surface hyperechogenic layer. In viviparous reptiles, one can observe the development and the activity of the foetuses [5].

Our observations of the reproductive apparatus of Red eared slider (*Trachemis scripta elegans*) indicated preovulatory follicle stasis (POFS) which was detected also in Veiled chameleon (*Chameleon calyptratus*).

The examination of the Leopard gecko (*Eublepharis macularius*) females allowed us to observe the presence of round follicles and after mating, the formation and growth of eggs up to their laying. The mean size of eggs increased from 18 × 9.3 mm up to 28.4 × 13.7 mm 3days before laying when it was possible to observe them with the naked eye through the abdominal wall.

The reproductive cycle of a young Gila monster (*Heloderma suspectum*) female at first mating observed during our study was unsuccessful, although its eggs started to develop and reached a size of 13.8 × 24.3 mm. However, they were subsequently absorbed and round follicles appeared again.

In one viviparous Prairie rattlesnake (*Crotalus viridis*) we observed unfertilised eggs which were retained for almost four months, but decreased in size and finally were disposed of by the female.

The determination of gender in species with minimum sexual dimorphism can present a problem for both veterinarians and breeders and USG examination appears to be suitable for this purpose. In males one can observe the testes or hemipenises, and in females, round follicles in ovaries or oval eggs in the oviducts. Our examination of two male reptiles, one Gila monster lizard (*H. suspectum*) and one Prairie rattlesnake (*C. viridis*) confirmed the presence of hemipenises in these reptiles. It is a paired organ visible at the tail basis behind the cloaca. By this examination, one can identify males without manual probing and thus eliminate the risk of trauma to hemipenises or cloaca in these animals [7].

CONCLUSIONS

Our study investigating the possibility of the examination of individual internal organs in selected reptile species showed that USG imaging is most suitable for the examination of the liver, heart, gallbladder and reproductive organs. By this method, it is possible to evaluate some physiological parameters, such as size, position, echogenicity, blood flow by Doppler imaging and also to detect some pathological deviations.

In selected females of lizards and snakes, it was possible to observe the phases and course of gravidity, development and expulsion of unfertilized eggs by viviparous female of Prairie rattlesnake and unsuccessful gravidity in a young Gila monster lizard. This examination is non-invasive and may replace some other potentially risky approaches used for determination of gender.

On the basis of the above, one may conclude that USG imaging is a diagnostic tool suitable also for exotic animals

such as reptiles. However, it is necessary to observe all technical requirements and consider the anatomic-morphological specificities of individual reptile species.

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BEHAVIOUR PROBLEMS OF CATS REARED INDIVIDUALLY OR IN COEXISTENCE WITH OTHER ANIMALS (CATS, DOG)

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ABSTRACT

The aim of this study was to determine whether behaviour problems in indoor cats depend on the number of cats in a household or rearing one or more cats in a household together with a dog. The study was carried out on animals which were divided for the purpose of this study into 4 groups: (1) households with one cat; (2) households with two cats; (3) households with three or more cats; (4) households with one or more cats and a dog. Altogether 91 cats were included in the study. The practical part of this investigation was based on a questionnaire. It was observed that the probability of behaviour problems was not related unambiguously to the number of cats in a household or the company of a dog. The percentage of the occurrence of changed behaviour did not differ significantly between the groups.

Key words: behaviour problems; cat; dog; individual and group rearing

INTRODUCTION

Although Queen Victoria popularized cats as domestic animals, it has been only lately that cats have begun to adjust to indoor-only conditions. The fact is, that today cats are the most frequent pets found in American households. According to the Humane Society of the USA, there are 83.3 million dogs and 95.6 million cats kept as household pets in the country [7]. The increased population of humans living in cities and towns is probably one of the main reasons for the increasing popularity of cats. Cats are better equipped for life in a smaller space, require less care and can coexist easier with humans in these hectic times.

Cat owners who try to adjust cats to their own lifestyle without considering the essence of existence of these animals and their needs, eventually find out that they have at home an unhappy cat which behaves in the way considered inappropriate by the owner. The social behaviour of cats is closely related to behaviour problems of these animals. The cat owners should try to understand how domestication has affected the behaviour of these animals. Each cat has its

own specific story, a repertoire of experiences which affect its own perception of the world. First we have to understand cats in general before we can comprehend the laws according to which the individual cats will act and behave [7].

One of the reasons for keeping cats exclusively indoors is the fact that owners try to prevent conflicts which may develop when a new cat tries to integrate into the territory in the garden of its owner which already belongs to a neighbour's cat. On the other hand, if the cat has to stay throughout its life in the relatively small space of our households, this can induce stress without the presence of another animal. However, although people have kept cats in apartments for more than 30 years, only a few systemic studies have been conducted with the aim of revealing whether cats consider such "prisons" as stressful [5].

Barry and Crowell-Davis [3] described the relationship of two castrated indoor cats in their study which focused on differences in the behaviour within genders. They observed that there were no significant differences between genders in friendly and aggressive behaviour. However, in households with two females, no mutual cleaning of cats was observed, but in the case of households with two tomcats the animals spent more time in close proximity [3].

Consequently, the life of an indoor cat is considerably affected by its owner as it was indicated by Adamelli et al. [1] who investigated the cat — man relationship with respect to their quality of life in a group of 62 cats. The quality was evaluated by means of a questionnaire which focused on the care of the animal, its behaviour, characteristic features of the owner and his/her cat, simple physical examination of cat and Lexington's test of pet attachment. This test revealed a medium quality of life of 87.2 % of the cats. Although the quality of life of these cats was good and they were in good physical condition, only 16.1 % showed no abnormal behaviour. Scientists who performed this study concluded that the quality of a cat's life is affected considerably by coexistence with other animals. All analysed aspects (care, behaviour, physical condition) were eventually affected by the owners. Other characteristics (gonadectomy, age at obtaining the animal, coexistence with other cats) which affected the animals to a lesser but still significant extent, were determined by performed testing [1].

In order to understand the behaviour of cats, particularly with the aim of finding out more about their ability to coexist with other cats, dogs or people, one should first gain an understanding of the development of their social behaviour in the process of domestication of a solitary wild cat compared to the domestic cat, the animal we know today.

The aim of this study was to determine whether behaviour problems and inappropriate behaviour of indoor cats depends on the number of cats in the household or their coexistence with a dog, by means of a questionnaire.

MATERIALS AND METHODS

The subject of this investigation involved indoor cats divided for the purpose of this study into the following 4 groups: (1) households with one cat (n = 10); (2) households with two cats (n = 7); (3) households with three or more cats (n = 11); and (4) households with one or more cats living under one roof with a dog (n = 5). Altogether 91 cats were involved in the study. All of them were cats kept exclusively indoors without the possibility of free movement outside. This eliminated the influence of the external environment on their behaviour which the owner normally is unable to observe. The households/breeding units were selected at random.

The practical part of the study was based on a questionnaire. Also we selected written and electronic approaches in order to obtain additional relevant data.

The questionnaire started with questions seeking information about the owner of the animal(s) and the animal(s) themselves. The questions were divided to the following groups: Cat owner; Relationship with household members; Health state; Environment; Daily activities; Observed behavioural changes. If any question was not clearly answered or the answer was incomplete or too brief, we sent supplemental questions to the responders that helped to elucidate aspects concerning the behaviour of cat(s). The respondents were also asked to provide a scheme of his/her apartment/house which constituted the territory of the cat(s), together with a description where the cat(s) exhibited various forms of the behaviour of interest.

RESULTS

The information obtained through questionnaires was processed and is summarised in Tables 1 and 2.

Table 1. Percentage evaluation of results

	Behaviour problems		Unsuitable toileting behaviour associated with organ disease	Cat – dog relationship
	Aggression to man, cats; marking; other	Territorial /maternal aggression; marking		
Group 1 (one cat)	40 %	40 %, of that 20 % involved single occurrence	–	–
Group 2 (two cats)	43 %	29 %	–	–
Group 3 (3 and more cats)	55 %	45 %	27 %	–
Group 4 (cat/cats + dog)	20 %	20 %	–	100 % neutral to positive

Table 2. Descriptive evaluation of results

	Aggression to man, cats; other	Territorial/maternal aggression; marking; other	Unsuitable toileting behaviour associated with organ disease
Group 1 (one cat)	Aggression to man; vocalisation	Territorial aggression; single display of territorial aggression; urine-marking (bed)	-
Group 2 (two cats)	Aggression to man and cats; urine-marking	Territorial aggression; marking and vocalisation in oestrus	-
Group 3 (3 and more cats)	Aggression to man and among cats; elimination disorders, fear; pica	Territorial aggression; aggression and marking in oestrus; maternal aggression	Diabetes mellitus and FLUDT
Group 4 (cat/cats + dog)	Petting aggression to man	Territorial aggression	-

FLUDT — feline lower urinary tract disease

DISCUSSION

Bradshaw [4] in his most relevant book titled "Cat sense", stated that domestication not only suppressed the internal distrust of people in wild cats, but also reduced their alertness toward other cats. According to this author, the social behaviour of cats probably began to develop when they discovered food storages by means of which humans made the concentrated food sources available to cats. Each cat which maintained natural distances from all members of its own species, was not able to utilize these new sources as effectively as cats capable of recognising the mutual relations and use them for their own benefit. Another advantage involved the rearing of the young. Two and more cats which gathered their kittens were able to take care of them much better than isolated cats which had to leave their young when they went to hunt. Life in a family also provides the cats the opportunity to learn one from another which is more advantageous than learning individually.

The first group evaluated in our study included households with one cat. In this group both aggression to man and cats (40 %) and territorial aggression (40 %) were observed. Although the proportion of animals with aggression to man and cats in this group was lower in comparison with groups two and three of cats, this cannot be considered proof that cats reared alone, i. e. without the presence of a feline "rival", will not display problem behaviour which is in agreement with the observations by Bradshaw [4].

The occurrence of behaviour problems (43 % and 29 %), detected in households with two cats in our study can again support the correctness of other conclusions made by Bradshaw [4] and of additional studies mentioned in this section. However, behavioural problems in the form of aggression were observed also in unrelated cats (two intact and two spayed) and in two related male cats. Unsuitable behaviour was associated with manifestation of oestrus in cats and therefore we consider behaviour problems a more important indicator of the relationship between two cats.

According to Bradshaw [4], the fact that two cats have the same owner does not necessarily mean that they will get along well with one another. Many cats observe the principal rule of cat society, namely to proceed cautiously when approaching any cat which is not part of the family. However, the majority of owners are ignorant of this rule and carelessly obtains another cat in belief that these two animals will soon become good friends. Although this may apply in general to dogs, the cats will more likely only tolerate each other [4].

Cat societies are matriarchal and are not so strongly developed as those of dogs. Each social unit begins with one female and its progeny and if there is abundance of food, daughters will stay with their mother even after having their own young and cats will share the care of them. A typical small colony consists of a mother and her adult daughters with their last litters and one or two tomcats. Any important situation, such as the death of the dominant female, may result in disturbed relationships between some cats, increases the incidence of aggressive behaviour and may lead to division of the colony into two or more smaller groups.

Information about relations between related cats in colonies and the unrelated ones presented by Bradshaw [4] are also useful for the evaluation of our results. Their practical validity is confirmed by our results obtained in the group with three or more cats, where the occurrence of behaviour problems was the highest (55 % and 45 %). In agreement with the above author, in one household with six cats which were unrelated (with the exception of one pair) and were brought together at different times from different conditions the parallel occurrence of aggression to man and cats and territorial and maternal aggression was the highest. Differences related to increasing number of cats (from 3 up to 12) were also evident. We observed higher occurrence of undesirable urination associated either with behaviour problem or oestrus when six or more cats were kept together.

The common denominator of breeding centres with higher number of cats was unsuitable but understandably also natural behaviour associated with the reproduction cycle and artificial making of cat societies which included the following: sexual aggression of breeding males and females, unwanted elimination in the form of marking in oestrus and maternal aggression related to protection of the litter.

Feuerstein and Terkel [6] investigated the relationship of cats and dogs living together under the same roof. This study was based on a questionnaire and the direct observation of the animals. The results revealed that both species were capable of developing relatively friendly relationship with another species and the gender had only a little effect on the character of this relationship. Also, adopting the cat before the adoption of a dog contributes to the development of a basis for a friendly relationship provided that their first contact takes place at a young age (cat up to 6 months of age, and a dog up to one year). According to these authors it is possible to conclude that the mutual exposure of one species to another at an early age facilitates the mutual recognition of body language by these animals and the subsequent development of a friendly relationship [6].

The results of Feuerstein and Terkel [6], as well as ours, disprove the opinion that a cat and a dog in one household cannot get along well with each other. In all households in group 4, the relationship of cat and dog was either neutral or positive and owners observed that the animals were involved in a hunting imitation play. As far as problem behaviour was concerned, only in 20 % of households the owners observed aggression to cats which were brought to the household for a short time only. This is however understandable behaviour of the indoor cat which coexists in one room apartment with two owners and a dog. Additional behaviour problems were observed in 20 % of the households in the form of petting aggression.

When a new cat was brought to the household, changed behaviour in the form of aggression was observed in 43 % of animals from group 2, which lasted from two days up to one week, and in 45 % of cats from group 3 associated with undesirable urination which lasted two to three days, either in resident or newly introduced cats. Our results resemble those of Levine et al. [8] who reported that introducing another cat into a household with several other cats, result-

ed in displays of aggressive behaviour in about half of them manifested by scratching and biting.

The study by Amat et al. [2], dealing with main risk factors associated with behaviour problems in animals, showed that 47 % of owners of 336 cats who visited the veterinary clinic because of behaviour problems, complained about aggressive behaviour and 39 % reported undesirable elimination. Of all aggressive conflicts, 64 % involved aggressive behaviour against cats and 36 % were conflicts with man, most frequently with owners. The most frequent forms of aggression to man were play aggression and stroking aggression. The majority of problems with elimination included urination (59 %), urination and defecation (32 %) and defecation alone (9 %), and the most frequent reason was toilet aversion (63.4 %). Indoor cats exhibited more behaviour problems than cats with access to the exterior and the aggressiveness occurred more frequently in households with one cat [2]. Our results agree with the observations of the above authors [2] with one exception, namely that the object of aggression to man were toward the owners. According to our results, the owners was a target of such behaviour in only three out of seven cases.

The summary of the detected behaviour problems indicates that the most frequent behavioural problem in all groups was aggression to man or other cats and inappropriate toileting behaviour rated as second. Less frequent or rare problems were vocalisation, pica (sucking microtene bags) and fear.

CONCLUSIONS

On the basis of our results, we can conclude that the probability of the occurrence of behaviour problems was not related unambiguously to the number of cats in a household (breeding centre) nor to its coexistence with a dog. The percentage of occurrence of changed behaviour following the introduction of a new cat did not differ markedly between the groups.

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LIPID BASED FORMULATIONS OF BIOPHARMACEUTICS CLASSIFICATION SYSTEM (BCS) CLASS II DRUGS: STRATEGY, FORMULATIONS, METHODS AND SATURATION

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ABSTRACT

Active ingredients in pharmaceuticals differ by their physico-chemical properties and their bioavailability therefore varies. The most frequently used and most convenient way of administration of medicines is oral, however many drugs are little soluble in water. Thus they are not sufficiently effective and suitable for such administration. For this reason a system of lipid based formulations (LBF) was developed. Series of formulations were prepared and tested in water and biorelevant media. On the basis of selection criteria, there were selected formulations with the best emulsification potential, good dispersion in the environment and physical stability. Samples of structurally different drugs included in the Class II of the Biopharmaceutics classification system (BCS) were obtained, namely Griseofulvin, Glibenclamide, Carbamazepine, Haloperidol, Itraconazol, Triclosan, Praziquantel and Rifaximin, for testing of maximal saturation in formulations prepared from commercially available excipients. Methods were developed for preparation of formulations, observation of emulsification and its description, determination of maximum solubility of drug samples in the respective formulation and subsequent analysis. Saturation of formulations with drugs showed that formulations 80 % XA and 20 % Xh, 35 % XF and 65 % Xh were best able to dissolve the drugs which supports the hypothesis that it is desirable to identify limited series of formulations which could be generally applied for this purpose.

Key words: biopharmaceutics classification system – class II drugs; lipid formulations; maximum saturation; spontaneous emulsification

INTRODUCTION

Biopharmaceutics classification system (BCS)

Biopharmaceutics classification system classifies drugs according to their solubility in water and membrane per-

Note: *Due to confidentiality of information obtained from Astellas Pharma Europe BV we cannot disclose identity of the tested excipients.*

meability into four classes described in Table 1. Drugs included in the BCS II class are generally classified as drugs with low solubility but high permeability. These drug little dissolve during their passage through the gastrointestinal (GI) tract [13] and therefore are not completely absorbed. Their bioavailability is therefore limited by their low solubility in water which means that even a small increase in solubility may result in considerable increase in bioavailability [6]. With preliminary dissolution of such drugs in lipids, surfactants or their mixtures we can avoid to the dissolution step which limits absorption of these drugs from the GI tract [2]. It is important to achieve adequate absorption from the water environment. This is the reason why stress is laid on the potential of excipients to produce spontaneous emulsions and form micro- or nano-droplets.

Table 1. Biopharmaceutics classification system (BCS)

Class	Permeability	Solubility
I	High	High
II	High	Low
III	Low	High
IV	Low	Low

Formulations

Lipid-Based delivery systems range from simple oil solutions to complex mixtures of oils, surfactants and co-solvents [8]. They have a high potential for increasing solu-

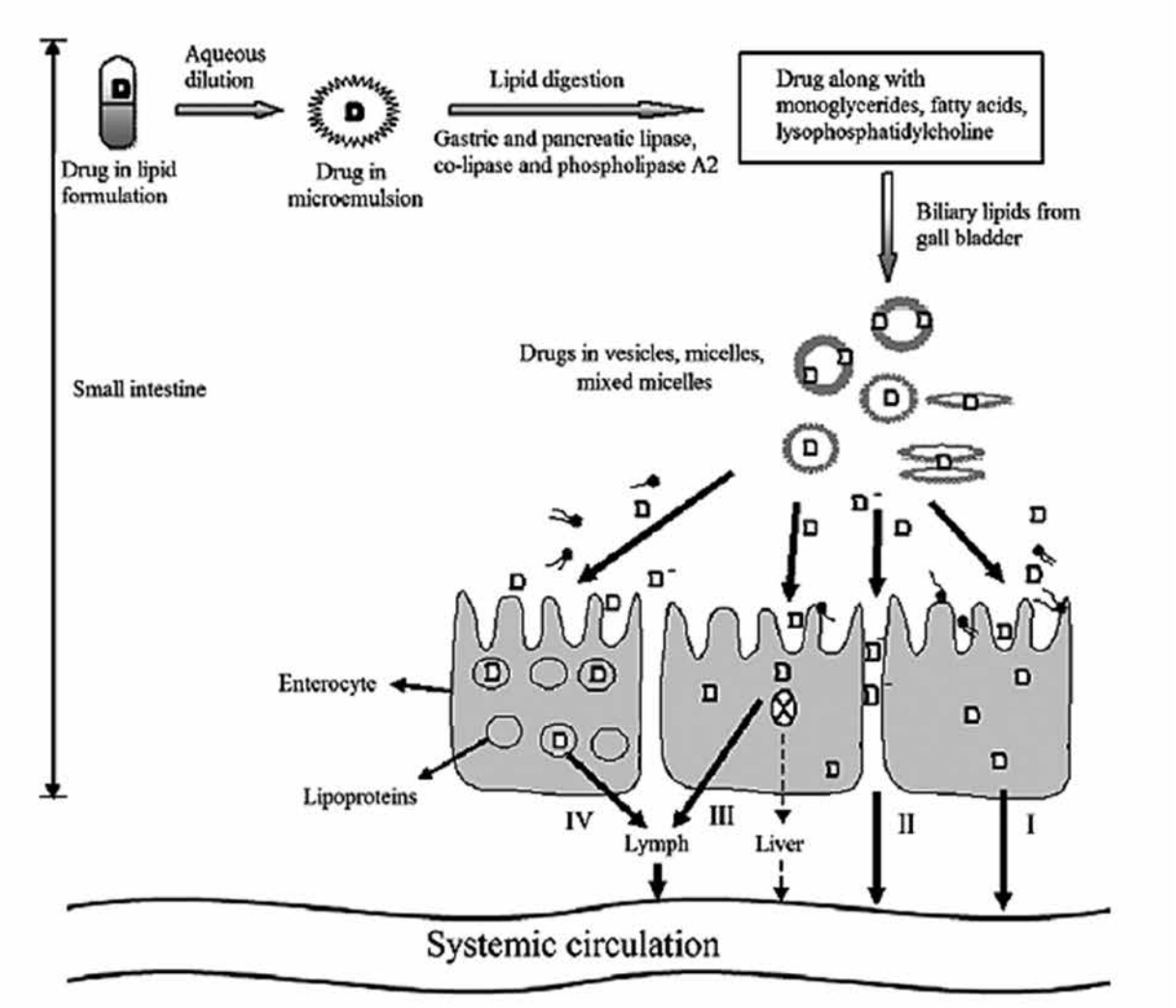


Fig. 1. Scheme of intestinal transport of drug from LBF [5].

bility of BCS class II drugs. Drugs in LBF are already in a soluble form and thus the dissolution step is omitted in the GI tract. Moreover, these formulations should be able to form emulsions in the water environment and maintain the drug in the solubilized state without its precipitation. In the GI tract, the LBF systems increase absorption of drugs by accelerating the dissolution process facilitating the formation of solubilised phases by reduction the particle size to the molecular level [3, 9] changing drug uptake, efflux and disposition by altering enterocyte-based transport, [11, 14] and enhancing drug transport to the systemic circulation via intestinal lymphatic system [1, 4, 10].

The aim of the study was to identify a limited series (mixtures of) excipients that can be used for oral administration of a range of structurally different poorly water soluble drugs and study their potential as simplified strategy formulation.

Toxicity of excipients

Excipients are essential components of drug products. They may be also potential toxicants. Examples of known excipients-induced toxicities include renal failure and death caused by diethylene glycol, or cardiotoxicity induced by propylene glycol [7]. Safety of excipients used in this project was supported by extensive toxicological evaluations and precedence of use in approved pharmaceutical products. Their identification as GRAS (Generally Recognized As Safe), or inclusion in FDA (Food and Drug Administration) database of non-active substance should provide the guarantee of their status [12].

MATERIAL AND METHODS

The following additives were tested: XA, Xf, Xg, Xh, XB, Xi, Xj, XD, XE, XC, Xk, Xo, Xm, XF. The biorelevant medium was prepared using the following: SIF powder original, 36 % HCl, sodium chloride, sodium dihydrogen phosphate dehydrate, sodium hydroxide. Methanol (MeOH), tetrahydrofuran (THF), acetonitrile (AcN) and ammonium formate were used for preparation of solutions for UPLC (Ultra Performance Liquid Chromatography). Additional material was used for preparation of formulation, manipulation with samples and analysis as follows: stirring bars, serum and injection bottles (10 and 20 ml), BD Plastipak syringes 1 ml, 2 ml, 5 ml, 20 ml, Transferpipette®, Plastibrand® pipette tips, Eppendorf combi-tips, HPLC (High Pressure Liquid Chromatography) bottles, GHP Acrodisc® 13mm syringe filter with 0.2 µm GHP membrane, BEH C18 chromatographic column (50 × 2.1 mm; 1.7 mm particles), Waters Acquity UPLC® System, and model drugs: Griseofulvine, Praziquantel, Rifaximin, Itraconazol, Haloperidol, Carbamazepine, Glibenclamide and Triclosan.

Preparation of formulations

The selected excipients were warmed up in a water bath at 55 °C for 5—10 min and homogenised. Required quantities of the relevant excipients were weighed into serum bottles (20 ml) and mixed (magnetic mixer, 300 rpm) for 15 min in a water bath. After mixing the stirring bar was removed, nitrogen was introduced into bottles and the bottles were closed.

Emulsification potential — water, FaSSGF and FaSSIF (Fasted State Simulated Gastric and Intestinal Fluid)

Behaviour was observed at laboratory temperature. 5 ml of medium was transferred into injection bottles (10 ml) and added 50 µl of formulation by means of 1 ml injection syringe. Observed was the content of bottles during the first contact with the medium for the presence of spontaneous emulsification. Subsequently, was added a stirring bar and the bottle was closed. Rotation started at 100 rpm and increased every minute by 100 rpm until emulsion formed. Rotation speed was one of main criteria for selection of formulation. Observations were recorded in protocols.

The simulated gastric fluid contained 30 mg SIF powder original in 0.5 l (17 mmol.l^{-1} solution, pH = 1.6). The simulated intestinal fluid was prepared by dissolving 1.12 g SIF powder original in 0.5 l FaSSIF buffer solution (pH = 6.5) containing NaOH (5 mmol.l^{-1}), $NaH_2PO_4 . 2H_2O$ (14 mmol.l^{-1}) and NaCl (52 mmol.l^{-1}1).

UPLC screening

For the screening were prepared 100 ml solutions of formulations (7 µl.ml^{-1}) and drugs (0.1 mg.ml^{-1}) in tetrahydrofuran.

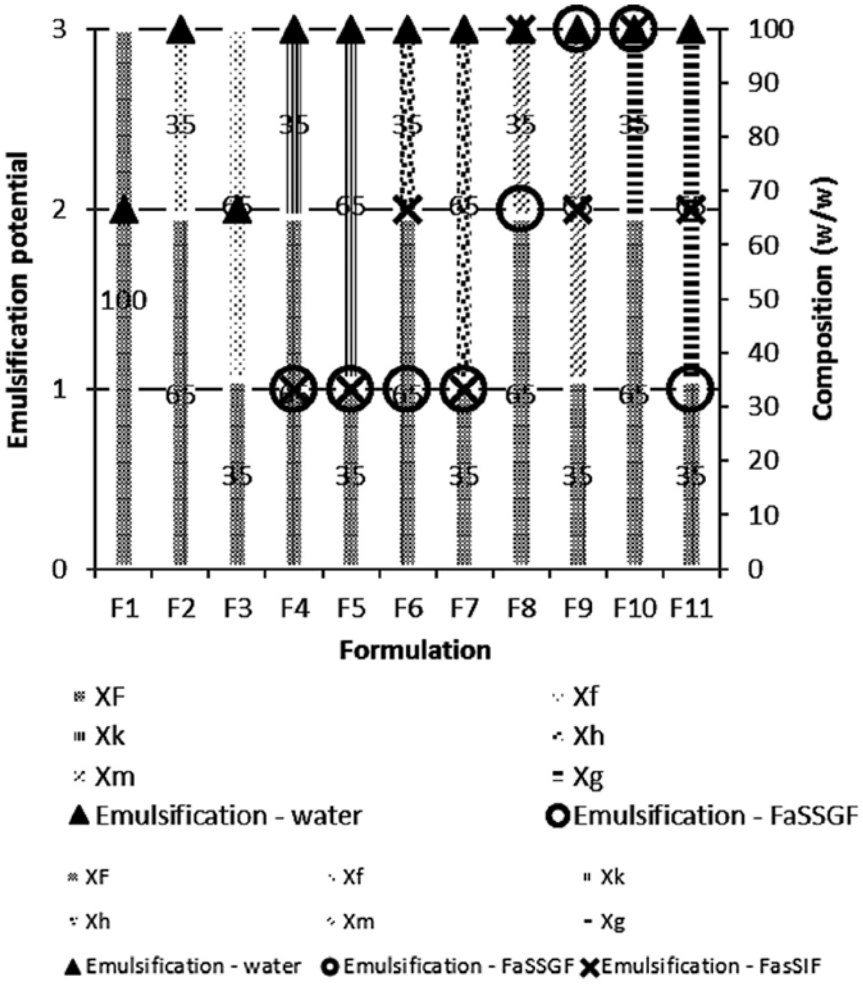
Instrumental methods: gradient analysis. Column temperature: 40 °C. UV spectrum was observed in the range 200—400 nm using PDA (Photodiode Array Detector) detector.

Table 2. Description of instrumental gradient method

No.	pH buffer	Modifier	Column	Runtime [min]	Flow rate [ml.min^{-1}]	Inj. volume [µl]
1	3	AcN	BEH C18	1.5	0.8	4
13	3	AcN	BEH C18	5.5	0.8	4

Time [min]	% A (buffer)	% B Modifier
0	95	5
1.0/5.0	10	90
1.01/5.01	95	5
1.50/5.50	95	5

Week needle wash: 10 % ACN
Strong needle wash: 70 % CAN
Seal wash: 10 % MeOH
pH buffer: ammonium formate 20 mmol.l^{-1}



0 = more than 500 rpm — no potential; 1 = 400–500 rpm — unsatisfactory potential;
2 = 200—300 rpm — satisfactory potential; 3 = 100 rpm — the best emulsification potential

Fig. 2. Example of composition (w/w) of formulations and of their emulsification potential

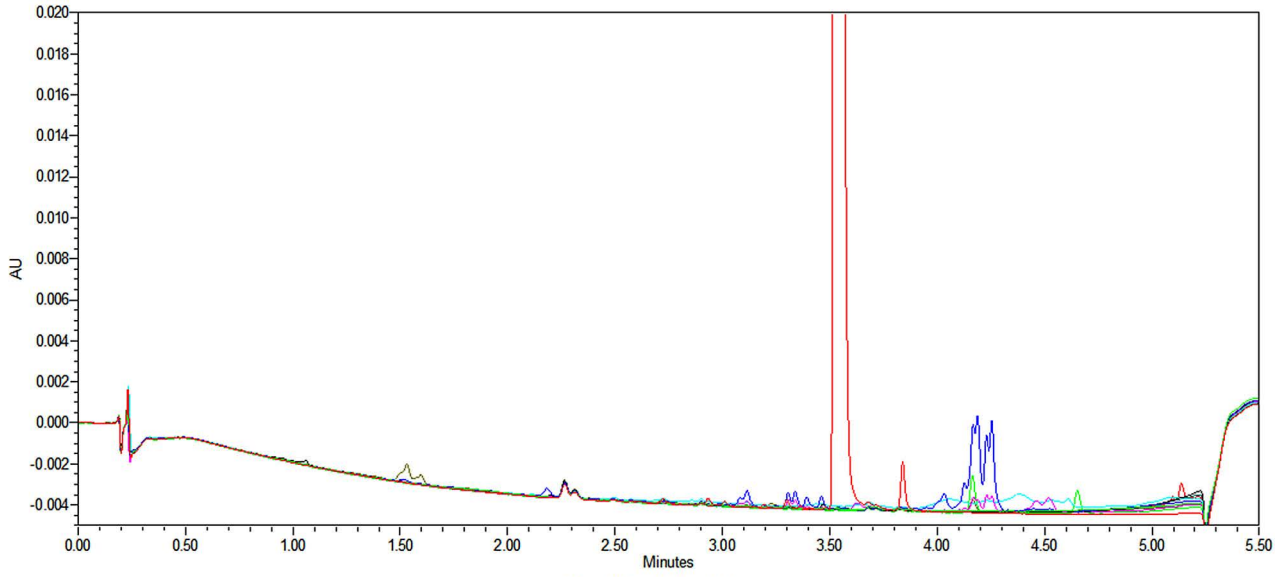


Fig. 3. Example of obtained chromatograms: screening of excipients and Itraconazol using Method 13

Itraconazol (the highest peak — red); XA — green; XCf — blue; XCh — blue; XB — red
Xi — green; XF — blue; Xk — brown; Xg — red; Xm — green

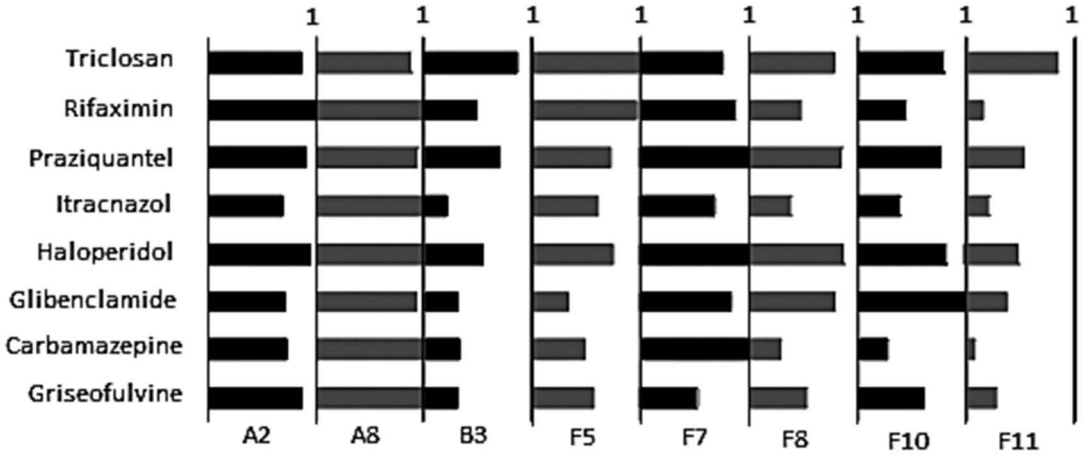


Fig. 4. Relative solubility of drugs in the tested formulations

Maximum saturation

The experiment was carried out in duplicate. Five ml of formulation was added into a glass 10 ml bottle containing 125 mg of the API and the stirring bar. The content was mixed at 350 rpm for 20 hours at 50 °C. Subsequently, the mixing was continued at laboratory temperature at 100 rpm for 20 hours. If the drug dissolved completely, additional quantity of drug was added and the procedure was repeated. Analysis was carried out using 1 ml of sample withdrawn by means of a 2 ml syringe.

The sample was filtered into a HPLC bottle through GHP Acrodisc® 13 mm syringe filter equipped with 0.2 µm GHP membrane and was diluted with tetrahydrofuran to a required concentration. Fresh reference standards were prepared by weighing accurate amounts of drugs and dissolving it in THF. THF was used as a blank. Then, 1 µl of sample was injected to Waters Acquity UPLC ® System using Method 13.

RESULTS AND DISCUSSION

Formulations

Series of formulations were prepared (A, B, C, E, F) and their physical properties (viscosity, phase, homogeneity, colour) were described. To each of the formulations a factor was assigned according to rotational speed which resulted in formation of emulsion and this factor together with the stability and degree of dispersity were the main criteria for selection of formulations for subsequent study. Several formulations were unstable, e.g. the phases separated in water environment. Also some of them exhibited inadequate emulsification potential and degree of dispersity. In this way we were able to reduce the number of prospective formulations.

The following formulations were selected for studies in FaSSGF and FaSSIF (Fasted State Simulated Gastric and Intestinal Fluid): A1; A2; A3; A8; A9; B3; B4; C2; C3; C9; C13; E7; F4–F11.

The following formulations were selected for studies of maximum saturation: A2; A8; B3; F5; F7; F8; F10; F11.

Waters Acquity UPLC screening method

All drugs and excipients were analysed using Method 1 and Method 13 (Waters Acquity UPLC ® System). These methods differ in the length of analysis. The longer Method 13 appeared more suitable for all drugs and excipients. On the basis of UV spectra of individual drugs (Fig. 3) we selected optimum wavelength for analysis of additional samples at their saturation.

Maximum saturation

Fig. 4 illustrates relative solubility of drugs in tested formulations. As the maximum solubility of drugs in individual formulations differed (see Table 3), a factor 1 was used to indicate 100 % solubility. Thus the drug with factor 1 means the best solubility. Maximum saturation may be related to the composition of formulation (e.g. 12.7-fold difference between solubility of Carbamazepine in F7 and F11). It should be noted that relative solubility of all drugs in formulation A8 was relatively high.

Haloperidol, Carbamazepine and Praziquantel reached the highest concentration in F7: 14.1 mg.ml^{-1}, 70.0 mg.ml^{-1} and 56.4 mg.ml^{-1}, respectively. Griseofulvine and Itraconazol in A8: 10.2 mg.ml^{-1} and 3.3 mg.ml^{-1}, respectively. The highest concentration of Triclosan was reached in F5:

Table 3. Solubility of drugs in water and in suitable formulations

Molecule	Concentration		Molecule	Concentration	
	Water [mg.l^{-1}]	Formulation [mg.ml^{-1}]		Water [mg.l^{-1}]	Formulation [mg.ml^{-1}]
Griseofulvine	8.6	A8: 10.2	Glibenclamide	4.0	G4: 15.8
Carbamazepine	17.7	F7: 70.0	Praziquantel	400	F7: 56.4
Haloperidol	14.0	F7: 14.1	Rifaximin	7.4	A2: 109.1
Itraconazol	insoluble	A8: 3.3	Triclosan	6.05	F5: 533.4

533.4 mg.ml^{-1}; of Rifaximin in A2: 109.1 mg.ml^{-1}; of Glibenclamide in G4: 15.8 mg.ml^{-1}. Formulation G4 was experimental and was not mentioned in materials and methods above.

The solubility of drugs in the selected formulations was increased considerably and with some formulations was higher by several orders of magnitude in comparison with their solubility in water (Table 3).

CONCLUSION

For improvement of the oral bio-availability of poorly water soluble drug substances, adjustment of the Lipid based formulations, prepared from mixtures of commercially available excipients with self-emulsifying properties in aqueous environment was investigated. Observed was emulsification potential of various formulations in water, FaSSGF and FaSSIF with respect to solubility of eight structurally different drugs. On the basis of selection criteria and relevant properties, eight formulations were selected for study of their ability to dissolve drugs.

Maximum saturation of drugs was determined by means of UPLC and previous treatment and dilutions of samples. Formulations A8 and F7 showed generally the best ability to dissolve almost all tested drugs which supports the hypothesis to identify a limited series of formulations generally suitable for this purpose.

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