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PERIODONTAL DISEASE IN ASSOCIATION WITH SYSTEMIC DISEASES IN THE DOG

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ABSTRACT

Periodontal disease (PD) is the second most common disease affecting dogs in UK veterinary practices. Veterinary and human literature suggests that periodontal disease may be associated with bacteraemia and a chronic, systemic release of inflammatory mediators which produce direct or immune-mediated changes elsewhere in the body. Thirty canine periodontal patient's electronic medical histories were analysed for comorbidities. The findings were analysed overall to identify any possible associations. Seventy three percent of these dogs had comorbidities, most commonly haematopoietic, cardiovascular, musculoskeletal and hepatic systems were involved. The most prevalent comorbidities were: high liver enzymes, heart murmur, mitral valve disease, and monocytosis. Other interesting comorbidities were: endocarditis, neutrophilia, submandibular lymph node enlargement and arthritis. Periodontal disease patients had a higher prevalence of disease when compared to the disease prevalence data for UK pet dogs in general. Mitral valve disease was over 17 times more likely in the periodontal disease patients. Comorbidity disease prevalence also increased with more severe periodontal disease stages. This study supports an association between periodontal disease and systemic diseases in the dog. Further studies should focus on confirming a cause and effect relationship. Until then, these data may be useful for veterinarians to examine periodontal patients for concurrent diseases and can be used as a tool to promote dental disease prevention to pet owners.

Key words: comorbidity; dog; periodontitis; periodontal disease; systemic disease

INTRODUCTION

Periodontal disease (PD) is the second most frequently seen disease in dogs in a veterinary practice in the United Kingdom [17]. Eighty percent of dogs show signs of periodontal disease by three years of age [29] and the incidence of the disease increases considerably with advancing age, causing significant oral pain and suffering [22]. Pet owners

are currently recommended to brush their dog's teeth once per day, although compliance to this oral hygiene recommendation is low [12, 15].

Periodontal disease may act as a source of bacteraemia and chronic inflammation causing a chronic systemic release of inflammatory mediators, bacterial and cellular by-products which may lead to direct or immune-mediated changes elsewhere in the body.

There is evidence in both the human and veterinary literature of an association between periodontal disease and systemic diseases. In humans, 57 systemic conditions are being researched in connection to periodontal disease; most of which are the result of chronic inflammatory disease [14], including cardiovascular diseases such as atherosclerosis, stroke and coronary heart disease [1, 16,], endocarditis [8], rheumatoid arthritis [2, 4, 5,], diabetes [6, 26] and pregnancy complications such as preeclampsia [3, 21], low birth weight [9] and premature delivery [28].

Less literature is available on dogs, although, mitral valve disease, atherosclerosis [19], histologic changes in the myocardium [7], endocarditis [11, 25], chronic kidney disease [10, 24], hepatic pathology [19], diabetes [13, 18, 20] and meningoencephalomyelitis [27] have been reported.

The aim of this study was to select canine periodontal patients, grade the severity of the periodontal disease, identify concurrent systemic diseases in each patient and analyse the overall findings to identify potential associations.

MATERIALS AND METHODS

Sample selection

Gatehouse Veterinary Centre in Lavister, a small animal first opinion veterinary practice in Wales, UK, provided access to their canine periodontal patient medical histories. The 30 most recent periodontal patient dental examination patients were selected, and their electronic medical histories analysed for concurrent diseases. The sample included 30 dogs of 18 breeds, aged 2 to 16 years of which 13 were females and 17 males.

Stage of periodontal disease

The patient's dental charts were analysed to determine the level of severity of periodontal disease. Guidelines for staging were outlined on the dental charts to allow the veterinary surgeons to categorise each stage in the same way.

Table 1. Periodontal patient medical histories

	Breed	Age in years	Gender	Neuter status	PD stage	Comorbidities
1	Cross	11	Male	Neutered	1	Dermatitis
2	Cavalier King Charles spaniel	9	Male	Neutered	1	Severe mitral valve disease, Chronic heart failure
3	Cavalier King Charles spaniel	9	Male	Neutered	1	Mitral valve disease, Heart murmur, Otitis externa, Cranial cruciate ligament rupture
4	Shetland sheep dog	5	Male	Neutered	1	Heart murmur
5	Miniature Schnauzer	8	Male	Intact	1	None
6	Border terrier	5	Male	Neutered	1	None
7	Yorkshire terrier	5	Male	Neutered	1	None
8	Terrier	2	Male	Intact	1	None
9	Border terrier	13	Male	Neutered	2	None
10	West Highland terrier	10	Female	Neutered	2	High total protein, High liver enzymes, Arthritis of the stifle, Carnassial abscess

Table 1. Continues

	Breed	Age in years	Gender	Neuter status	PD stage	Comorbidities
11	Cavalier King Charles spaniel	10	Male	Intact	2	Anal adenoma
12	Miniature Poodle	8	Female	Neutered	2	High bile acids, Diffuse hepatic changes, Liver disease, Hepatic vascular dysplasia, Splenic reactive lymphoid hyperplasia
13	Dachshund	8	Female	Neutered	2	Intervertebral disc disease, Anal gland impaction, Sebaceous cyst
14	Cross	8	Female	Intact	2	Erythrocytosis, Reticulocytosis
15	Bull terrier	8	Male	Neutered	2	Fibroadnexal hamartoma
16	Coton de tulear	7	Male	Neutered	2	Lameness
17	Jack Russel terrier	9	Female	Neutered	2	None
18	Labradoodle	4	Male	Neutered	2	None
19	Jack Russel terrier	13	Female	Neutered	3	None
20	Jack Russel terrier	16	Male	Neutered	3	High liver enzymes, High total protein, Osteoarthritis of the elbow
21	Cross	15	Female	Intact	3	High liver enzymes, Squamous cell carcinoma under tongue
22	Jack Russel terrier	14	Male	Neutered	3	Erythrocytosis
23	Lhasa apso	14	Female	Neutered	3	High urea, Thrombocytosis, Cystitis,
24	West Highland terrier	12	Male	Neutered	3	Monocytosis, Neutrophilia, Lameness, Thrombocytosis
25	Bichon-Frise	11	Female	Neutered	3	Heart murmur, High liver enzymes, Lymphopenia, Lameness, Thrombocytosis
26	Miniature poodle	11	Female	Neutered	3	Mitral valve disease, Endocarditis with valvular and septal lesions, Heart murmur, Monocytosis, Submandibular lymph node enlargement
27	Cocker Spaniel	10	Female	Intact	3	Arthritis of the elbow, Monocytosis
28	Yorkshire terrier	10	Female	Neutered	3	High liver enzymes, Erythrocytosis, Leukopenia
29	Jack Russel terrier	8	Female	Neutered	3	Reticulocytosis
30	Chihuahua	6	Male	Neutered	3	Heart murmur, Intervertebral disc disease

Table 2. The most common organ systems affected in PD patients and their corresponding comorbidities

Organ System	Comorbidity	Number of PD patients affected
Haematopoietic	Monocytosis	3
	Thrombocytosis	3
	Erythrocytosis	3
	Reticulocytosis	2
	Leukopenia	1
	Lymphopenia	1
	Neutrophilia	1
		Total: 14
Cardiovascular	Heart murmur	5
	Mitral valve disease	3
	Chronic heart failure	1
	Endocarditis	1
		Total: 10
Musculoskeletal	Lameness	3
	Arthritis	2
	Intervertebral disc disease	2
	Cruciate rupture	1
	Osteoarthritis	1
		Total: 9
Hepatic	High liver enzymes in blood	5
	Hepatic vascular dysplasia	1
	High bile acids in blood	1
	Liver disease	1
		Total: 8
Urinary	High total protein in the blood	2
	Cystitis	1
	Urea in the blood	1
		Total: 4
Tumour	Anal adenoma	1
	Fibroadnexal hamartoma	1
	Squamous cell carcinoma under tongue	1
		Total: 3
Glandular	Anal gland impaction	1
	Sebaceous cyst	1
		Total: 2
Lymphoid	Splenic reactive lymphoid hyperplasia	1
	Submandibular lymph node enlargement	1
		Total: 2
Dermatologic	Dermatitis	1
	Otitis externa	1
		Total: 2
Dental	Carnassial abscess	1
		Total: 1

Stage 1 was characterised by marginal gingivitis, involving a red line with some oedema, Stage 2 was characterised by swelling and some bleeding and stage 3 was characterised by severe inflammation and spontaneous bleeding [23].

Medical history analysis

Using the electronic medical histories, haematology and biochemistry test results, pathology laboratory reports, dental records and consultation and surgery notes were analysed to identify comorbidities. This was facilitated by the practice recommending pre-anaesthetic blood tests for patients before dental examinations.

RESULTS AND DISCUSSION

The periodontal disease patient sample involved a wide range of breeds, ages, genders and periodontal disease stages. From the sample, 73 % of the periodontal patients were found to have one or more comorbidities (Table 1).

There were common associations in the periodontal patient samples. Certain organ systems were affected more frequently than others. The haematopoietic and cardiovascular systems were the most prevalent systems affected (Table 2) and included heart murmur, mitral valve disease, chronic heart failure, endocarditis and monocytosis amongst other comorbidities.

Table 3. Top 10 most common comorbidities in periodontal patients

Top 10 most common Comorbidities	Number of patients affected
High liver enzymes	5
Murmur	5
Mitral valve disease	3
Monocytosis	3
Lameness	3
Thrombocytosis	3
Erythrocytosis	3
Arthritis	2
Intervertebral disc disease	2
Reticulocytosis	2

The most common comorbidities were: high liver enzymes, heart murmur, mitral valve disease, monocytosis and lameness (Table 3). Other interesting concurrent diseases included: neutrophilia, submandibular lymph node enlargement and arthritis. These findings potentially reflect chronic, systemic inflammation.

There was a significantly higher disease prevalence in periodontal disease patients in the study when compared to UK pet dog data [17] (Table 4). In periodontal patients, mitral valve disease was over 17 times more likely, heart disease generally was over 6 times more likely and heart murmur was over 4 times more likely. This suggests that periodontal disease could be an important factor in the development of systemic diseases. The comorbidity prevalence increased with more severe periodontal disease.

Fifty percent of patients with stage 1 periodontal disease had comorbidities, 70 % of patients with stage 2

Table 4. Disease prevalence [%] in the UK dog population compared to periodontal patients

Comorbidity	Disease prevalence data for UK pet dogs [17]	Disease prevalence in periodontal disease patients
Heart murmur	3.94	16.7
Heart disease	0.88	6.7
Mitral valve disease	0.57	10
Lameness	1.7	10
Arthritis	2.83	6.7
Hepatopathy	0.49	3.33

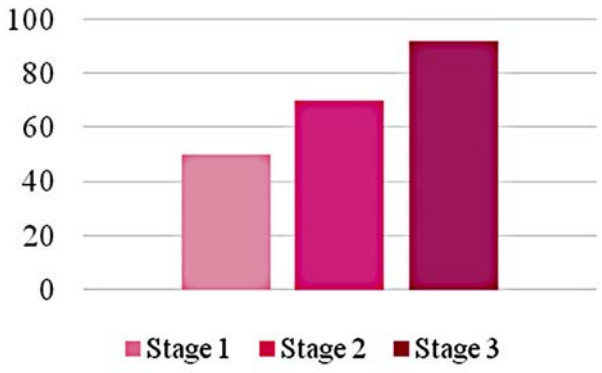

Fig. 1. Percentage of patients with comorbidities according to the periodontal disease severity

had comorbidities and 92 % of stage 3 had comorbidities (Fig. 1). This suggests that the more severe the periodontal disease becomes, the greater the chance of systemic disease developing.

DISCUSSION

The findings support an association between periodontal disease and systemic diseases in the dog. The data also support current literature which links canine periodontal disease to heart disease [7, 11, 19, 25] and hepatic pathology [19] and can be related to human associations such as: arthritis [2, 4, 5] and endocarditis [8].

This study suggests that periodontal disease could be an important factor in the development of systemic diseases. The identification of associations between periodontal disease and systemic diseases suggest that periodontal disease could be used as an early warning tool to detect concurrent diseases. Comorbidities could also be diagnosed and treated sooner resulting in improved welfare and longevity.

This study focused on an association as opposed to identifying a causal relationship. Future studies should work towards identifying a cause and effect relationship, for example by comparing microbiology samples from the periodontium and affected systemic organs. This would support the idea that treating and preventing periodontal disease may prevent and treat comorbidities such as endocarditis.

Until then, this data might be useful for veterinary surgeons to examine periodontal patients for concurrent diseases and can be used as a tool to promote dental hygiene recommendations to pet owners. It is currently recommended to brush a dog's teeth once per day using toothpaste especially made for dogs. Regular veterinary checkups are important to identify early signs of periodontal disease and veterinary treatment may be required to prevent its development.

CONCLUSIONS

Concurrent systemic diseases were identified in 73 % of the periodontal patients sampled. The cardiovascular and haematopoietic systems were particularly affected. The most common comorbidities were high liver enzymes, heart murmur and mitral valve disease amongst others. The comorbidity incidence increased with PD severity and there was significantly higher disease prevalences in the periodontal patient samples compared to the UK pet dog data. This study supports an association between periodontal disease and systemic diseases in the dog.

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ANTIOXIDANT ENZYMES OF HONEYBEE LARVAE EXPOSED TO OXAMYL

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ABSTRACT

Oxamyl is a carbamate insecticide used to control a broad spectrum of insects. It can also affect non-targeted organisms when applied incorrectly. The world food production depends partially on honeybee pollination abilities and therefore it is directly linked to the health of bees. The success of the colony development depends, among other factors, on the health of the larvae. The first 6 days are crucial for their development. In this stage, the worker larvae grow exponentially and may be exposed to xenobiotics via their diet. In this study, we investigated the effect of oxamyl on honeybee larvae (*Apis mellifera*) by monitoring the changes in their antioxidant enzyme system. The activities of superoxide dismutase, catalase and glutathione-S-transferase were determined in the homogenates of *in vitro* reared honeybee larvae after their single dietary exposure to oxamyl at doses of 1.25, 2.5, 5, 10 and 20 µg a.i./larva (a. i.—active ingredient). The doses of oxamyl did not cause statistically significant changes in the activities of the enzymes. Even a slight activation of these enzymes protected the larvae from the adverse effects of the reactive oxygen species (ROS). Marked changes in both the enzyme activity and the content of lipid peroxidation products were observed at the oxamyl dose of 10 µg a. i./larva. This fact may indicate a potential oxidative damage to the larvae. These results allowed us to assume that the toxic effects of oxamyl involves not only the inhibition of acetylcholine esterase but is also associated with ROS production.

Key words: antioxidant enzyme system; honeybee; larvae; oxamyl

INTRODUCTION

The honeybee (*Apis mellifera*) is undoubtedly the economically most important insect. Up to 35 % of the world agricultural production depends upon the pollination of crop plants by bees [9]. The pollination capabilities of bees are directly conditional on the health of honeybee colony. This is the reason why the health of bees is currently a subject of considerable attention. The decreasing tendency of

bee keeping and survival of bee colonies is associated with environmental abuse [1]. As a sensitive biosensor of environmental changes, the bee colony reacts even to small deviations caused by pollutants. Sublethal effects of plant protection products can be manifested by the changed behaviour of bees when: searching for food, memory disorders, ability to learn, rejection of food, loss of orientation and similar situations [16, 24]. The flying worker bees directly affected by insecticide may not have enough energy to return to the bee hive [21]. Those which survived the contact with such pollutants carry them back to the hive. There, the xenobiotics and their residues may be stored for considerable periods and may accumulate in the wax and honey. These compounds which accumulated in the hive, can induce in a short time, negative long-lasting effects on the colony behaviour [12].

By means of contaminated food and wax, all developmental stages of the honeybee can be exposed to a broad spectrum of xenobiotics. The presence of multiple residues can result in mutual interactions even at sublethal concentrations and cause a permanent "pesticide" stress manifested by delayed development of the larvae or shortened life of the bees [19].

Reactive oxygen species (ROS) are the side product of aerobic metabolism. The antioxidant system and antioxidant enzymes as its part constitute one of the protective mechanisms against oxidation damage. Antioxidant enzymes are involved in important physiological processes and can affect the health and survival of bees, their cognitive abilities, immune response to pathogens and longevity [5, 15]. If the free radicals are capable of overcoming the protective antioxidant system of the insect, an oxidation stress arises. The potential oxidation damage increases the demands of the insects on oxygen due to their way of life and the food rich in pro-oxidants. Carbamate insecticides cause reversible inhibition of acetylcholine esterase. According to a number of authors the primary toxic effect of carbamates may increase as a result of their ability to generate reactive oxygen species [17, 23]. The action and effects of such insecticides may be elucidated also on the basis of changes in the enzyme system of the exposed individuals.

The aim of this study was to evaluate the effects of oxamyl on the activity of antioxidant enzymes in honeybee larvae under *in vitro* conditions, 72 hours after their single exposure to this insecticide [18].

MATERIALS AND METHODS

Honeybee (*Apis mellifera carnica*) larvae were obtained from three healthy queen-right colonies reared in an experimental apiary of the University of Veterinary Medicine and Pharmacy in Košice, SR, during the summer of 2017. Synchronised first instar larvae were transferred to a laboratory where they were reared under *in vitro* conditions according to the method of A u p i n e l et al. [2] and OECD 237 [18]. Each experimental group comprised 12 larvae from the transferred colonies (three parallel groups, 12 larvae in each). The larvae were maintained in a 48-well plate in commercial grafting cells of 9 mm internal diameter (ref CNE/3, Nicotplast, France). Throughout the acute oral test (D1–D7), the larvae were kept in an enclosed box at 34.5 °C and a relative humidity of 90 ± 5 % (Sicco, Germany). With the exception of D2, they were fed a diet adjusted to the needs of the larvae, depending on the developmental stage [18]. The diet was prepared using royal jelly (Institute of Apiculture, Liptovský Hrádok, Slovakia), D-glucose, D-fructose, yeast extract and distilled water:

Diet A (D1): 50 % fresh royal jelly + 50 % water solution containing
2 % yeast extract, 12 % glucose and 12 % fructose;

Diet B (D3): 50 % fresh royal jelly + 50 % water solution containing
3 % yeast extract, 15 % glucose and 15 % fructose;

Diet C (D4—D6): 50 % fresh royal jelly + 50 % water solution containing
4 % yeast extract, 18 % glucose and 18 % fructose.

The stock solution of oxamyl in a glucose-fructose solution (6.6 mg a .i./500 µl Glu-Fru solution) was prepared on the day of administration (D4). By serial dilution of the stock solution with the fresh diet we prepared five dilutions with the final contents of oxamyl in the feed rations of: 1.25; 2.5; 5; 10; and 20 µg a. i./larva (a. i.—active ingredient). Dimethoate (organophosphate) at the concentration of 8.8 µg a. i./larva was used as a positive control. For the validity of the test, the following was required: cumulative mortality between days 4 and 7 of larvae on the control plate ≤ 15 %; mortality of larvae on Day 7 in the positive control ≥ 50 % [18].

On the first day of the test (D1), the larvae were transferred with a special grafting tool to the surface of the grafting cell with Diet A (20 µl). On D4, the larvae from the experimental groups were fed diet C with the respective

doses of oxamyl. The control group received a diet with the addition of distilled water while the positive control group received a diet with dimethoate. On D5 and D6, larvae of all groups received diet C. The test was terminated on day 7 (D7). The mortality of the larvae was recorded on D5, D6 and D7. The larvae that did not move nor reacted to the touch were considered dead. On the basis of mortality, the 72h LD50 was calculated using ToxRat software.

The D7-survived larvae were frozen (–50 °C) and later homogenized in 5 mmol·l^{-1} TRIS-HCl buffer solution (pH 7.8) containing 0.15 mol·l^{-1} KCl, 1 mmol·l^{-1} Na2EDTA and 2 mmol·l^{-1} glutathione using a homogeniser Ultra-Turrax T25 (Germany). The homogenates (25 % w/v) were centrifuged at 105 00 g and °C, for 1 hour (Beckman L8-60, USA). They were stored at –50 °C (LTF 325 Arctiko, Denmark) until the enzyme analysis.

The total proteins were determined by the method of Bradford [4]. The determination of the activity of superoxide dismutase (SOD) was based on the measurement of the inhibition rate of cytochrome c reduction at 550 nm. The xantin/xantinoxidase system was responsible for the formation of the superoxide radical in the reaction mixture [8]. The activity of catalase (CAT) was based on the determination of the decrease in hydrogen peroxide in the reaction mixture at 240 nm [22]. The activity of glutathione-S-transferase (GST) was determined by the measurement of the increment conjugate of reduced glutathione at 340 nm with 1-chloro-2,4-dinitrobenzene as a substrate [11]. Thiobarbituric acid reactive substances (TBARS), the products of lipid peroxidation, gave a colour reaction with thiobarbituric acid at the development of compounds absorbing light at 535 nm [10]. The specific activity of enzymes was expressed in U·mg^{-1} protein. The chemicals used were of the highest analytical purity and were purchased from the following companies: Sigma, Merck and Boehringer.

The results of the enzyme analysis are presented as means ± SD (n = 3). The statistical evaluation was carried out by the Student t-test ($P < 0.05$ was considered significant).

RESULTS

The aim of this study was to observe the effect of the carbamate insecticide oxamyl on the antioxidant system of honeybee larvae *in vitro*. The oxamyl doses were selected on the basis of preliminary testing. The LD50 calculated on the basis of larvae mortality was 7.15 µg a. i./larva.

Enzymes are generally considered a sensitive parameter indicating exposure of an organism to xenobiotics. The most important biomarkers are enzymes that participate in the development of oxidative stress or catalyse detoxication processes in the exposed organism.

Superoxide dismutase catalyses dismutation of the superoxide radical to hydrogen peroxide and oxygen. The results of our study showed that the specific activity of SOD in bee larvae from the experimental groups did not change significantly in comparison with the control. A slightly decreased activity of SOD in comparison with the control was recorded at the oxamyl dose reaching 2.5 µg a. i./larva. With the increasing dose of oxamyl, the activity of this enzyme gradually increased. The highest specific activity was determined at the dose of 10 µg a. i./larva. Exposure to the dose of 20 µg a. i./larva caused a decrease in the activity of SOD (Tab. 1). Catalase is the most effective eliminator of hydrogen peroxide. The catalase activities failed to show significant changes (Tab. 1). The changes observed in individual experimental groups copied the changes in the activities of SOD.

Glutathione-S-transferase is a primary detoxifying enzyme which participates in biotransformation of xenobiotics by means of conjugation reactions with glutathione. With an increasing dose of oxamyl, the specific activity of

Table 1. Specific activity of superoxide dismutase and catalase in homogenates of honeybee larvae following single exposure to the insecticide oxamyl

Oxamyl dose [µg a. i./larva]	**SOD** [U·mg^{-1}]	**CAT** [U·mg^{-1}]
0	52 ± 11	475 ± 43
1.25	71 ± 46	439 ± 100
2.5	41 ± 6	537 ± 200
5	55 ± 20	452 ± 85
10	100 ± 40	680 ± 180
20	70 ± 0,0	541 ± 0,0
Dimethoate (positive control)		
8.8	**67 ± 37**	**439 ± 100**

SOD—superoxide dismutase; CAT—catalase; a. i.—active ingredient. The values are presented as the arithmetic means ± SD (n = 3)

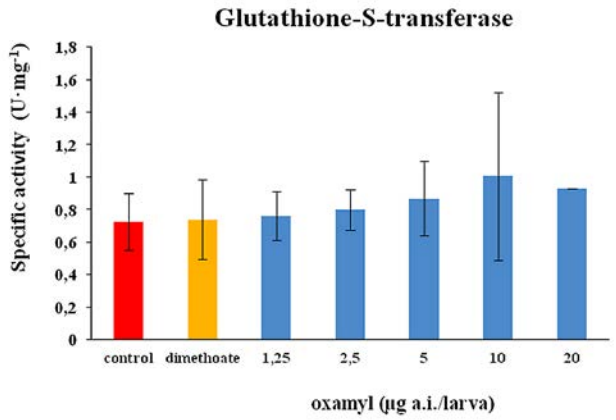

Fig. 1. Specific activity of GST in the homogenates of honeybee larvae following a single exposure to the insecticide oxamyl

GST—glutathione-S-transferase; dimethoate—positive control; a. i.—active ingredient; the values are presented as the arithmetic means ± SD (n = 3)

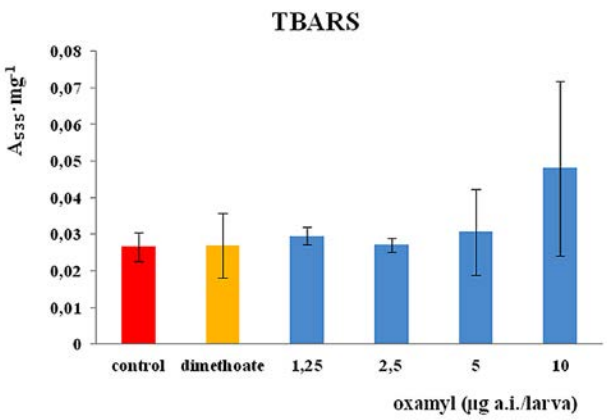

Fig. 2. Content of TBARS in the homogenates of honeybee larvae following a single exposure to the insecticide oxamyl

TBARS—thiobarbituric acid reactive substances; dimethoate—positive control; a.i.—active ingredient. A535.mg^{-1}—absorbance.mg^{-1} protein. The values are presented as the arithmetic means ± SD (n = 3)

GST increased. The highest activity (1.006 U·mg^{-1}) was determined in larvae exposed to the dose of 10 µg a.i/larva (Fig. 1).

The products of lipid peroxidation react with thiobarbituric acid to provide coloured products. Their quantity manifests the degree of damage to the lipids due to undesirable oxidative changes. A marked increase in the content of TBARS was observed in the group exposed to the action of oxamyl at a dose of 10 µg a. i./larva (Fig. 2).

DISCUSSION

Oxamyl acts as a reversible inhibitor of acetylcholine esterase which results in the accumulation of acetylcholine. The primary toxic effect of oxamyl may be supported by the development of reactive oxygen species as was described also in association with the action of other carbamates [23]. The increased production of ROS in insects is associated with the detoxication processes [7]. Antioxidant enzymes play an important role in the regulation of the level of ROS and are produced by cells as a response to stress. The SOD and CAT constitute the primary protection system of an organisms against oxidation stress. GST is an early marker of the induction of the detoxication system and contributes also to the protection of cells against oxidative damage [3]. The superoxide that serves as a substrate of SOD is responsible for the direct damage to macromolecules but also as a source of additional ROS. The role of SOD is to keep the level of superoxide in the cell low. Inhibition of its activity may result in increased production of ROS depending on the degree of oxidation stress. A slight decrease in the activity of SOD was recorded at the dose of oxamyl reaching 2.5 µg a. i./larva. However, in general the changes in the activity of SOD in homogenates of honeybee larvae showed no significant changes (Tab. 1). The presence of CAT was confirmed in the honey. Its probable role consists in the maintenance of H2O2 in honey under the toxic level. High activity of CAT was observed also in bees and can be affected by the presence of environmental contaminants [5]. The slight increase in the activity of CAT observed in our experiment (Tab. 1) indicated that the larvae were exposed to higher concentration of hydrogen peroxide originating from the diet or produced following the exposure to xenobiotics, i. e. oxamyl.

The important function of GST involves the detoxification of insecticides and secondary metabolites and protection against oxidative stress [3]. Some GST isoenzymes exhibit peroxidase activity [6]. This activity is especially important for invertebrates because they lack selenium-dependent glutathione peroxidase [25]. The GST of the delta class participate in the resistance of insects to insecticides.

The mechanism of this resistance at the metabolic level involves the induction of detoxification enzymes capable of transforming xenobiotics to less toxic and more soluble compounds that can then be eliminated [14]. Our study showed a gradual increase in the activity of GST (from 0.739 to 1.006 $U \cdot mg^{-1}$) with increasing doses of oxamyl in comparison with the control (0.724 $U \cdot mg^{-1}$). An increased specific activity of GST was observed in honeybees exposed to permetrin, deltametrin and flumetrin [20]. The GST of the class sigma showed high affinity to products of lipid peroxidation. Because they are located in the metabolically active tissues of insects, one may assume that these enzymes play an important role in the protection of insects against oxidative stress [7].

In our study, the activity of enzymes was determined on day D7, 72 hours following the single exposure to oxamyl in homogenates of the larvae *in vitro*. The characteristic feature of this larval stage is the highest content of lipids [13]. Lipids are considered as molecules most susceptible to oxidative damage. An increased level of TBARS was determined already at the dose of oxamyl reaching 10 μg a. i./larva. Our results allowed us to conclude that the toxic action of oxamyl involves not only the inhibition of acetylcholine esterase but also the production of ROS. However, the doses of oxamyl caused no damage to the larval tissues. The adverse effects of pesticides on *in vitro* larvae was frequently manifested by a decreased survival rate and weight loss [2]. Y a n g et al. [26] observed that sublethal doses of imidacloprid had no effect on the larvae but interfered with memory and the ability to learn in adult honeybees developed from these larvae.

CONCLUSIONS

The first 6 days of development of worker bee larvae are of key importance because of their exponential growth during this stage. Exposure to residues of pesticides in the diet and wax can have undesirable effects. The determination of enzyme activities carried out in our study allowed us to conclude that the antioxidant enzyme system of larvae reacted to the doses of oxamyl in their diet. Already moderate induction of these enzymes ensured the protection of the larva against the harmful action of ROS. Only the highest tested dose of oxamyl induced an increased content of products of lipid peroxidation which can indicate potential oxidative damage to larval tissues. As the success of the bee colony depends on the health of developing larvae, the potential adverse effects of pesticides on larval stages should become a part of any overall analysis. The results of our study are a small contribution to this effort.

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HISTOLOGICAL SCORING SYSTEMS IN THE CARTILAGE REPAIR OF SHEEP

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ABSTRACT

Researchers around the world use histological analysis to provide the most detailed morphological information of articular cartilage repair and it predominantly relies on the use of histological scoring systems which are important tools for valid evaluations. Due to hyaline cartilage complex structure and avascular nature, damaged cartilage does not heal spontaneously and it is still a challenge to regenerate and restore its tissue function. The aim of this study was to investigate the quality of regenerated cartilage by using three different histological scoring systems; O'Driscoll, Pineda and Wakitani which are all classic scores described for such animal studies. We used an *in vivo* ovine model in which a full thickness chondral defect was created and then implanted with the biomaterial (polyhydroxybutyrate/chitosan; PHB/CHIT). The results of this histological analysis demonstrated that the cartilage repaired tissues received scores indicating that the majority of the regenerated tissue resembled hyaline-like cartilage. After six months of repair the regenerated cartilage showed characteristics like good surface continuity, uniformed stained extracellular matrix, clearly visible zones and cellular proliferation. In conclusion, this study may be used to investigate and improve the regenerative capacity of hyaline cartilage in preclinical models and it also sheds further light on both the evaluation and methods used for the regeneration of damaged cartilage.

Key words: animal model; articular cartilage; cartilage repair; histology; regeneration; scoring system

INTRODUCTION

Cartilage is a flexible, sustainable form of tissue composed of cells called chondrocytes and a highly specialized extracellular matrix. The extracellular matrix has unique biomechanical properties and is composed of ground substance and fibers. The matrix contains high concentrations of glycosaminoglycans (GAG) and proteoglycans (PG) and these can interact with elastic and collagen fibres. The composition of the matrix components and cells can create variations and produce three different kinds of cartilage adapted to local biochemical needs [10]. Hyaline cartilage can be commonly found in the ribs, nose, larynx, and trachea. Hyaline cartilage on the articular surfaces of bones (the surfaces in joints) is called articular cartilage (AC). Healthy AC provides a smooth, cushioning and low friction surface for joints [20], and is composed of four layers based on the structure of the extracellular matrix and cell morphology: a) superficial zone, b) intermediate zone, c) radial zone, and d) calcified cartilage zone [14]. It is known that AC can tolerate considerable intense and repetitive stress but it has a limited ability of repair. This inability to heal is attributable to a few factors, such as the chondrocytes immobility, limited ability of mature chondrocytes to proliferate and the avascularity of cartilage. Degradation of AC result in osteoarthritis, even minor injuries of AC might lead to progressive damage and osteoarthritic joint degeneration. It is one of the global leading causes of significant pain, disability and immobility [12, 13]. Globally, osteoarthritis affects around 3.3 % to 3.6 % of the world population. It is the 11th most debilitating disease around the world since it causes moderate to severe disability in 43 million people [3, 4]. In attempts to regenerate or repair AC many methods has been developed by researchers and surgeons whose goal is to create and restore the function of hyaline cartilage.

Nowadays tissue engineering is able to create live tissue and is used to replace or improve biological function; it can be done by the usage of combinations of cells, biomaterials, biochemical and physio-chemical factors. The methods used for cartilage repair and regeneration do not perfectly restore cartilage but there are very promising results seen in repairing cartilage from traumatic injuries or cartilaginous diseases.

At this time, there are several different clinical methods available for cartilage repair which includes: autologous chondrocyte implantation (ACI), bone marrow stimulation, osteoarticular transfer system or mosaicplasty, osteochondral plug transplantation, penetration of the subchondral bone, matrix-induced autologous chondrocyte implantation, microfracture and most recently the next generation of ACI which involve scaffolds or cell-seeded scaffolds. There has recently been a significant expansion in biomaterial technologies, cell sources, scaffolds, molecular and genetic manipulations for the creation of functional tissue replacements to treat damaged cartilage [13]. It has been shown that implantation of acellular biomaterials significantly enhances the regeneration of cartilage compared to natural healing. Some advantages that acellular biomaterials offer includes: no costs for cell cultures, lack of donor-site morbidity, not as many regulatory issues, and one-stage surgical procedures.

Natural biomaterials used *in vivo* includes chitosan, collagen, hyaluronic acid and alginate. Also, synthetic biomaterials exists such as polyvinyl alcohol, poly(lactic-co-glycolic acid) and polycaprolactone. Multi-layered biomaterials are made to combine the beneficial properties of these materials, for example β-tricalcium phosphate-hydroxyapatite/hyaluronateatelocollagen and type 1 collagen-hyaluronic acid-fibrinogen hydrogel. These biologics are natural factors which induce proliferation and differentiation of cells [19]. The goal of cartilage repair techniques is to restore the function to the injured tissue as well as reduce pain.

To evaluate the success and outcome of cartilage regeneration one of the most important measures is the histological quality of the cartilaginous tissue. Nowadays a variety of histological scoring systems are used to evaluate and describe the quality of *in vivo* repaired tissues. Histological analysis is an excellent tool for a valid and objective evaluation of repair tissue dependent on the use of established scoring systems such as: O'Driscoll, Pineda and Wakitani. These systems are among the most commonly used for experimental cartilage repair and histological analysis is considered the gold standard method for evaluation of cartilaginous repair. The O'Driscoll score was the first histological grading system for articular cartilage defects and it was used to assess the effects of periosteal grafts in rabbits in the treatment of full thickness chondral defects [16]. This scoring system includes parameters such as: cellular morphology, safranin-O staining of the matrix, surface regularity, structural integrity, thickness, bonding to the

adjacent cartilage, freedom from cellular changes of degeneration (hypocellularity), chondrocyte clustering, and freedom from degenerative changes in adjacent cartilage [17]. When using the O'Driscoll score it is one of a few systems that allows for the assessment of the integration of the repaired tissue with its surroundings. Nevertheless, these extra added points do not significantly affect the final score and the scoring system is often used in modified versions [21]. The score value in this complex system ranges from 0-24 points where 0 is no signs of cartilage repair and 24 is complete regeneration, the maximum points value for one parameter range between 2—4 [17].

Stephen Pineda and co-workers introduced a semi-quantitative scale for histologic grading, composed of four parameters in order to evaluate the natural healing process of articular cartilage repair. A rabbit model was used by drilling defects into the articular cartilage of the knee and the samples stained with Safranin-O staining [18]. The simple Pineda scoring system scale contains four parameters; filling of the defect in percentage, reconstitution of the osteochondral junction, staining of the matrix, and cell morphology. The score value in the Pineda system ranges from 0—14 points, where 0 is complete regeneration and 14 is no repair, the maximum point values for one parameter range between 2—4. It means that the system is the inverse in which a low score value represents a good repair response [17].

The Wakitani system is an elementary scoring system developed by Shigeyuki Wakitani and co-workers in 1994. The system was used for histological evaluation of toluidine stained samples from a study where the authors investigated the effect of periosteum or bone marrow derived mesenchymal cells for the repair of large, full-thickness defects of articular cartilage. Rabbits were used as the animal models in which a chondral defect was created in the medial femoral condyle [24]. The Wakitani scoring system scale contains five parameters: cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration of donor with host cartilage. The score values range from 0—14 points where 0 is complete regeneration and 14 is no repair, the maximum point values for one parameter range between 2—4, similar to the Pineda score [17].

The aim of this study was to evaluate cartilage repair after the creation of a full thickness defect and implantation of biomaterial in the sheep stifle joint by using different established histological scoring systems (O´Driscoll, Pineda, Wakitani). These scoring systems histologically investigate the regenerated articular cartilage after implantation with a polyhydroxybutyrate/chitosan (PHB/CHIT) scaffold.

MATERIALS AND METHODS

Preparation of polyhydroxybutyrate/chitosan implants

The scaffold polyhydroxybutyrate/chitosan (PHB/CHIT) composite was prepared according to [9]. PHB (GoodFellow), dissolved in propylene carbonate were mutually mixed with chitosan (Sigma Aldrich) dissolved in 1 % acetic acid at a ratio equal to 1 : 1. For precipitation the same volumes of differently concentrated biopolymer solutions were used. The mixing was accomplished by using a magnetic stirrer at 400 rpm for 10 minutes, thereafter acetone was added to the suspension for the complete precipitation of the biopolymers. The final product was filtered and washed with distilled water. Subsequently it was moulded into cylindrical forms that were 10 mm in diameter and 10 mm in height and lyophilized (Ilshin) for 6 hours. Sterilization of the implants were done at 121 °C in an autoclave. Observation of the polyhydroxybutyrate/chitosan mixture appearance demonstrated a macroporous microstructure of the spongy-like biopolymer composite implanted with high fractions of irregularly shaped macropores with sizes up to 100 μm and micropores < 20 μm (Fig. 1) [23].

Fig. 1. Polyhydroxybutyrate/chitosan biomaterial

Sheep cartilage sample collection

Animals

Five clinically healthy ewes of the crossbreed Merino and Valachian were used. The age of the sheep ranged from 1.5—2 years with an average body weight of 50.7 kg ± 1.9 kg. They were housed at the Clinic for Ruminants at the University of Veterinary Medicine and Pharmacy in Kosice where they were kept in free stalls. Water was freely available and they were fed hay and concentrates. The animals got 14 days to acclimatize to their new environment before the scheduled surgery. They also underwent a standard preoperative clinical examination before being included in the study. The health status of the sheep were evaluated daily after the surgical intervention and the animals were sacrificed six months after the surgical procedure. The experimental animals from the farm PD Agro Michalovce (Slovak Republic) and the experimental protocol were approved and obtained from the State Veterinary and Food Administration of the Slovak Republic No. 3508/17-221.

Anaesthesia and postoperative care

Prior to the surgery, the sheep had been fasted, e. g. food and water were withheld for 12 hours. The sheep were premedicated with buthorphanol (0.1 mg.kg^{-1} IM, Butomidor, Richter Pharma, Wels, Austria) and medetomidine 0.02 mg.kg^{-1} IM (Cepetor, CP-Pharma Handelsgesellschaft, GmbH, Burgdorf, Germany) for induction and for maintenance of anaesthesia ketamine 8 mg.kg^{-1} IV (Ketamidor, Richter Pharma, Wels, Austria) was used. Post surgically all sheep were given NSAID flunixin meglumine (2.2 mg.kg^{-1} IM, Flunixin a. u. v., Norbrook, Newry, UK) 1× daily for 7 days and broad spectrum antibiotic oxytetracyclinum dihydricum (20 mg.kg^{-1} IM, Alamycin LA a. u. v., Norbrook, Newry, UK) 1× every second day.

Surgical process

To induce the traumatic defect in the articular cartilage of the left stifle joint, a skin incision was placed over the left lateral side from the medial patellar ligament distal to the tibial tuberosity. After incising subcutaneous tissue and the superficial fascia, the medial femoral condyle was exposed. The joint was flexed and partially luxated to allow for the creation of a full thickness defect in the articular cartilage by using a drill machine. The full thickness defect was created at an exact location in the distal epiphysis of the femur (trochlea femoris sinister) with a diameter of 6 mm and a depth of 2 mm. At the site of the defect, drilling Kirschner wires were used to reach the space of the bone marrow area and after that, the defect was filled with a biopolymer implant (Fig. 2). The sheep were sacrificed 6 months after implantation and the cartilage samples were obtained from biopsies (diameter 8 mm and a depth of 10 mm) harvested from the healing defect by using an Osteochondral Autograft Transfer System (OATS, Arthrex, USA). Also, biopsy removal was performed in the same way to get control samples from the original healthy cartilage but taken from the opposite side of the trochlea.

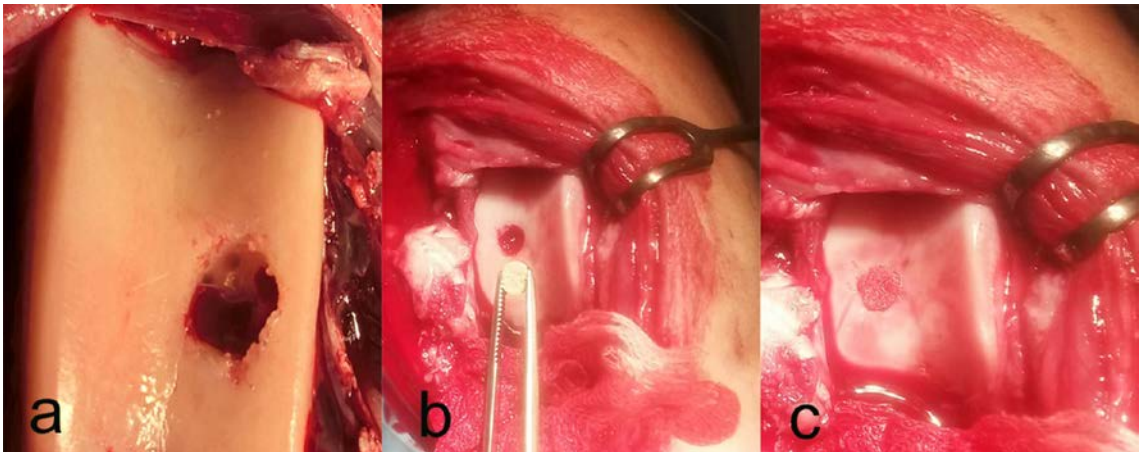


Fig. 2. Surgical procedure

a) Inducing of articular full thickness cartilage defect; (b) Inserting of scaffold into prepared articular defect; (c) Articular defect with implanted scaffold

Histological preparation

Hematoxylin-Eosin staining

Immediately after the animals were sacrificed the cartilage samples were dissected. Straightaway after harvesting the samples they were fixed in 10 % neutral formalin and decalcified with chelaton; after that dehydration was performed in a 70—100 % ethanol series and the next step was paraffin embedding. When embedded in paraffin, the cartilage samples were cut into 7 μm thick pieces. These sections were stained for routine histological study with haematoxylin and eosin (H&E) for the histological evaluation under the light microscope (Olympus CX23). By using this staining technique, it is possible to evaluate the overall structure including the tissue repair, cell morphology, bone cartilage interference, abnormal calcification and tidemark [5].

Alcian blue staining

The deparaffinized and hydrated sections were submerged in 3 % acetic acid solution for 3 minutes and then washed in tap water. Sections were stained with Alcian blue solution (Sigma Aldrich, USA, 1 % solution in 3 % of acetic acid, pH 2.5) and counterstained with Nuclear Fast Red solution (Merck Millipore, USA) for staining of the chondrocyte's nucleus. Afterwards the sections were washed in tap water, dehydrated, cleared and mounted in medium (Entellan, Merck Millipore, USA). This staining was used for the evaluation of the glycosaminoglycans in the matrix of the cartilage, it also displayed the cellular morphology and collagen deposition.

Evaluation of cartilage samples

The histological grading and observations were carried out on 50 articular cartilage samples, five sheep from which five slides with two sections on each slide were available for the study, meaning that 10 samples from each animal were used. These samples were acquired from a cartilage repair investigation and taken from regions across the trochlea in which full thickness chondral defects were created using an *in vivo* ovine model. These were then given scores, e. g. graded with the Pineda, O'Driscoll and Wakitani systems [16, 18, 24]. The Pineda system on a scale from 0 (complete regeneration) to 14 (no repair), the O'Driscoll system on a scale from 0 (no signs of cartilage repair) to 24 (complete regeneration), and the Wakitani system on a scale from 0 (complete regeneration) to 14 (no repair). These evaluations were done on all cartilage samples from the trochlea under light-microscopy and the data were obtained, the samples were scored with individual scores from each system according the three different scoring systems. Before starting with the evaluation of the experimental cartilage samples it was important to become familiar by means of self-education by studying the appearance of physiological articular cartilage, fibrocartilage and degenerated cartilage. The samples were not presented to the observer in a blinded order.

Statistical analysis of the data were performed using the programme GraphPad Prism 6.0. One-way and two-way ANOVA tests were used for analysis. Values of $P < 0.05$ were deemed statistically significant.

RESULTS

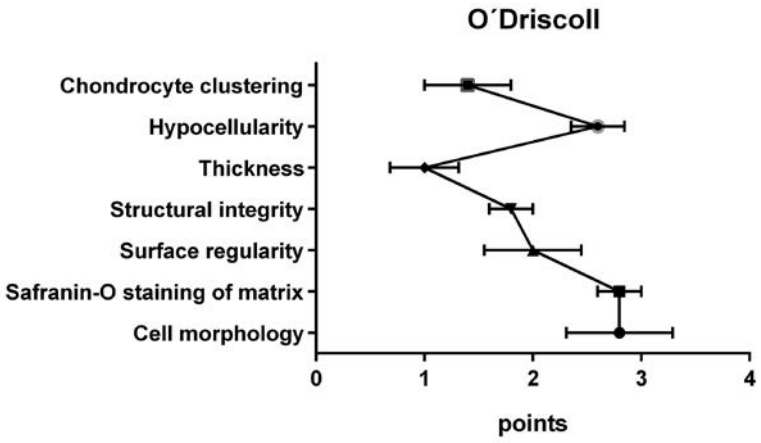
Histological scoring of cartilage repair

The examination of the histological sections from the trochlea with its full thickness chondral defects showed that articular cartilage regeneration was observed in some animals. The experimental group displayed evidence of newly formed repaired tissue with clearly visible zones in the defect site and hyaline-like cartilage formation demonstrated by Alcian blue and H&E staining. These histological observations of the repaired tissues were also reflected

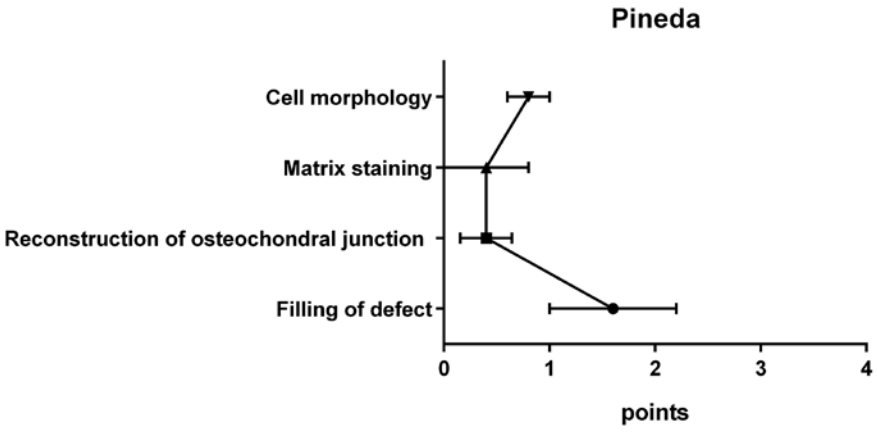
Table 1. Results of the histologic evaluation of cartilage repaired tissue for the complex O'Driscoll together with simple Pineda and Wakitani semi-quantitative scoring systems

Score	Sheep	Mean	Range	Median total score
Pineda	1	2.2	2—3	3.06
	2	3.8	2—4	
	3	5.8	5—7	
	4	2.3	1—3	
	5	1.2	1—2	
Wakitani	1	3.4	2—4	3.36
	2	2.8	2—4	
	3	5.9	5—7	
	4	2.5	1—3	
	5	2.2	2—3	
O'Driscoll	1	14.3	14—16	14.28
	2	15.0	15	
	3	9.3	7—11	
	4	14.8	14—16	
	5	18.0	18	

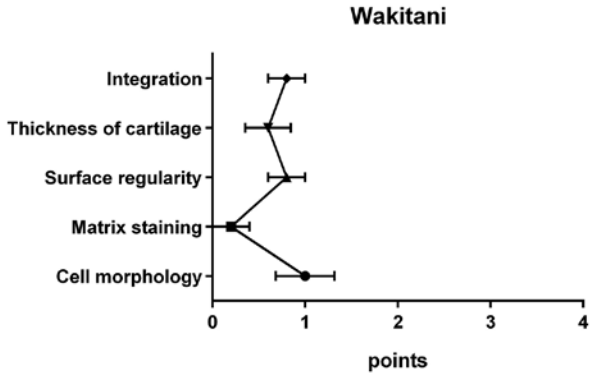
Results are expressed as the mean value for each sheep



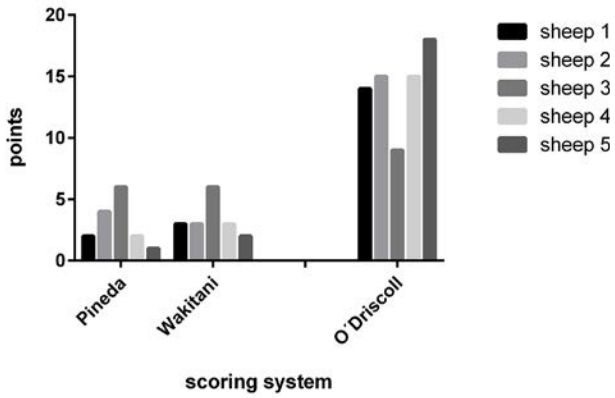
Graph 1. The mean score for each evaluated parameter scored with O'Driscoll



Graph 2. The mean score for each evaluated parameter scored with Pineda



Graph 3. The mean score for each evaluated parameter scored with



Graph 4. Results of the evaluation according the different histological scoring systems

in terms of the histological scoring. The trochlear sections that were graded with the Pineda and Wakitani scoring systems showed low scores, however the result from the O'Driscoll system revealed a higher score (Tab. 1; Graph 4). Since the samples only consisted of the defect and not adjacent cartilage, a modified O'Driscoll score was used and the parameters, bonding to the adjacent cartilage and freedom from degenerative changes in adjacent cartilage was not used in this evaluation. The trochlear defect graded with a modified O'Driscoll system on a scale from 0 (no signs of cartilage repair) to 19 points (complete regeneration) got a total mean score of 14.28 points and suggested a satisfactory result with hyaline-like tissue (Graph 1).

When the trochlear defect was graded with the Pineda system on a scale from 0 (complete regeneration) to 14 points (no repair) got a total mean score of 3.06 points, this low value represented a good repaired response. When scoring with the Wakitani system the parameter, integration of donor with host adjacent cartilage was evaluated on macroslides. Grading with the Wakitani system showed a total mean score of 3.36 points; the system was similar to the Pineda and graded on a scale from 0 (complete regeneration) to 14 points (no repair). The result of 3.36 points correlated with the Pineda score results and reflected a good repair (Graphs 2 and 3).

Assessing the data obtained by scoring with the different systems, the individual score per sheep varies when using the same system. Once the five sheep were scored with the Pineda system, the individual scores for the sheep ranged between 1.2—5.8 points. The scores for the sheep when graded with Wakitani system ranged between 2.2—5.9 points and for the O'Driscoll system, the scores ranged between 9.3—18.0 points (Tab. 1).

In the control samples harvested from healthy articular cartilage all zones were clearly visible. The superficial or tangential zone had the lowest content of PGs, since the chondrocytes synthesize high concentrations of collagen and only low concentrations of proteoglycan, thus making it the zone with the highest water content and the Alcian blue was less intense in these area. The superficial zone of the trochlea showed chondrocytes lying parallel to the joint surface with an oval shape instead of the flat elongated appearance that were usually seen. In the transitional, intermediate or middle layers round and slightly larger chondrocytes surrounded by extracellular matrix were seen. The collagen fibres were thick and arranged randomly, the layers were also more intensively stained with Alcian blue which indicated a higher proteoglycan content. It is viewable that the cell density in this zone was lower and they were embedded in an abundant extracellular matrix. The cell density in the transitional zone was lower which indicated mature cartilage with predominantly spheroid-shaped cells embedded in an abundant extracellular matrix. The deep or radial zone consisted of large chondrocytes most of them were grouped in radial columns and the arrangement was perpendicular to the surface. Noticeable was that this zone contained the highest content of proteoglycans and had the lowest water content. The layer of calcified cartilage was a calcified zone that should have been clearly separated from the zone by a tidemark; in this case the tidemark was not visible. The collagen fibrils were arranged perpendicular to the articular surface and they penetrated from the deep zone through the calcified cartilage into the subchondral bone. As can be seen, the mineralized zone only contained a small volume of cells embedded in the calcified matrix; this part showed a very low metabolic activity (Fig. 3).

Microscopic evaluation of the repaired tissue within the defect site of the trochlea

The histological findings of the regenerated cartilage following the implantation of PHB/CHIT in the experimental group showed an overall good result of the newly formed repaired tissue. After six months of repair some of the sections from trochlear repaired tissue showed that the defect was repaired completely by hyaline cartilage. In these regions the microscopic appearance of the cartilage was normal cartilage, e.g. hyaline cartilage in which all the zones could be clearly identified. The regenerated cartilage displayed a good surface continuity showing a smooth intact articular surface. In some regions though, the surface was uneven and several layers even separated from the cartilage surface. Probably these layers represented parts of the synovial membrane. At higher magnifications, the chondrocytes in the superficial zone had a flat and elongated appearance and could be seen parallel to the surface. The result was intact, homogenous cartilage which exhibited a uniform stained extracellular matrix. There were no signs of hypocellularity and the cartilage were arranged into all the zones that was clearly visible and unaffected. In the deep zone the chondrocytes were arranged in vertical columns separated by a thin layer of extracellular matrix (Fig. 4).

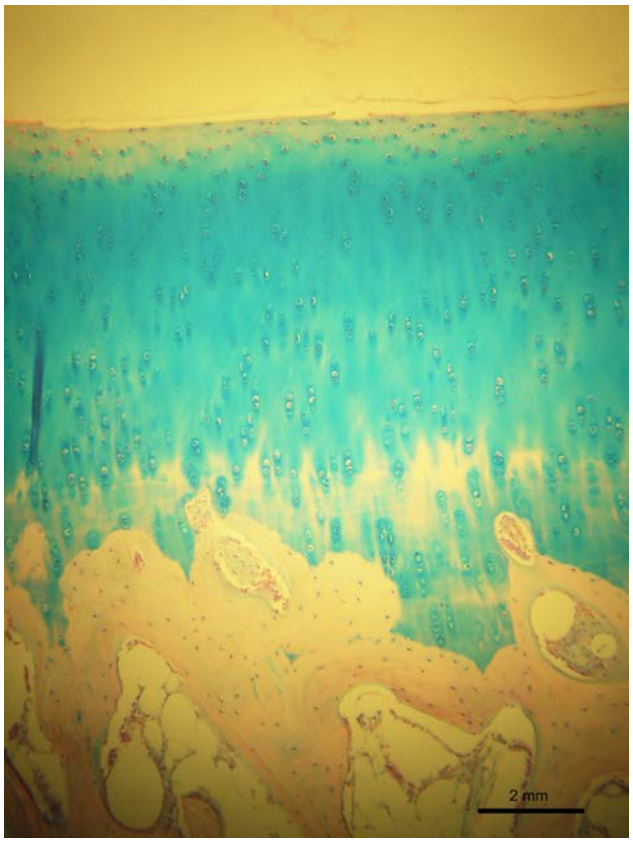


Fig. 3. Histological section of articular cartilage, control group, stained with Alcian blue to demonstrate GAGs content

In another sample from the trochlea, the zonal arrangement could not be clearly observed, the surface showed good continuity but in the superficial layer the cells was small, elongated and the appearance of chondrocytes were lost. In the transitional layer cell proliferation was present; it was composed of large round chondrocytes that could be seen either singly or in pairs. In the deep part of the regenerated tissue the cells were more disorganised and they were not grouped into discrete columns (Fig. 5).

In one of the five animals, the trochlear sections showed that the cells were more disorganised and the zones could not be clearly observed (Graph 4). There were also clustering cells in the cartilage and the chondrocytes were not arranged into four zones; instead they formed these clusters that could be seen throughout the cartilage. Surface fibrillations were observed; this superficial fibrillation of the articular cartilage resulted in the loss of smoothness and it also showed signs of degeneration. Fissures that extended into both the superficial and transitional zones of the cartilage were observed. The affected cartilage demonstrated loss of Alcian blue staining reaching from the superficial zone into the transitional zone in which it was more manifested due to the proteoglycan loss. Changes could be observed in the superficial zone either as oblique splits or horizontal separation of the collagen bundles. This repaired tissue was of lower quality and differentiated from hyaline cartilage by its coarser fibrillary nature (Fig. 6).

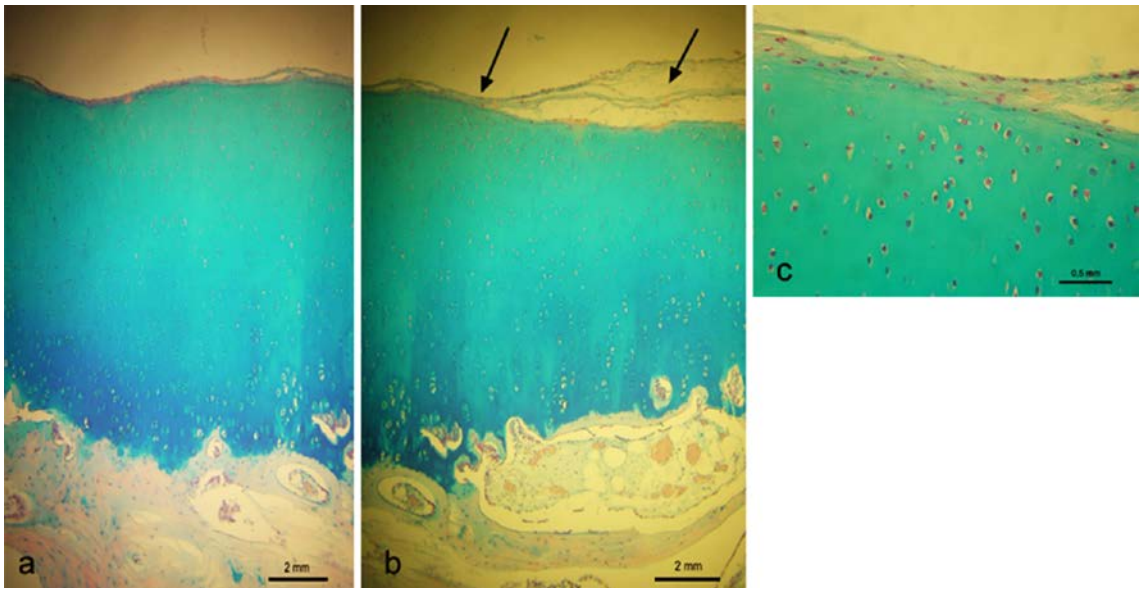


Fig. 4. Microphotograph of articular cartilage, experimental group

a) Microscopic appearance of the cartilage was normal and all zones clearly visible; b) + c) in some regions the surface was uneven and several layers were separated from the cartilage surface (arrows)

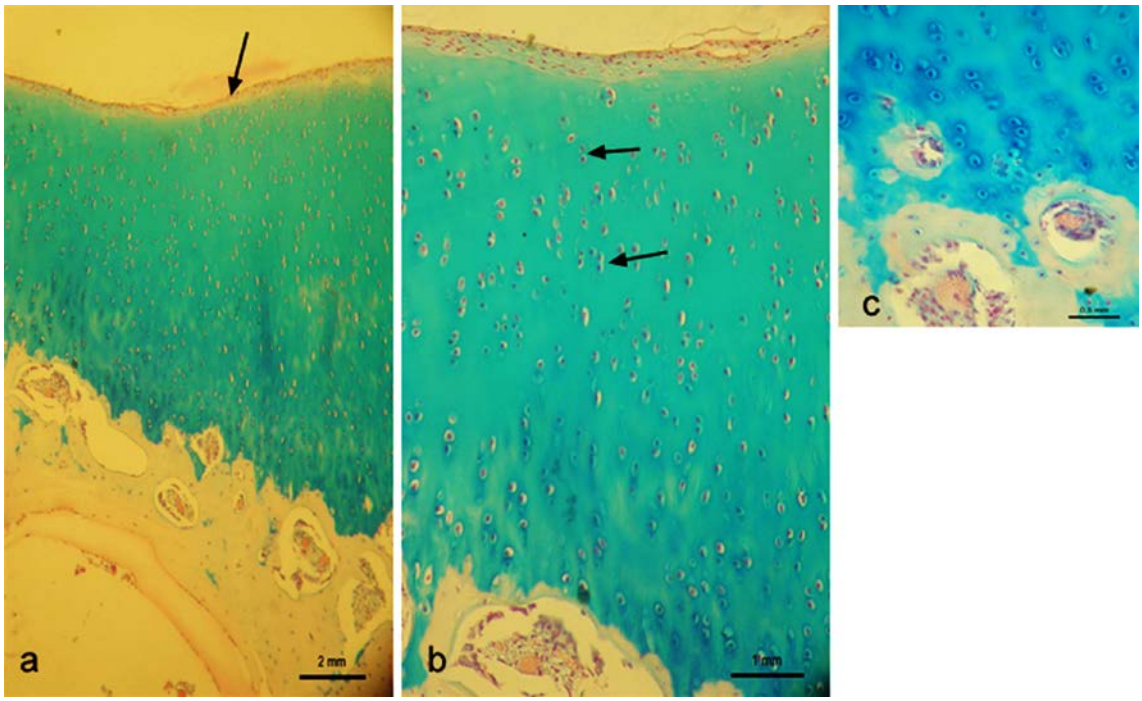


Fig. 5. Microphotograph of articular cartilage, experimental group

a) The defect was repaired with hyaline cartilage, which showed good surface continuity (arrow); b) cell proliferation was present (arrows), but zones were not clearly visible; c) chondrocytes were not grouped into a columnar organization

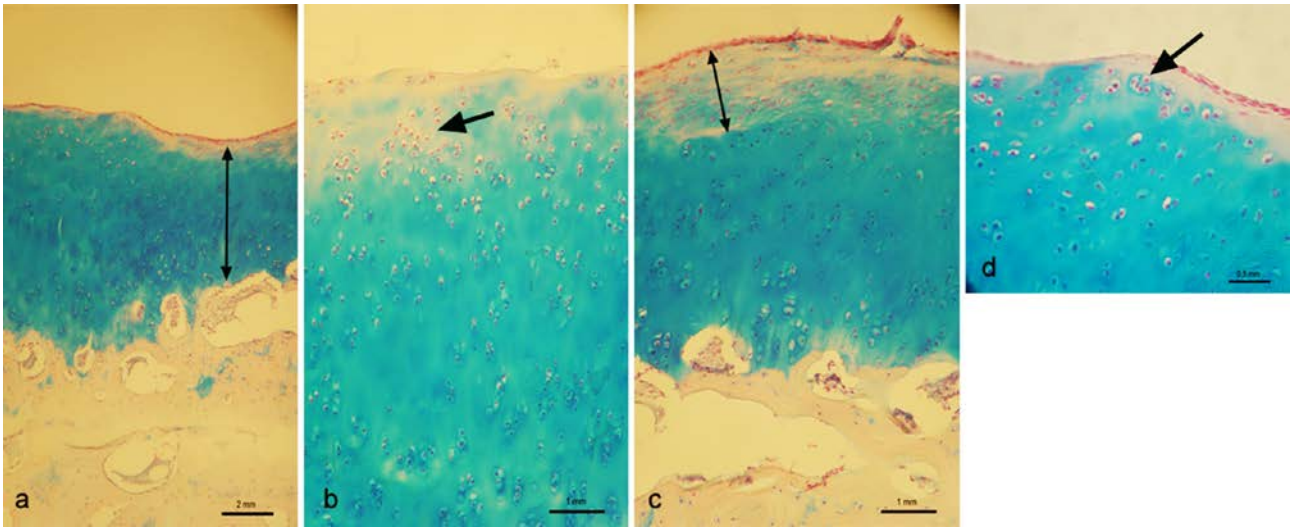


Fig. 6. Microphotograph of articular cartilage, experimental group

a) the defect was repaired incomplete, under the level of adjacent cartilage, in this zone cells were more disorganised; b) in some regions the cartilage showed decreased and uneven staining of proteoglycans (arrow); c) surface fibrillation; d) arrangement of chondrocytes in small clusters (arrow)

DISCUSSION

Histological analysis significantly contributes to both the assessment of cartilage damaged and the success of cartilage regeneration when evaluating cartilage repaired tissues and its quality. R u t g e r s et al. reported that using more than one scoring system will give more information about the histological characteristics of the regenerated tissue [21].

A correlation can be seen between each three of these systems (O'Driscoll, Pineda, Wakitani) when it comes to

the cartilaginous repaired tissue and it suggests an overall good morphology for the regenerated cartilage. It was concluded by Moojen et al. that both O'Driscoll and Pineda scores were reliable semi-quantitative cartilage scoring systems with good correlation [11]. The values from all of the three scoring systems showed similar results even though the parameters and values were different; attention-getting differences between these three scores were the number of single criteria included. Pineda and Wakitani scores were classified as elementary systems since these systems only consists of 4 or 5 single criteria for evaluation, while the O'Driscoll score belonged to a complex system with 9 single criteria for evaluation. Orth and Dadry showed in a study which included the same three systems used in our work, that no significant disparity in reproducibility and reliability could be detected between the complex and elementary scores. Due to this study the authors suggested that these histological scoring systems represent a relatively potent tool for the evaluation of articular cartilage repair. However, it is important to note that the complex scoring systems will provide more descriptive information when it comes to the nature or character of the regenerated tissue, particularly about cellular and structural properties or the presence of degenerative changes [17]. Meaning that, for example O'Driscoll and Pineda, are validated scores which may increase the comparability of information and therefore stimulate consistency between the two studies.

During the histological analysis, there were a few characteristics of this repaired tissue noted, such as cluster formation, loss of matrix staining and some zones were not clearly visible; therefore it resulted in a lower score in comparison to the others.

Cartilage of lower quality, e.g. fibrocartilaginous repaired tissue may degenerate after a few years and does not tolerate mechanical loads over time, and it is connected with the risk of developing secondary osteoarthritis [2]. It is also important to note that hyaline cartilage repaired may still fail if mechanical irritation persists due to malalignment, poor defect fill or if the surface is disintegrated. Many characteristics are just as vital as the extracellular matrix composition when determining the quality of the repaired tissue. Examples of important characteristics are: cell organisations, viable cells with zonal specific arrangements, density of cells, surface integrity, cartilage-bone integration, and the orientation and organisation of the collagen fibrils [15].

The evaluation of the histologically stained sections provided a method to determine how closely the morphology and organisation of t″4he repaired cartilaginous tissue resembles normal physiological articular cartilage and it is considered the gold standard for this purpose. For preclinical and clinical repair of cartilage, histological scoring provides a crucial outcome measure [5, 17].

Animal models in cartilage repair studies have been of a major importance for the development of effective treatments of cartilage injuries. For the introduction of new treatments and the use of them in clinical practice, *in vivo* animal studies are necessary for closing the gap between *in vitro* experiments and human clinical studies [6]. In the literature, it can be found that numerous large animal models may be used in cartilage repair studies. The ovine stifle joint defect model is widely accepted as an adequate model and are commonly used for the assessment of the efficacy of strategies for cartilage regeneration because of the anatomical similarity between their stifle joint and the human knee joint. Another advantage is the possibility to use arthroscopy for a "second look" which is considered beneficial. There are also limitations with the ovine model; the thickness of the articular cartilage in sheep is variable and can range between 0.4–1.6 mm [22]. This could lead to variable results within a study since this variability makes the volume of the defect created in the cartilage and subchondral bone likely to be different between individual animals. In other studies, the volume of cartilage and subchondral bone involvement has varied significantly and therefore the standard deviation between published studies are prominent. Smaller animals such as rodents or rabbits are generally accepted and used as a small animal model in initial lines of investigation. Nevertheless, in the final preclinical evaluation of a reconstruction technique for articular cartilage, often it requires a confirmation in large animal models [12]. At the moment, no perfect preclinical animal model exists but the authors concluded that the ovine is a readily accessible model for cartilage repair studies despite the previous mentioned limitations [1].

The terminal goal of all cartilage repair methods is to regenerate a repaired tissue with corresponding structure, biochemical and biomechanical characteristics of normal physiological hyaline cartilage. Studies have indicated that scientific advances in the area of tissue engineering have made important steps towards the development of normal articular cartilage repair. Especially, research has demon-

strated the potentiality of biomaterials physico-chemical character significantly influencing the differentiation, proliferation and matrix deposition by progenitor cells [8].

By filling an osteochondral defect with biomaterial, it may act as a template for the regeneration of new hyaline cartilage tissue. The idea is to use marrow stimulating technique such as subchondral drilling and implant biomaterial to guide and stimulate regeneration of the articular cartilage. In a review, P o t et al. concluded that on the whole, clinical studies showed improved cartilage regeneration when using biomaterials for implantation after bone marrow stimulation in animal models, but the authors also concluded that there is still room for enhancement regarding the clinical outcome and tissue quality [19]. Their conclusion is in line with this study in which we observed overall satisfactory results but also less satisfactory results in some aspects. Ideally, biomaterial should achieve regeneration by stimulating the recruitment of cells from the bone marrow and provide the biochemical and physical guidance that direct the cells to regenerate and built up the zones of articular cartilage to exhibit normal morphology [7].

A weakness of this study was the small number of animals making the number of samples available for scoring and evaluation limited. Also, not all parameters in the O'Driscoll score were used for grading in this study. The parameters bonding to the adjacent cartilage and freedom from degenerative changes in adjacent cartilage was not used in this evaluation since the samples only consisted of the defect. This might lead to an overestimation of the quality of the repaired tissue. To reach high objectivity in a study using histological scoring systems H o e m a n n et al. stated that two to three independent blinded readers is advisable to perform the evaluations [5]. To translate results from animal studies to human studies are always delicate matters; the cartilage in sheep is thinner compared to human cartilage and the created defects are only possible to make in the lower range of what is judged clinically significant for humans. For research purposes and further assessment of cartilage repaired tissues alternatives to histological scoring and analysis should be considered to get more information about the cartilage quality. Noticeable is also that translational values of animal models to clinical trials will depend on the comparability to the clinical situation, which in this case was the short follow up time. Many studies (also this one) investigated cartilage repaired tissues up to six months and this is a limiting factor for the translational value since clinical improvements in humans often observed up to one and a half year after surgery [19].

Histological analysis of repaired tissues from large animal models can also be supplemented with analyses of the regenerated tissue biochemistry [5].

The overall results presented by histological scoring indicated that hyaline-like tissue was formed in most of the sheep. In one sheep out of five less satisfactory results were observed. The results suggest that a total score can indicate a level of regeneration and quality of the repaired tissues. Histological analysis remains the best method for scoring and describing the properties of the structure and morphological health of hyaline cartilage. Both complex scoring systems such as O'Driscoll and the elementary inverse systems of Pineda and Wakitani are well suited to describe the complex structures of animal cartilage repair.

CONCLUSIONS

In conclusion, the results of this animal study suggests that the scoring and histological findings demonstrated an overall successful *in vivo* repair and regeneration of articular cartilage. The discovered results provide further knowledge that could help improve the field of cartilage tissue engineering and regeneration associated with cartilage damage in an ovine animal model.

ACKNOWLEDGEMENT

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RADIOCAESIUM IN HONEY FROM KOŠICE AND PREŠOV REGIONS IN EASTERN SLOVAKIA

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ABSTRACT

The activity of radiocaesium was measured in 50 samples of blossom and honeydew honey obtained in the years 2017 and 2018 from beekeepers from various localities of Košice and Prešov regions, eastern Slovakia. The measurements were carried out by gamma spectrometry in Marinelli-type vessels of volume 450 ml without any previous processing of samples. The activity of radiocaesium in honey samples were low, below the detection level of 0.2 Bq.kg^{-1} in the majority of samples, and was near the levels measured before the Chernobyl disaster. Higher values were detected in honeydew honey compared to the blossom honey. Consumption of honey from the regions presents no risk of exposure to ^{137}Cs.

Key words: blossom honey; honeydew honey; radiocaesium; Slovakia

INTRODUCTION

Since the times of the most famous antique doctor and father of medicine Hippocrates, honey has been recommended as a miracle and universal medicine suitable for the treatment of many diseases [29]. It has had a valued place in traditional medicine for centuries. Honey has been reported to have an inhibitory effect on around 60 species of bacteria including aerobes, anaerobes, Gram-positives and Gram-negatives [19, 20]. In addition to antibacterial and antifungal effects, natural honey has demonstrated antiviral effects [1, 10]. The mechanisms of antimicrobial activity of honey are different from antibiotics, which destroy the bacteria's cell wall or inhibit intracellular metabolic pathways. The antibacterial activity is related to four properties of honey. First, honey draws moisture out of the environment and thus dehydrates bacteria. The sugar content of honey is also high enough to hinder the growth of

microbes, but the sugar content alone is not the sole reason for honey's antibacterial properties [27]. Second, the pH of honey is between 3.2 and 4.5, and this acidity is low enough to inhibit the growth of most microorganisms. Hydrogen peroxide produced by the glucose oxidase is the third and probably the most important antibacterial component, although some authors believe the non-peroxide activity to be more important. Lastly, several phytochemical factors for antibacterial activity have been identified in honey [1, 10]. It stimulates the central nervous system and human psychics, improves the quality of sleep, acts as a source of energy and supports digestion, facilitates the treatment of the liver, ulcerous diseases of the stomach and duodenum. Honey is also effective in healing of wounds, but the exact mechanism of this effect is yet to be elucidated [10]. Studies carried out on animals showed honey's effect against cardiovascular risk factors [31]. Honey also decreases venous blood pressure and inhibits oxidative stress, enhances detoxification of an organism, supports the immune system and increases performance in general [10].

Honey is classified as blossom honey (nectar of plants) or honeydew honey (secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants). Honeydew honey is generally characterised by higher values of electric conductivity, pH, acidity and ash content, darker colour, higher oligosaccharides content and lower content of monosaccharides than the blossom honey. The antioxidant and antibacterial properties of honeydew honey are higher than those of most blossom honeys [22].

Honey is relatively free of adverse effects. Allergy to honey is rare, but there could be an allergic reaction to either pollen or bee proteins in honey. Theoretical risk of rise in blood glucose levels may always be there when applied to large open wound in diabetics [10].

Honeybees have been used as monitors of a variety of environmental contaminants, including trace metals, low level radioactivity and pesticides [14]. Due to their ability to act as unique environmental samplers of pollutants they can be intentionally placed into the regions with increased air pollution which is then reflected in the content of xenobiotics in bee bodies and their products, particularly in honey, propolis, and most frequently in pollen [14, 28].

Flower pollen is a good indicator of air contamination. By simply fitting a pollen trap to a honeybee hive, and measuring the radiocaesium content of the pollen species present, it is possible to produce models to give accurate and precise estimations of concentrations in leaves and flowers of plants within the forage area [18]. Because the beeswax is used to produce honeycomb cells, it also reflects the cumulated environmental pollution. In Austria they were able to detect in this way substances that have been banned for 13 years. On the contrary, honey is exceptionally protected by bees and thus it constitutes a pure product obtained also under worse conditions [30].

Contamination of the environment by artificial radionuclides occurred as a result of nuclear weapons testing in the fifties to sixties of the 20th century and the Chernobyl accident in 1986. Ipatyev et al. [15] focused on the influence of this accident on the forest ecosystem in the period of 1986—1994. They observed that oak trees exhibited stronger ^{137}Cs absorption capacities than pine trees. Due to the time lapse and the character of the Chernobyl accident, of the artificial radionuclides only ^{137}Cs with physical halftime decay of 30.1 years were detected recently in the soil in Slovakia [16]. According to Šaro and Tolgyessy [24] caesium is the radionuclide which passes to the food chain due to surface contamination of soil and crops. Caesium is retained in the surface layer of soil and can be taken up partially by the roots of plants. It is its subsequent accumulation in plants that is the main subject of concern. The radiocaesium then enters the digestion tract through the food chain [5].

The aim of this study was to determine the activity of radiocaesium in individual samples of blossom and honeydew honey collected from various locations in eastern Slovakia and compare the maximum values with the maximum acceptable level specified by the relevant legislative provisions [6].

MATERIALS AND METHODS

Fifty samples of honey (42 blossom and 8 honeydew honey) were collected in the years 2017 and 2018 from the Prešov and Košice regions in eastern Slovakia. The samples were provided by honeybee keepers that participate actively in production of honey and honeybee products.

Gamma-ray spectrometric measurements were used for assaying gamma-ray emitting radionuclides. This measurement is based on the use of detectors of the semiconductor type that are used for qualitative analysis of energy of the ionizing radiation of certain radionuclide but also

for quantitative analysis of the content of radionuclide in the sample based on emission of photons of specific energy. This method is suitable for measurement in environmental samples with low radioactivity. As it requires no preparation of the sample material, the measurements were carried out on the samples of honey that were not subjected to any previous processing.

The samples were transferred to Marinelli beakers with a volume of 450 ml and the measurements were performed using a gamma spectrometer (Canberra) equipped with Ge detector (GC 4020 with 40 % effectiveness and 1.8 keV resolution) and DSA-LX analyser according to the manufacturer's instructions. All measurements were carried out at the Veterinary and Pharmaceutical University in Brno, Czech Republic. The presented relative standard uncertainties ua were determined according to JCGM 100:2008, 1st edn. [11].

RESULTS AND DISCUSSION

Of the 50 samples of honey, activities of ^{137}CS in 35 samples were below the detection limit of 0.2 Bq.kg^{-1}. Table 1 shows the activities of ^{137}Cs measured in honey samples that exceeded this level (n = 15). Results are presented as Activity concentration ± combined standard uncertainties ua [11].

The radioactivity level in all honeydew honey samples measured (n = 8) exceeded 1.72 Bq.kg^{-1}. The highest ^{137}Cs activity was detected in honeydew honey (2.33 ± 0.05 Bq.kg^{-1}) that originated from the location Branisko. The highest ^{137}Cs activity in blossom honey was detected in rape honey from Nižná Šebastová.

Examination of honeys from industrially polluted regions did not show the presence of pollutants which, however, was not true for the bodies of bees nor the bee poison where various contaminants were found. This indicated that bees are able to produce pure honey, even from polluted sources, and thus function as a biological filter retaining the contaminants and not passing them to this product. Despite that, trace amounts of pesticides were detected in honeys that the bees were not able to remove by their gastrointestinal tract [7].

Nectar (blossom) honeys are produced during the spring and the early summer, normally over short periods of time. The nectar secretion pattern is very abundant and fast, and the blossom honey production is completed quickly. Production of honeydew honeys takes longer as it probably begins as blossom honey early in spring but is mixed with honeydew secretions toward the end of summer [9]. Because of the delayed collection of honeydew honey, this honey is always a little more contaminated than the blossom honey. This was reflected also in our results. Additional sources of contamination may be polluted water or artificial fertilizers.

The use of bees as indicators is important particularly in big cities and around highways as bees obtain nectar and pollen from plants that take up minerals from soil and thus serve as indicators of heavy metals. Bees also belong to the most accurate indicators of air quality because the trapped pollen may be successfully analysed. The more accurate assessment of environmental pollution of a relevant region requires presence of several bee colonies.

One should also mention the fact that the movement of xenobiotics throughout the plant is not so intensive, and after the pollutants are taken up by roots they accumulate for some time in the root system before they pass to the

Table 1. Activities of ^{137}Cs in the samples that exceeded the detection limit of 0.2 Bq.kg^{-1}

Type of honey	Location	Activity concentration [Bq.kg^{-1}] ± combined standard uncertainties ua [11]
Honeydew	Branisko	2.33 ± 0.05
Honeydew	Podhradík	2.05 ± 0.01
Honeydew	Ľubotín	1.82 ± 0.02
Honeydew	Šindliar	1.91 ± 0.04
Honeydew	Helcmanovce	2.09 ± 0.14
Honeydew	Košice	1.73 ± 0.03
Honeydew	Kavečany	1.72 ± 0.11
Honeydew	Krompachy	2.26 ± 0.13
Blossom (rape)	Šalgovík	0.21 ± 0.03
Blossom (rape)	Nižná Šebastová	0.75 ± 0.02
Blossom (rape)	Košice	0.27 ± 0.05
Blossom (buckwheat)	Kendice	0.33 ± 0.02
Blossom (linden)	Lipovec	0.52 ± 0.03
Blossom (sunflower)	Lačnov	0.27 ± 0.02
Blossom (meadow)	Malý Šariš	0.23 ± 0.01

honeydew or nectar. This indicates that it is more relevant to determine the real content of pollutants in the air than in the soil [23].

Franić and Branica [12] conducted long-term investigations of ^{134}Cs and ^{137}Cs activity concentrations in multifloral and chestnut honey sampled in northwest Croatia. For both radionuclides, the activity concentrations peaked in May 1986, decreasing exponentially until the mid-1990s, when they fell under the detection limit for both radionuclides. After the Fukushima-Daiichi accident in 2011, the presence of both radionuclides in honey was detected once again. The observed $^{134}Cs/^{137}Cs$ activity ratio in honey was similar to the ratio found in other environmental samples. The estimated collective effective doses for the Croatian population incurred by honey consumption indicated that honey was not a critical pathway for the transfer of ^{134}Cs and ^{137}Cs from fallout to humans [12].

There were large variations in the radionuclide deposition levels. Of the artificial radionuclides associated with the Chernobyl accident, only ^{137}Cs occurred recently in the soil in Slovakia [16]. This radionuclide rapidly integrated into the biological cycle, similar as potassium [13].

The proportion of ^{137}Cs available to plants can vary in soils with high content of minerals depending on many factors. Under certain conditions, it can even increase with depth [25]. Through consumption of plants it can pass to the muscles of animals. In the period of 1998—2008, ^{137}Cs activity in the muscles from wild boars hunted in the Ravensburg district (southern Germany) varied from less than 5 up to 8 266 $Bq.kg^{-1}$ in dependence on season, atmospheric conditions and other factors [26]. After a gradual decrease of ^{137}Cs in game meat in the nineties in north-eastern Moravia, its levels unexpectedly increased after floods and exceeded the acceptable limit of 600 $Bq.kg^{-1}$ [17]. Despite such high levels in the surrounding states, samples of wild boar meat did not even approach the acceptable level of ^{137}Cs [2, 16].

Before the Chernobyl accident that occurred on 26th April 1986, the activity of ^{137}Cs in honey varied around 2 $Bq.kg^{-1}$ as a result of nuclear weapons tests [8]. Bunzl et al. [4] started with measurements of the activity of radionuclides in honey and pollen in the region of Munich in week intervals up to August 1986. At the beginning of May, the specific activity of ^{137}Cs exceeded 600 $Bq.kg^{-1}$ but in the course of May it decreased to 200 $Bq.kg^{-1}$. The activity of ^{137}Cs was higher in pollen in comparison with honey. Borawska et al. [3] determined radioactivity of honey 12 years following Chernobyl. The activity of 39 samples ranged between 0.5 and 46.3 $Bq.kg^{-1}$. The highest mean level (24.3 $Bq.kg^{-1}$) was measured in heather honey and the lowest (0.6 $Bq.kg^{-1}$) in blossom honey. Between June 2001 and December 2004, the ^{137}Cs activity was investigated in a total of 336 samples of different varieties of honey from the Liguria region of Northern Italy. The purpose of this study was to define (a) residual radioactive contamination following the Chernobyl accident and ^{137}Cs long-term decline, (b) correlation between ^{137}Cs activity and different honey varieties, and (c) correlation between ^{137}Cs activity and the prevailing geomorphological configuration in the collection areas. The results were compared according to the place of the nectar collection and the type of honey. Significantly higher concentrations of ^{137}Cs were measured in the valley of the river Stura (mean level 8.45 $Bq.kg^{-1}$). In this region the heaviest rains occurred when the post-accident radioactive cloud passed over this area. The mean ^{137}Cs specific activity was 4.33 ± 5.04 S.D. $Bq.kg^{-1}$. The levels of ^{137}Cs in chestnut honey were significantly higher than in other types of honey which was ascribed to the extensive, superficial and deep, root apparatus of the tree. Honey samples from acidic argillite soils, which withhold radionuclides after deposition and slowly release them to plants, also showed higher ^{137}Cs activity. The authors used the data obtained in the study to calculate the half-life decay of ^{137}Cs in honey. They obtained the following results: acacia honey 437 days (1.199 years); chestnut honey 577 days (1.58 years) and honeydew honey 394 days (1.08 years) [21].

Comparison of our results showed higher concentration of radioactive caesium in samples of honeydew honey in comparison with blossom honey. Similar results were presented by Borawska et al. [3]. In the case of raw materials, semi-finished products and products originating exclusively or partially from the forest ecosystem one should consider that potentially increased levels of radiocaesium may occur even without changes in the current radiation situation in Slovakia. The activities of ^{137}Cs determined in the samples of honey examined in our study correspond to the activities measure before the Chernobyl accident.

CONCLUSIONS

The activities of radiocaesium in honey samples determined in this study were very low and were far below

the acceptable radioactivity limit in food set by legislation for persistent contamination after the Chernobyl accident (600 $Bq.kg^{-1}$). In the majority of samples the radiocaesium activity was below the detection limit of 0.2 $Bq.kg^{-1}$. This implies that consumption of honey originating from the investigated Slovak regions poses no risk to the human health resulting from post-Chernobyl radiocaesium contamination.

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DETECTION OF CHROMOSOMAL BREAKS INDUCED BY THIACLOPRID IN HUMAN LYPMHOCYTES AND DETECTION OF DOUBLE-STRAND BREAKS BASED ON γH2AX HISTONE PHOSPHORYLATION

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ABSTRACT

Thiacloprid, a neonicotinoid insecticide, is widely used to control various species of pests in the current agriculture of today. The potential genotoxic effects of thiacloprid on human peripheral blood lymphocytes were investigated *in vitro* by chromosome aberrations (CA), and double-strand breaks (DSB), which were detected by the phosphorylation of γH2AX histone. Human peripheral blood lymphocytes were exposed to 30, 60, 120, 240, 480 μg.ml^{-1} doses for the last 24 and 48 hours of culture. Thiacloprid increased CA at the concentrations of 240, 480 μg.ml^{-1} ($P < 0.05$), but these results did not confirm genotoxicity. The mitotic index (MI) was important to us; it served as a basis for the confirmation of the cytotoxicity of this insecticide. During 48 hours of culture, at the concentration of 480 μg.ml^{-1}, its value rapidly decreased (0.42) ($P < 0.001$), which did not allow us to analyse the results because of the high cytotoxic response.

Key words: acetylcholinesterase; γH2AX histone; chromosomal aberrations; neonicotinoids; phosphorylation; thiacloprid

INTRODUCTION

Humans have inhabited the Earth for more than 3 million years, while the insects have existed for at least 250 million years. Nowadays we can only guess that our ancestors used various methods against invasive insects, such as setting up smoke fires, rubbing the skin with mud or dust, using bile of a green lizard to protect apples against worms and decay, extracts from spice and tobacco, soapy water, white powder, vinegar, turpentine, fish oil, salt brine, lye and many others [10]. However, now we have more effective methods to control the insects. These methods are chemical compounds—insecticides. Neonicotinoids are the most successful new class of insecticides which are referred to as orthosteric modulators nAChR [3]. These chemical com-

pounds are derived from nithiazine containing a nithromethylene functional group. As it was discovered in 1984, the effect of nithiazine lies in postsynaptic agonic action on acetylcholine receptor, the same case as nicotin. Nithiazine had the required specificity (i. e. low toxicity for mammals), but was not photostable, so it was not commercially successful [8, 9]. The invention of imidacloprid, the most important neonicotinoid insecticide, was launched by the discovery of 1-(6-chloro-3-pyridylmethyl)-2-nitromethylenimidazolidine [5]. Its favourable characteristics made it a commercial blockbuster, followed by clothianidin, dinotefuran, acetamiprid, thiamethoxam and thiacloprid.

The reason for the development of this new insecticide class was that they would be more effective and less harmful for non-target organisms than their predecessors – nicotinoids. Thus, the mechanism of their action is blocking the insect post-synaptic nicotinic acetylcholine receptors, which should make them non-toxic compounds for vertebrates. After blocking the receptors, the transfer of impulses is impossible and so the organism is subjected to paralysis, even death. Later the neonicotinoids got under criticism for their possible adverse or toxic effects.

The aim of our study was to evaluate chromosome aberrations induced by thiacloprid and detect double-strand breaks on the basis of γH2AX phosphorylation in human peripheral blood lymphocytes *in vitro*.

MATERIALS AND METHODS

Detection of chromosomal breaks induced by thiacloprid in human lymphocytes

The whole blood culture of a 21-year-old woman was performed in 5 ml of a culture medium during 72 hours in a thermostat with a temperature set on 38 °C. The components of the culture medium were RPMI 1640 containing L-glutamine and HEPES 15 μmol (GE Healthcare Hyclone Lab, Utah, USA); 15 % foetal calf serum (BoFeS, Sigma, Chemical Co. St. Louis, MO, USA), antibiotics (100 U.ml^{-1} penicillin, 0.1 mg.ml^{-1} streptomycin and 0.25 μg.ml^{-1} amphotericin) and the last component was mitogen phytohemagglutinin (PHA, 180 μg.ml^{-1}, Welcome, Dartfordt, England)—a *Phaseolus vulgaris* extract. The dedifferentiation (transformation to the immature cells—lymphoblasts, which divide and enter into mitosis) was carried out by PHA. Ethyl methanesulphonate (EMS, Sigma, St. Louis, MO, USA, 250 μg.ml^{-1}), mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM) and thiacloprid at the concentrations of 30, 60, 120, 240 and 480 μg.ml^{-1} were added for the last 24 and 48 h of the culture. Colchicine at the concentration of 5 μg.ml^{-1} (Merck, Darmstadt, Germany)—a *Colchicum autumnale* alkaloid—was added for the last 90 minutes of the culture. Colchicine is a mitotic poison specifically inhibiting the formation of a mitotic spindle and thus stopping cell division in the metaphase of mitosis, during which the chromosomes are in the most condensed state and suitable for analysis. The activity of colchicine lasts until the addition of a fixation solution. After processing the cell cultures by fixation, we stained the slides according to Giemsa. Aberrations and MI were statistically evaluated by the χ-square test.

Detection of double-strand breaks on the basis of γH2AX histone phosphorylation

Thiacloprid was added to the lymphocyte cultures at the concentration of 480 μg.ml^{-1}. To detect the γH2AX histone, we used the Anti-gamma H2A.X primary antibody (phosphor S139, ab26350) and the Goat Anti-Mouse IgG H&L secondary antibody (Alexa Fluor®488, ab150113). Finally, the staining of the nuclei was carried out by DAPI + VECTASHIELD. The evaluation was performed manually by the fluorescence microscope NIKON Eclipse Ni-U with the Progres® camera and the METAFER automatic analysis module.

RESULTS

The results of the 24 and 48-hour cultures are summarized in Tables 1 and 2. During both 24 and 48-hour cultures we detected statistically insignificant results at the first 3 concentrations. At 240 and 480 μg.ml^{-1}of thiacloprid, a slight DNA damage occurred ($P < 0.05$), probably as a result of cytotoxic effects. Since we had not detected a statistically significant increase in chromosome aberrations depending on the lowest dose, the direct genotoxic effect of the given insecticide based on the conventional cytogenetic analysis had not been confirmed. Cytotoxicity was confirmed at the concentration of 480 μg.ml^{-1}during the 48-hour culture. The rapid decrease of the MI resulted in the fact that it was not possible to examine enough metaphases as a result of the high cytotoxic reaction. The result of the

other part of our study (detection of double-strand breaks by the phosphorylated γH2AX histone) was negative. In this part of the experiment we focused on testing the ability of the highest concentration of thiacloprid (480 μg.ml^{-1}) to induce γH2AX foci after the 4-hour exposure of human lymphocytes. The phosphorylated γH2AX histone is nowadays considered to be a sensitive and quantitative marker of the double-strand breaks. After the automatic evaluation by the Metafer software, we concluded that human lymphocytes did not contain a statistically significant occurrence of γH2AX foci which would refer to double-strand breaks caused by thiacloprid. The results of the detection are depicted in Figures 1 and 2.

Table 1. Results of chromosome aberrations after the exposure to human lymphocytes in peripheral blood during 24 hours

Donor	Metaphase number	Types of CA					% Breaks ± SD	% MI
		G	CB	IB	CE	IE		
Control	100	1	1	–	–	–	1.0 ± 0.01	2.6
Thiacloprid [μg.ml^{-1}]								
30	100	5	2	–	–	–	2.0 ± 0.14	2.0
60	100	6	3	–	–	–	3.0 ± 0.17	1.7
120	100	8	3	1	–	–	4.0 ± 0.20	1.3*
240	100	8	5	2	–	–	7.0 ± 0.26*	1.0**
480	100	12	7	1	–	–	8.0 ± 0.27*	1.0**
EMS 250 μg.ml^{-1}	**100**	**10**	**10**	**2**	**1**	**–**	**16 ± 0.35*** **	**0.9*** **

Statistical significance: *—P < 0.05; **—P < 0.01; ***—P < 0.001; Control—positive control; EMS = ethylmethylsulfonate [250 μg.ml^{-1}]—positive control; G—gap; CB—chromatid break; IB—isochromatid (chromosomal) break; CE—chromatid exchange; IE—isochromatid (chromosome) exchange

Table 2. Results of chromosome aberrations after the exposure to human lymphocytes in peripheral blood during 48 hours

Donor	Metaphase number	Types of CA					% Breaks ± SD	% MI
		G	CB	IB	CE	IE		
Control	100	3	2	–	–	–	2 ± 0.141	2.4
Thiacloprid [μg.ml^{-1}]								
30	100	6	3	–	–	–	3 ± 0.17	2.0
60	100	8	6	1	–	–	7± 0.26	1.7
120	100	12	7	1	–	–	8 ± 0.27	1.5
240	100	13	8	3	–	–	11 ± 0.31*	1.1*
480	ND	–	–	–	–	–	–	0.42***
MMC 0.4 μg.ml^{-1}	**100**	**12**	**10**	**–**	**2**	**–**	**14 ± 0.35****	**0.9****

Statistical significance: *—P < 0.05; **—P < 0.01; ***—P < 0.001; MMC—mitomycin; C—positive control; G—gap; CB—chromatid break; IB—isochromatid (chromosomal) break; CE— chromatid exchange; IE—isochromatid (chromosome) exchange; ND—none detected

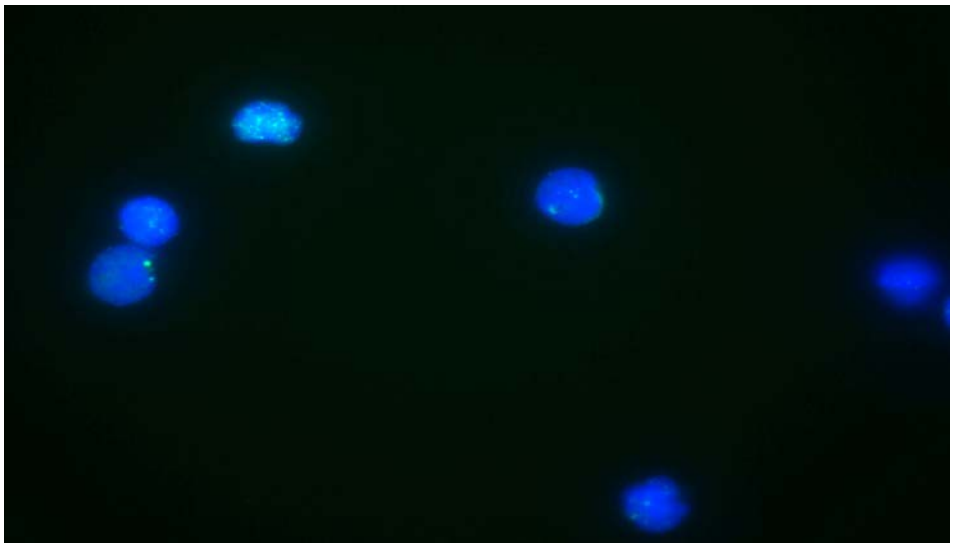

Fig. 1. Representative picture of a positive control depicting green foci stained by Alexa Fluor®488

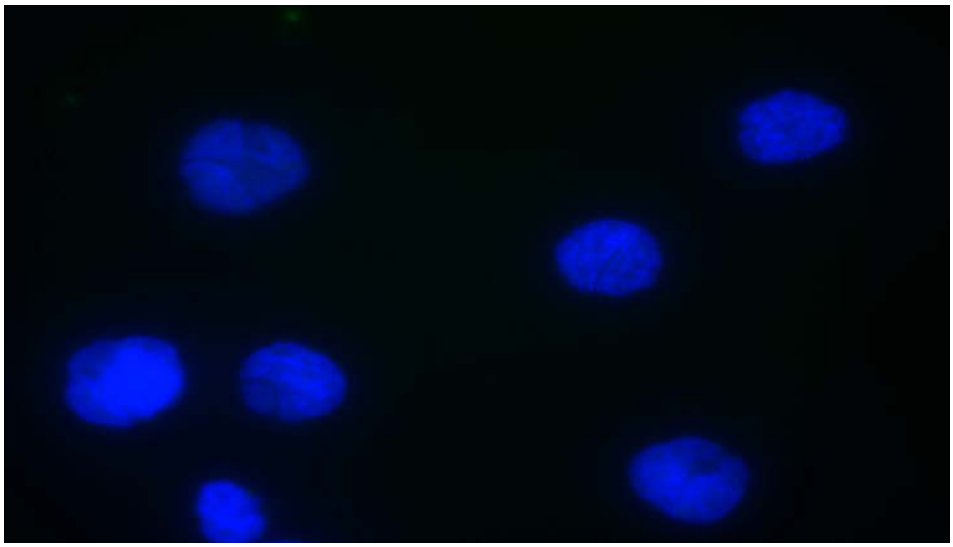

Fig. 2. Representative picture of a lymphocyte exposed to thiacloprid—no foci

DISCUSSION

Structural chromosome aberrations represent irreversible changes in the chromosome structure, which could have serious genetic consequences for a cell or an organism. In our study we focused on the detection of chromosomal breaks induced by thiacloprid in human lymphocytes. The results of our study did not confirm the genotoxic effect of thiacloprid, however, the highest tested concentration (480 $\mu g.ml^{-1}$) exerted a cytotoxic effect after the extended time of exposure.

The cytotoxicity of thiacloprid was confirmed in the *in vitro* experiment in cells of the Chinese hamster V79 at the concentration up to 750 $\mu g.ml^{-1}$. No other statistically significant growth of cells with chromosome abberations were observed at other concentrations [7]. On the contrary, the study of Kocaman et al. [6] confirmed the genotoxic effect of thiacloprid on human lymphocytes at the concentrations of 75, 150 and 300 $\mu g.ml^{-1}$. The other study of Calderon-Segura et al. [1] focused on human lymphocytes, confirmed the results of the previous experiment. In the study the authors compared the genotoxic effects of commercial preparations, such as Calypso (thiacloprid), Poncho (clothianidin), Gaucho (imidacloprid) and Jade (imidacloprid) and they detected that Jade exerted the strongest genotoxic effect.

The other aim of our study was to detect the induction of the phosphorylated γH2AX. Nowadays, the phosphorylated γH2AX histone is considered to be a sensitive and quantitative marker of the double-strand breaks.

The results of our experiment corresponded with the study of Galdíková et al. [4], which was focused on the detection of the γH2AX induction after the exposure of the human cell line HT 29 to thiacloprid by the immunofluorescence method. The insecticide was used at the concentrations of 60, 120, 240 and 480 $\mu g.ml^{-1}$. The results did not demonstrate the increased frequency of the DSB by using the immunocytochemical method with a microscopic visualization. Cavas et al. [2] examined the genotoxic effect of another representative of neonicotinoids—acetamiprid—on the human intestinal cells CaCo–2. However, acetamiprid did not induce the formation of the γH2AX foci at the same rate as the H_2O_2 positive control.

CONCLUSIONS

The results of this study showed that thiacloprid exerted cytotoxic activity at the concentration of 480 $\mu g.ml^{-1}$ at the 48-hour duration of incubation. The genotoxic or clastogenic effect on the human lymphocytes was not confirmed. By the automatic statistical evaluation using Metafer we made a conclusion that the chosen neonicotinoid (thiacloprid) did not induce statistically significant frequencies of the γH2AX foci.

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THE PREVALENCE AND ASSEMBLAGES OF *GIARDIA DUODENALIS* IN DOGS: A SYSTEMATIC REVIEW IN EUROPE

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ABSTRACT

***Giardiasis* is one of the most frequent causes of diarrhoeic diseases in the world. *Giardia* cysts are most commonly transferred via ingestion of contaminated water or food. On the basis of genetic characteristics *Giardia duodenalis* is classified in eight assemblages A—H. Zoonotic assemblages A and B are increasingly found in isolates from dogs which may constitute the reservoir of human giardiasis. This article presents a brief review of *G. duodenalis* assemblages detected in dogs that were documented in European countries.**

Key words: assemblages; dogs; *Giardia duodenalis*; giardiasis

INTRODUCTION

Giardia duodenalis is a cosmopolitan unicellular parasitic protozoan that infects domestic and free living animals including humans [47]. The World Health Organisation (WHO) includes *G. duodenalis* among the so-called "Neglected Tropical Diseases" [45]. Throughout the world as many as 200 million cases of giardiasis are diagnosed annually [17, 22].

On the basis of genetic differences, *G. duodenalis* is classified in eight assemblages [15]. Assemblages A and B are considered zoonotic and were confirmed in humans but also in a broad range of hosts [6, 7]. The remaining 6 assemblages are mostly host-specific; C and D are typical of dogs and other canines; assemblage E was identified in ungulates including cattle, sheep and goats; F in cats; G in rats, and H in marine mammals [3, 44]. Dogs may be infected also by assemblages A or B and present potential risk of transfer of this infection to humans [15]. In order to guarantee the correct identification of assemblages and confirmation of the potential zoonotic transmission of *G. duodenalis*, the use of molecular methods is recommended. The PCR method for identification of *G. duodenalis* has on average 92 % sensitivity and 100 % specificity [49].

The life cycle of *G. duodenalis* is simple. The host is most often infected by the faecal-oral route through contaminated food, water or direct physical contact [22]. *G. duodenalis* occurs in two forms, as the trophozoite or the cyst. The trophozoite is an actively motile, vegetative, flagellated pear-

shaped stage existing in the small intestinal lumen. Survival of this parasite in the outer environment is ensured by the oval cysts that constitute the infectious stage. After the ingestion of cysts, excystation occurs under the action of digestive enzymes and low pH. Each four-nuclei cyst releases two-nuclei trophozoites. By means of a suction disk, the trophozoites adhere to enterocytes in the proximal part of the duodenum and multiply by longitudinal binary fission. Under the action of bile salts their encystation occurs as they transit toward the colon. The cysts that are eliminated to the outer environment with faeces are fully infectious [1]. Younger age categories are more frequently affected by giardiasis and exhibit more pronounced clinical signs. The signs are observed within 2 weeks after ingestion of infectious cysts. Duodenal microvilli in the affected individuals are shortened and thickened which results in a maladsorption syndrome [16, 53]. The characteristic signs include disorders in resorption of fats and fat-soluble vitamins (steatorrhea). The decreased enzymatic activity in the host causes disorders of absorption of zinc which becomes a part of surface antigens of the parasite. Zinc is an element irreplaceable in immunological reactions and its deficiency can have an immunosuppressive effect on the host organism [53]. Clinical signs are frequently non-specific and the infection may have a latent course. Alternating periods of diarrhoea and constipation are observed and the faeces mostly contain mucus and fat, but no blood. In some patients the disease may be chronic and last several months; even years [14]. Giardiasis has been recognised as an important risk factor for long-term syndromes, for example post-infection irritable bowel and chronic fatigue syndromes. Recent studies indicate that extra-intestinal consequences of giardiasis were also observed including arthritis or hypersensitive skin reaction [11, 33].

The aim of this study was to present a review of the prevalence and assemblages of *Giardia duodenalis* in dogs in European countries with respect to the zoonotic potential of this parasite.

OCCURRENCE OF *G. DUODENALIS* IN EUROPE

Giardia spp. is a well-known agent of one of the most extensively spread intestinal infections in temperate, tropical and subtropical zones. It has been assessed that it causes annually 280 million of diarrhoeal diseases throughout the world [58]. *G. duodenalis* is capable of infecting a broad range of hosts including humans and domestic and wild living animals. Investigations of various populations of dogs have revealed a different prevalence dependent on the age. The highest prevalence (46—50 %) was observed in dogs under 1 year of age [13, 24]. In some European countries the prevalence of giardiasis in dog breeding centres reached 100 %. On the other hand, only 10 % positivity was detected in dogs kept under good hygiene conditions [26]. The previous studies that involved groups of dogs of different age and breeding conditions confirmed differences in the prevalence of giardiasis. The following text and Table 1 provides a summary of results obtained in various European countries including Slovakia.

In 1999, Letková et al. [32] reported 10.1 % prevalence of *G. duodenalis* in dogs in Košice, eastern Slovakia. In 2007, Szabová et al. [54] detected the occurrence of *G. duodenalis* in 5 districts of eastern Slovakia (Michalovce, Trebišov, Košice, Poprad and Bardejov). Samples of examined faeces originated from dogs living in households, dog shelters, quarantine stations and public grounds in Košice and Bardejov. Cysts were detected in the faeces of dogs in the district Trebišov (1.1 % positive samples) and Košice (1.7 %). Goldová et al. [19] confirmed 69.1 % and 36.9 % prevalence of giardiasis in dogs from a dog shelter; younger than 7 months and older than 7 months, respectively. Štrkolcová et al. [50] found a 33.3 % prevalence of *G. duodenalis* in dogs from shelters in the Košice district and 4.2 % in dogs from a Roma settlement. The studies cited above were based on the microscopic examination of faecal samples and molecular methods were not used.

Giardiasis in dogs as human companions can have serious consequences due to the zoonotic potential of this parasite [15]. The first infection of humans in Europe caused by specific canine genotype C was confirmed by the study conducted in Slovakia. This supports the potential role of dogs in zoonotic transfer of *G. duodenalis* assemblage C to humans [51].

In 1994, Hořejš and Koudela investigated the occurrence of giardiasis in dogs in the Czech Republic and detected an overall prevalence of 5—6 % [23]. In 2001, Svobodová and Doležil detected the presence of giardia cysts in 41.6 % of the dogs in a dog shelter in Ostrava [52]. Dubná et al. [12] investigated in 2007 the prevalence of parasites in dog faeces in the Prague city

Table 1. Review of assemblages of *G. duodenalis* diagnosed in dogs in Europe

Country	Assemblages	Source
Slovakia	–	Letková 1999 [32]; Szabová 2007 [54]; Goldová 2011 [19]; Štrkolcová 2014 [50], 2015 [51]
Czech Republic	-–	Hořejš and Koudela 1994 [23]; Svobodová and Doležil 2001 [52]; Dubná et al. 2007 [12]
Austria	A/AII/B/BIV	Joachim and Prosl 2005 [25]; Hinney et al. 2017 [21]; Lee et al. 2017 [30]
Poland	A/B/C/D/C + D	Bajer 2008 [2]; Stojecki et al. 2015 [49]; Piekarska et al. 2016 [43]
Hungary	C/D	Szénási et al. 2007 [55]
Germany	C/D/A + C/ C + D	Cirak and Bauer 2004 [8]; Leonhard et al. 2007 [31]; Pallant et al. 2015 [42]; Sommer et al. 2018 [48]
Serbia	–	Nikolić et al. 1993 [39], 2002 [36], 2008 [37], 2011 [38]
Romania	C/D/E/C + D	Mircean et al. 2012 [34]; Györke et al. 2016 [20]
Italy	C/D	Zanzani et al. 2014 [57]; Scaramozzino et al. 2018 [46]
Belgium	A/B	Claerebout et al. 2009 [9]
Great Britain	AIII/C/D	Upjohn et al. 2010 [56]
Bosnia	–	Omeragić et al. 2014 [40]
Estonia	–	Lassen et al. 2009 [28]
Sweden	A/C/D/C + D	Lebbad et al. 2010 [29]
Holland	A/C/D/C + D	Overgaauw et al. 2009 [41]
Croatia	C/D	Beck et al. 2012 [5]
Spain	AII/BIII/BIV/C/D	deLucio et al. 2017 [10]; Gill et al. 2017 [18]
Portugal	–	Neves et al. 2014 [35]
Greece	C/D	Kostopoulou et al. 2017 [27]

centre, agricultural areas and two shelters. They observed that the prevalence of *Giardia* spp. in Prague reached 0.1 %. The examination of 540 samples from rural areas showed a prevalence of 2.2 % and the *Giardia* spp. were one of the most frequently detected parasites.

The epizootological studies conducted in Austria in 1990–2005 showed that prevalence of giardiasis in dogs ranged between 22 and 36 % [25]. An extensive research conducted in 2015 in Vienna included the examination of 1001 samples of dog faeces. The diagnostic methods included the flotation method according to Faust and a rapid SNAP test for the proof of coproantigen. The flotation method detected cysts in 5.2 % of the samples and the 6.5 % of the samples were positive in the SNAP test [21]. Lee et al. [30] confirmed by molecular analysis the occurrence of assemblages A and B in Austria. The most frequent assemblages were AII and BIV.

Several studies investigated the prevalence of giardiasis in dogs in Poland. The total prevalence ranged from 1.9 to 36 % [2]. The prevalence of 5.6 % was determined in dogs in eastern Poland using a specific fluorescent staining and genotyping revealed the zoonotic assemblage A [49]. Piekarska et al. analysed isolates from domestic dogs in Wroclaw and identified assemblages D (9 %) and B (4.5 %), and assemblages C + D (4.5 %) in one mixed infection [43].

Szénási et al. investigated the prevalence of giardiasis in dogs from shelters in Hungary in relation to the method used. Microscopic examination indicated positivity in 14 samples (prevalence 7.5 %), while the ELISA test for the proof of coproantigen determined *G. duodenalis* in 58.8 % of the dogs. On the basis of sequencing the positive samples, they were classified in the assemblages C and D [55].

In Germany Cirak and Bauer observed 9.5 % occurrence of *G. duodenalis* cysts in domestic dogs [8]. Another study in south Germany investigated samples of faeces from asymptomatic dogs that were brought to veterinary clinics. *G. duodenalis* was detected in 55 of the 60 examined samples. Assemblage A was detected most frequently followed by mixed infections A + C and C + D. Individual assemblages C and D were proved only in 2 samples [31]. In a retrospective study, the results of parasitological examinations of faecal samples from 8,560 cats and 24,677 dogs between January 2003 and December 2010 in Germany were analysed. The examination of the faecal samples from dogs revealed stages of *Giardia* spp. (18. %). Dogs in the age groups up to 3 months and >3 up to 6 months of age showed significantly higher infection rates with *Giardia* spp. (37.5 % and 38.2 %, respectively) [4].

In the study by Sommer et al. [48] the prevalence of *Giardia duodenalis* infections in dogs and cats living in Germany were investigated using different diagnostic tests and the *Giardia* assemblages of infected animals were identified. All samples were investigated by enzyme-linked immunosorbent assay (ELISA), merthiolate-iodine-formalin concentration technique (MIFC) and zinc chloride flotation. ELISA-positive samples were additionally screened with a direct immunofluorescence assay (IFA). The samples from dogs tested positive for *Giardia* coproantigen (ELISA) in 30.6 %. The MIFC technique revealed *Giardia* cysts in 33.9 % of canine ELISA-positive samples, while using IFA, cysts were present in 90.4 % of the canine ELISA-positive samples. Dog-specific *Giardia* assemblages C and D were detected in 42 and 55 canine isolates, respectively. Two canine samples harboured the zoonotic assemblage A. According to the results of the study, *Giardia* is a common endoparasite in dogs and cats from Germany.

Publications from 1993 until 2011 [36—39] have confirmed that *G. duodenalis* was the most common intestinal protozoan parasite in dogs from the Belgrade area. Faecal samples from household, stray, farm and military working (kennel) dogs were investigated in three different studies. Significantly higher infection rates were found in stray, farm and military working dogs. With the intention to evaluate the correlation of *Giardia* infections in household dogs and their owners, faecal samples of all family members of households accommodating *Giardia* positive dogs were also screened for *Giardia* cysts in two of the three studies. Two people living in one household with an infected dog carried an infection with *G. duodenalis* as well. The finding supports a possible transmission of *Giardia* infections between human and canine cycles. However, a molecular analysis of the concerned samples would have been essential for a further statement on the zoonotic potential and the transmission dynamics arising from the investigated dog population. Human giardiasis was spread throughout Serbia with a higher incidence in the Northern part of the country [38].

A one-year study (2008—2009) in Romania investigated the prevalence of parasitic infections in sheepdogs, domestic and shelter dogs and dogs used for breeding. The prevalence of *G. duodenalis* in shelter dogs determined by the flotation method reached 16.5 % (27/164). Positivity in breeding dogs reached 7.2 %; in domestic dogs 4.8 % and in sheepdogs 4.3 % [34]. The more recent studies that used the PCR-RFLP method identified assemblages C, D and E. In one case, a mixed C + D infection was detected.

In Milano, Italy, of 37 examined samples of dog faeces, 11 were positive for *G. duodenalis*. In these dogs the PCR method identified assemblages C and D [57]. In another 3-year study that was carried out in Rome, *G. duodenalis* was the third most frequently diagnosed parasite with a prevalence of 20.5 %. The genotyping of the positive samples proved the presence of a typical canine assemblages C and D [46].

Studies in Belgium observed giardiasis in various dog categories. Prevalence in domestic dogs reached 9.3 % and dogs with gastrointestinal signs were positive in 18.1 % of cases while sheltered dog showed as high as 43.9 % positivity. In 80 % of the samples zoonotic assemblages A and B were identified [9].

The study conducted by Upjohn et al. investigated samples of faeces from 878 dogs in London and observed a prevalence of 21.0 %. The genotyping used identified assemblages C and D and in one sample a zoonotic subtype AIII [56].

The study conducted in 2013 in Tuzla municipality, Bosna and Hercegovina, determined the prevalence of *G. duodenalis* by the standard flotation method in the faeces from 134 dogs (79 from shelter, 44 with owners; 76 of dogs ≤6 months, 58 > 6 months) and detected the presence of *Giardia* spp. in 9/76 (11.84 %) of the ≤6 months old dogs and in 6/58 (10.34 %) of the dogs >6 months old [40].

Lassen et al. detected *Giardia* cysts in dogs in Estonia. During a 5-year study they determined a prevalence of 14 % in canine faecal samples 14 % [28].

The prevalence of *G. duodenalis* in faecal samples from farm and pet animals was investigated in Sweden. Forty samples from dogs were examined by the flotation method according to Faust and the microscopically positive ones were examined by PCR. The assemblages A, C, and D and one mixed infection caused by assemblages C + D were identified [29].

The examination of samples from 152 dogs in the Netherlands showed 15.2 % prevalence of *G. duodenalis*. The samples originated from veterinary clinics from a town and other rural areas. The ELISA method identified the coproantigen in 16 dogs and genotyping detected assemblages A, C and D and one mixed infection C + D [41].

A 9-year study (2007—2015) aimed at the determination of the prevalence of *G. duodenalis* in dogs was conducted in Zagreb. The flotation method according to Faust indicated the presence of cysts in 25.88 % of the samples. The subsequent study employing sequence analysis confirmed the assemblages C and D [5].

An epidemiological survey was conducted in Álava province in northern Spain with the aim of identifying the cysts of *G. duodenalis* in humans and animals. The fluorescence method was used for the proof of *Giardia* cysts in the samples. The total prevalence in dogs reached 29 % (16/55). Genotyping revealed the typical canine assemblage C [10]. In the same year, G i l l et al. identified assemblage D and sub-assemblages AII, BIII and BIV in sheltered dogs in the south of Spain. The circulating of these assemblages in dogs may present potential zoonotic risk [18].

In a recent study in Portugal, *G. duodenalis* was the most frequently detected parasite in dogs with gastrointestinal signs. The prevalence reached 15.5 %. No molecular methods were used for the identification of the assemblages [35].

Research published in Greece in 2017 revealed that the total prevalence of *G. duodenalis* in a study reached 25.2 %. Samples were examined first by the flotation method according to Faust and the positive isolates were subjected to genotyping. This proved the presence of assemblages C and D [27].

CONCLUSIONS

The diagnostic methods used for the proof of *G. duodenalis* are relatively simple. They include the basic microscopic examination for the presence of cysts in faecal samples; ELISA test for detection of coproantigen or the direct immunofluorescence method. However, only the molecular characterisation of *G. duodenalis* can guarantee the correct identification of assemblages and the confirmation of the potential zoonotic transmission. The PCR method for the identification of *G. duodenalis* has a mean 92 % sensitivity and 100 % specificity.

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OBSERVATION OF SEASONAL CHANGES OF SELECTED HEMATOLOGICAL PARAMETERS IN *TRACHEMYS* SPP.

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ABSTRACT

This study focused on observation of seasonal changes of selected haematological parameters in *Trachemys* spp. The experiment involved 6 turtles of the same species and approximately the same size. From September 2017 to December 2018 on a monthly basis, the samples of turtle blood were collected and blood smears were made, which were subsequently evaluated by the light microscopy. During the period of observation, we focused on the changes in the percentage of individual types of cells in a leucogram. The most significant differences in the percentage of leukocytes were observed during hibernation and during the summer period. The blood profiles revealed that the most heterophilic granulocytes were most abundant in the summer, when their values reached 50 % of the leukocytes. Significant changes in the percentage of heterophilic and basophilic granulocytes occurred during the period of hibernation. The number of heterocytes during hibernation decreased to 30 %. On the contrary, the number of basophils increased significantly to 33 %. Eosinophilic granulocytes, lymphocytes, and monocytes did not show such notable changes. The results of the study demonstrated that in turtles of the *Trachemys genus*, changes in their leucograms occurred during the period of hibernation. These changes are important for the evaluation of health condition of the turtles, determination of the prognosis and the treatment.

Key words: blood; blood cells; *Trachemys* spp.; turtle

INTRODUCTION

The red-eared sliders (*Trachemys scripta elegans*) are one of the favourite lizards kept by the public and frequently ending up as patients in a veterinary practice. Haematological and biochemical parameters of blood are used to evaluate the health of both humans and other animals. Several studies that focused on reptiles have described the general characteristics of their blood profile, but there are many species, the reference values of which, are still unknown or inaccurate. For the living individual the blood

profile presents the minimally invasive method which may help to evaluate its health; mainly if it is related to the determination of the relationship between such factors as environmental pollution or the occurrence of diseases. Such evaluations are dependent on the reliable reference values obtained from healthy animals. Blood parameters of reptiles may be influenced by many factors, such as age, sex, seasonality or reproduction [7].

In evaluation of haematological parameters in reptiles, the external factors, such as environmental conditions, which may enhance or suppress the response of an animal to a disease, should not be overlooked [15].

The aim of this study was to observe seasonal changes of selected haematological parameters in *Trachemys* spp., important for evaluation of their health, and to determine their prognosis and treatment.

MATERIALS AND METHODS

Materials

In our study we used the turtles of the *Trachemys scripta elegans* species as the objects of our examinations. We selected at random 6 turtles of approximately the same weight and size. The turtles were the possession of the AQUATERA club, a part of UVMP in Košice. The turtles were kept in indoor quarters with the following measurements: length 170 cm and width 80 cm. The average air temperature in the quarters was 26.5 °C throughout the year and the average temperature of the water was 24 to 25 °C. All of the animals were regularly fed fish, commercially produced feed, pinkies and salad twice a week. At the end of October, we started to prepare the turtles for their period of hibernation. With the falling temperature we reduced the amount of feed and in December, the turtles were placed in a room with a lower temperature, and feeding was stopped completely to cleanse their digestive tract. If the digestive tract had not been cleansed, both the intestines and their contents could have begun to rot. During the artificially simulated hibernation the turtles were replaced into plastic tanks. The water level in the tanks exceeded two levels of the width of the turtle shells. Firstly, the plastic turtle tanks were located in the room with the air temperature of 15 °C and water temperature of 13 °C. After one month spent in the room with the lowered temperature, the turtles were placed in the refrigerator. The hibernation period lasted three months with a constant temperature of 6 °C. The turtles would have been exposed to the same temperature decrease under their natural conditions. During hibernation the turtles did not receive feed. After the period of hibernation, they were returned back to their previous quarters. Gradually, we started to increase the temperature and serve the feed.

Sampling and processing of the samples

Blood samples were collected once a month between 8:00 and 10:00 a.m. Before sampling, the animals were subjected to a 3-day hunger strike to prevent the received feed from influencing the results of the experiments. The sampling was carried out from September 2017 to August 2018. During each sampling 0.2 ml of blood was withdrawn from each animal.

The sampling technique

Blood samples were taken from the subcarapaxial venous sinus. To drain the blood we used disposable syringes with the 2 ml volume and the needles of size 23 G (0,6 × 33 mm). The needle was injected dorsally behind the neck at approximately a 60° angle, while the syringe was held under a light vacuum [9]. The blood samples were processed immediately after the sampling.

Preparation of blood smears

The blood smear was prepared from the freshly drained blood immediately after the sampling. No anticoagulant preparations were used to prevent the damage of the blood elements or an obstruction in the differentiation of lymphocytes and counting the cells. To make blood smears we used degreased microscope slides.

Staining of blood smears

The blood smears were stained by the Diff-Quick staining technique, which is one of the most frequently used techniques in veterinary practice [15].

Evaluation of blood smears

The blood smears were evaluated under a light microscope of the type ZEISS Axio Lab. A1 in 1000x zoom with the use of immersion oil. The differential leukocyte count was determined manually from the stained blood smears. The first 100 leukocytes were counted.

RESULTS

Morphological differentiation of blood cells

We differentiated the blood cells according to many characteristics, such as size and shape of the cells. The individual granulocytes were differentiated according to the content of cytoplasm and the shape, colour and size of the granules. Other properties of the blood elements that helped us to identify the cells were the size, shape and structure of the nucleus, its colour and the content of chromatin.

We identified heterophilic granulocytes as large cells mostly of spherical or subspherical shape. The cytoplasm of the heterophils contained a huge amount of granules, which were of a spindle shape and a large size. The colour of the granules ranged from orange to brown-orange. The nucleus was always located eccentrically at the edge of a cell. The most common shape of the nucleus was oval, the signs of lobulalisation were sometimes visible, too. The content of the nucleus was of a violet colour (Fig. 1).

Eosinophilic granulocytes were identified as medium-size or large cells. The dominant feature of the cells was the presence of oval-shaped granules of brown-orange colour. The nucleus of an oval or round shape was present in the majority of the observed cells. The colour of the nucleus was dark violet or dark blue (Fig. 2).

Basophilic granulocytes were differentiated from other leukocytes by their intense colour. The cells were filled by blue-coloured granules of a different size. The nucleus was always located at the edge of a cell. During the summer period, the content of the basophilic cytoplasma was stained less intensively. During hibernation we observed a darker staining of the cytoplasmic structures (Fig. 3).

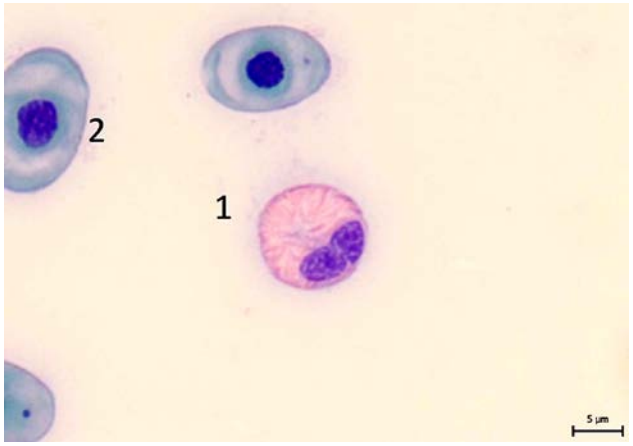

Fig. 1. Heterophil (*Trachemys scripta elegans*)
1—Heterophil; 2—Erythrocyte; Magn. ×1000; Diff-Quick staining

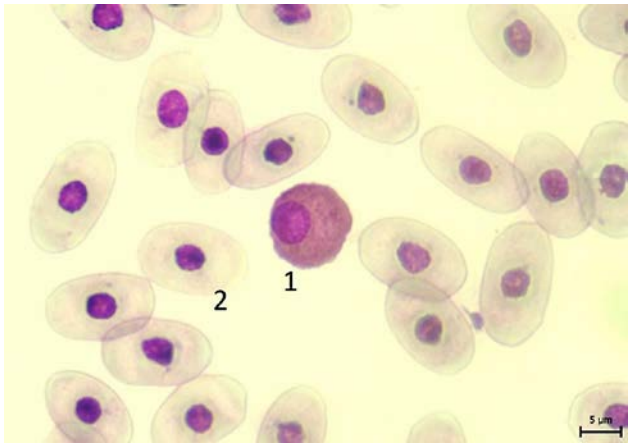

Fig. 2. Eosinophil (*Trachemys scripta elegans*)
1—Eosinophil; 2—Erythrocyte; Magn. ×1000; Diff-Quick staining

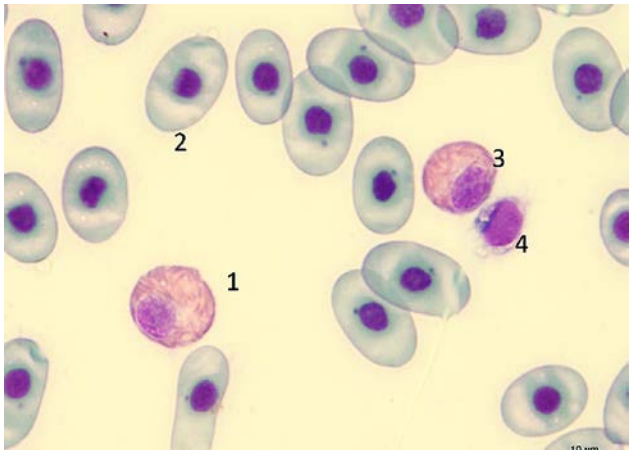

Fig. 3. Granulocytes (*Trachemys scripta elegans*)
1, 3—Heterophil; 2—Erythrocyte; 4—Basophil; Magn. ×1000; Diff-Quick staining

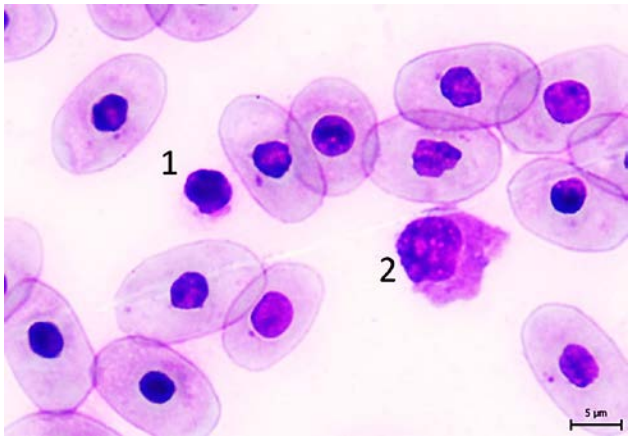

Fig. 4. Lymphocytes (*Trachemys scripta elegans*)
1—Small lymphocyte; 2—Monocyte; Magn. ×1000; Diff-Quick staining

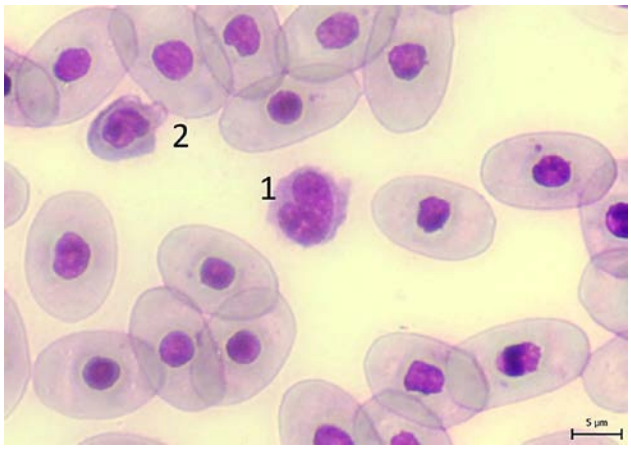

Fig. 5. Monocyte (*Trachemys scripta elegans*)
1—Monocyte with a typical kidney-shaped nucleus; 2—Erythrocytes. Magn. ×1000; Diff-Quick staining

Lymphocytes in the blood smear were differentiated on the basis of an absence of granules in the cytoplasma of the cells. A characteristic feature of the lymphocytes was their large nucleus surrounded by a narrow cytoplasmic rim. We identified the lymphocytes as small or medium-size cells (Fig. 4). The monocytes were identified as large cells. The nuclei of the monocytes were stained as basophilic and were of oval or kidney shaped (Fig. 5). The cytoplasm of the cells sometimes contained small azurophilic granules.

Determination of differential leukocyte count during the observation period

Fig. 6 captures the dynamics of changes in the percentage of heterophilic granulocytes in the leucogram during the period of observation. A significant decrease of the mentioned cells was observed during hibernation, i. e. from December to March, while the lowest value was observed in January (28 %). On the contrary, in the summer period, while the turtles showed the highest activity, we observed an increase in the heterophils. The highest percentage of heterophils was observed in September and October, when their values reached 53 %.

Fig. 7 depicts the average percentage of basophilic granulocytes in the leucogram during the period of observation. During hibernation, the significant increase of basophils in the peripheral blood was visible. The highest percentage of basophils was observed from December to March. During the summer we observed a decrease in the average percentage of basophils with the lowest value reached in May.

In the percentage of eosinophilic granulocytes we observed a slight variation during the whole observation period (Fig. 8). Higher values of eosinophils were observed during the period of hibernation, i. e. from December to March. From April to November the observed values were almost the same.

In the percentage of lymphocytes, we observed no significant differences in the leucogram during the whole observation period. A slight increase in their value was observed only in the time of preparation for hibernation and after hibernation (Fig. 9).

In Fig. 10 we present the percentage of monocytes in the peripheral blood. During the observation period, no significant changes in their value were observed. Neither hibernation, nor the summer period had an effect on the values of monocytes in the chosen species of turtles.

DISCUSSION

Nowadays we can find many published reports analysing the haematological or biological parameters of the

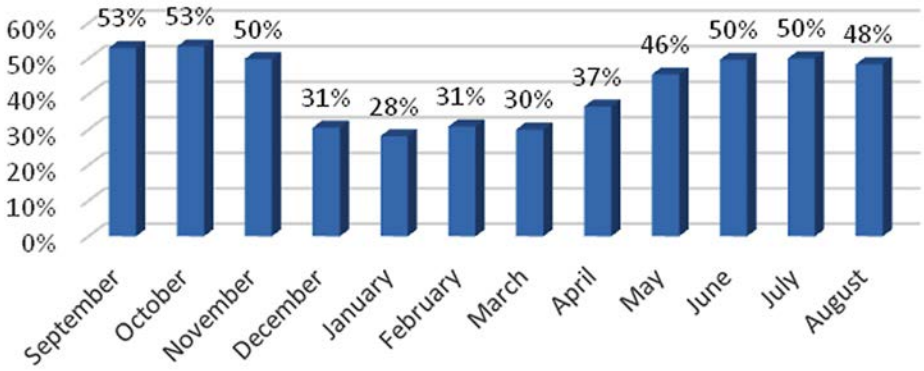

Fig. 6. The average percentage of heterophils during the observation period [%]

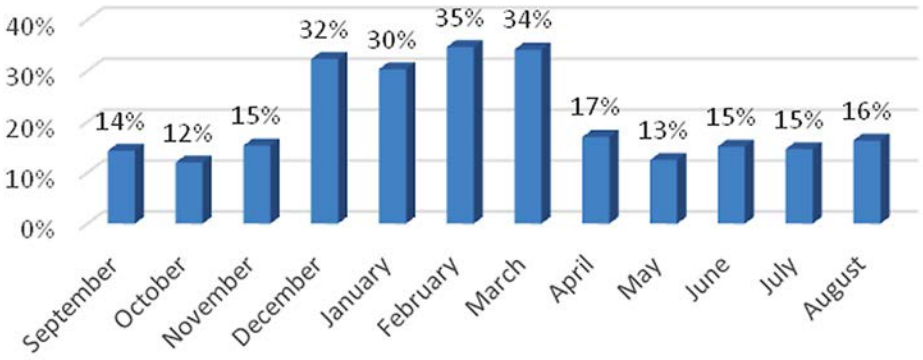

Fig. 7. The average percentage of basophils during the observation period [%]

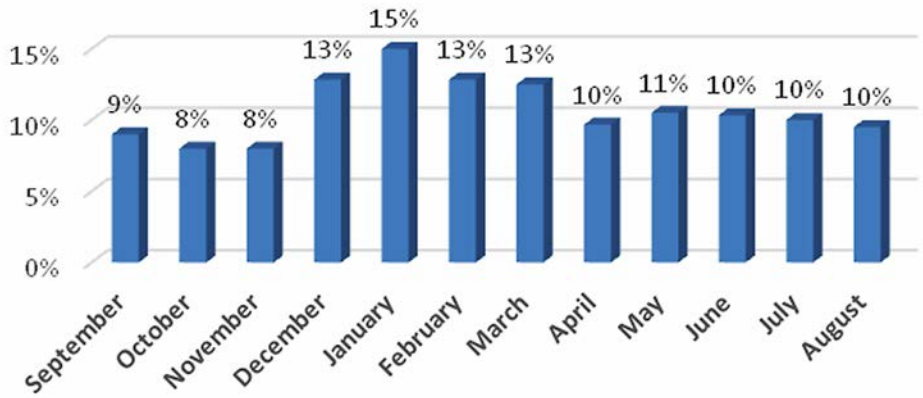

Fig. 8. The average percentage of eosinophils during the observation period [%]

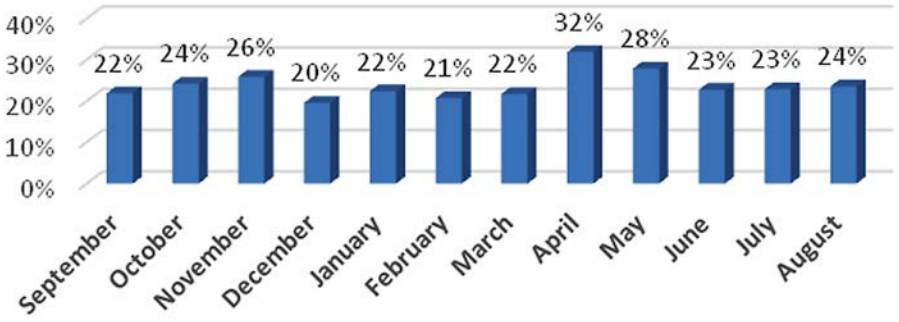

Fig. 9. The average percentage of lymphocytes during the observation period [%]

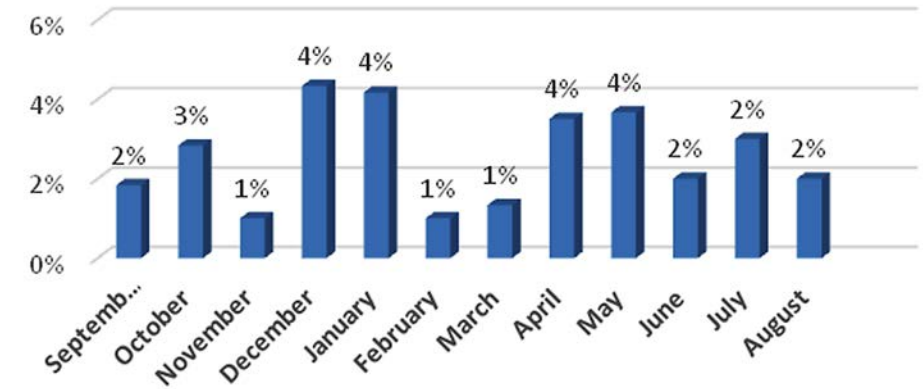

Fig. 10. The average percentage of monocytes during the observation period [%]

various species of turtles. These reports show that the haematological and biochemical parameters of blood may be influenced by sex, species, feed, hibernation, habitat, age and season [2]. Haematological results are useful for the evaluation of a physiological state of turtles providing relevant information for the diagnostics and determination of the prognosis of a disease [10]. For an accurate interpretation of a leucogram, it is necessary to use a method of manual counting of leukocytes in the blood smear due to the fact that reptile erythrocytes contain a nucleus misrepresenting the results while using an automatic cytometric method [14] .

Our experiment was focused on the effect of environmental factors on the haematological and biochemical parameters in *Trachemys scripta elegans*. The subject of our study was the effect of hibernation on the percentage of leucocytes in the leucogram. In our research, the differential count was significantly influenced by the season; differences based on sex were not observed. The decrease and increase of the percentage of individual types of leukocytes were observed in the period of hibernation as well as in the summer.

The most abundant type of cells in the leucogram were the heterophilic granulocytes during the summer period. However, we observed a significant decrease of heterophils during hibernation, when the temperature was lowered to 6 °C. During hibernation the number of basophilic granulocytes increased significantly. In the summer, the percentage of basophilic granulocytes decreased. Before and after hibernation we observed the increase of lymphocytes in the peripheral blood. Similar results were presented in the experiment of H e r n á n d e z et al. [6], who monitored the effect of the season on haematological parameters in *Trachemys scripta elegans*. The authors observed the increase in the number of heterophils during the summer, while in the winter, basophilic granulocytes had the highest percentage. Moreover, the percentage values of lymphocytes for both sexes increased in the winter period [6].

The research on *Mauremys leprosa* focused on haematological parameters which demonstrated that the percentage of heterophils ranged from 53.8 % up to 58.5 %; eosinophils from 35.3 to 32.6 %; lymphocytes from 6.3 % to 5.8 % and monocytes from 2 to 0 %. The percentage of heterophils was very similar to our own experiment. In comparison to our results, the number of eosinophils was significantly higher. Typical for this species is a low number of lymphocytes. Compared to our results, the number of lymphocytes was very low [7]. P i n - H u a n et al. [13] in the research of *Mauremys multica* focused on the effect of sex and season on haematological parameters and determined that the highest percentage of basophils was in the spring. The differential counts of heterophils and lymphocytes were significantly higher in the summer. The percentage values of monocytes were the lowest in the autumn. The white blood cells were significantly increased in the winter period, while the number of lymphocytes decreased. The results of this research corresponded with ours only partially—only in the percentage of heterophils and lymphocytes. The other experiment, in which the turtle species *Testudo graeca* and *Testudo hermanni* were observed, showed similar results as ours. The most abundant types of white blood cells were the heterophils in the summer. The basophils were the most numerous group of leukocytes in the winter [8]. The data in another study of various species of turtles presented a higher percentage of basophils in the peripheral blood in freshwater turtles and lower percentage in marine species [12].

Other previous studies revealed that the temperature had the effect not only on the cell-mediated, but also humoral immunity. In *Trachemys scripta elegans* the maximum antibody production and higher percentage of B-lymphocytes was observed at 28.8 °C; which may influence the immune response in turtles mainly during the hibernation period when the temperature in the environment is low [11]. Another study on the lymphocytes of *Trachemys scripta elegnas* claimed that both B- and T-lymphocytes make up from 38 to 45 % of leukocytes [16]. Our leucogram research did not confirm these results, since the lymphocytes made only 21 % of the overall number of leukocytes. We can find many studies dealing with haematological parameters of turtles. Many of them disagree, while others may confirm their results. The disagreeing results may occur due to the variety of turtle species, environmental effects, the effects of season, feed or sex. All of these factors should be considered in the evaluation of the turtles' health conditions. There are many publications on the classification of the white blood cells and many authors present different opinions.

The existence of an eosinophilic granulocyte in various species of reptiles is frequently discussed. However, some authors claim that there are no eosinophils in reptiles [1]. In our experiments the existence of eosinophils was confirmed. They were identified also in another studies on the

haematology of turtles [4, 5, 6]. Azurophils are one of the most discussed white blood cells. Some authors classify this type of cell as a developmental stage of monocytes and in relation to turtles they do not include this type into the differential white blood cell count [3]. In the evaluation of blood smears, no cells corresponding with the characteristics of azurophils were found.

CONCLUSIONS

The objective of our study was the evaluation of the effect of seasonal changes of the environment on haematological parameters of *Trachemys scripta elegans*. We evaluated the impact of environment temperature on the percentage of leukocytes in the peripheral blood of the turtles. Our results allowed us to conclude that the most significant changes in the leucogram were observed during the periods of hibernation and in the summer. During hibernation, the percentage of heterophilic granulocytes decreased to 30 %. In comparison to the reference value, which we determined to be 42 %, it is a significant change. On the other hand, the percentage of basophilic granulocytes during hibernation increased to 33 %—the reference value for basophils was 21 %. During hibernation, the number of eosinophilic granulocytes in the peripheral blood increased only mildly to 13 %. We observed changes in the leucogram also in the summer period, when the average percentage of heterophilic granulocytes increased to 50 % and the percentage of basophilic granulocytes decreased to 15 %. Lymphocytes showed only slight changes of the percentage to 22 % during hibernation. The monocytes were the cells on which the seasonality had no influence.

The detection of physiological changes in the leucogram related to seasonal changes in turtles may help to evaluate their health, identify pathological processes and determine the treatment of the turtles. This is important mainly due to inflammatory processes in the organism which may occur during hibernation or as a consequence of weakening of the organism related to the post-hibernation syndrome.

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OCCURRENCE OF *MALASSEZIA* SPP. ON HEALTHY HUMAN SKIN

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ABSTRACT

The genus *Malassezia* currently includes seventeen species that have been isolated from healthy and diseased human and other animal skin. *Malassezia* are implicated in a range of cutaneous diseases in humans: pityriasis versicolor, atopic or seborrheic dermatitis, dandruff, folliculitis and psoriasis. The outbreak of the disease depends on the interaction between the host immune system and *Malassezia* species. *Malassezia* stimulates both the cellular and humoral immune response in humans. Although *Malassezia* species have been associated with various dermatological diseases in people, the detailed pathological role of *Malassezia* remains obscured. *Malassezia* yeasts require lipids for their growth and therefore to a greater extent they colonize the sites with more sebaceous glands. The ecosystem on skin is complex and its balance depends on several factors. The aim of this study was to determine the presence of *Malassezia* yeasts in clinically normal skin of 42 healthy, randomly selected individuals of different ages. In the group of people examined, up to 30 persons (71.4 %) represented by children, adults and the elderly were positive to *Malassezia* yeasts. It has been shown that the back is an area with a higher incidence (66.7 %) of observed yeast compared to the head (40.5 %).

Key words: healthy skin; *Malassezia*; man; occurrence; yeasts

INTRODUCTION

The lipophilic yeasts *Malassezia* are usually members of the normal skin flora and mucosa in warm-blooded humans and other animals. At the same time *Malassezia* can act as an opportunistic organism that under certain conditions can become pathogenic and they may cause various diseases of skin or fungaemia [9]. The pathophysiology of *Malassezia*-caused or *Malassezia*-exacerbated skin conditions is largely unknown [26]. A range of skin microenvironmental factors, such as the bacterial microbiota, pH, salts, immune responses, biochemistry, and physiology, may play a role in the adherence and growth of *Malasse-*

zia species, favouring distinct genotypes depending on the geographical area and/or the skin sites involved [8].

Malassezia yeasts are associated with many dermatological disorders of the human skin, such as: atopic dermatitis, dandruff, folliculitis, pityriasis versicolor (PV) or seborrheic dermatitis and intravascular catheter-acquired infections [15]. They have participated in different skin disorders in animals, especially otitis externa and dermatitis [6]. In animals, only one species —*M. pachydermatis*, is important. All *Malassezia* species are lipid-dependent, except for *M. pachydermatis* that only one which is lipophilic. The genus *Malassezia* currently includes 17 species: *M. globosa, M. restricta, M. slooffiae, M. obtusa, M. furfur, M. sympodialis, M. japonica, M. yamatoensis, M. dermatis, M. pachydermatis, M. caprae, M. equina, M. nana, M. cuniculi, M. vespertilionis, M. brasiliensis* and *M. psittaci*. These can be divided into three groups according to the hosting organism: *Malassezia* occurring only in animals (*M. vespertilionis, M. brasiliensis, M. psittaci, M. caprae, M. equina, M. cuniculi* and *M. nana*), *Malassezia* occurring primarily in humans (*M. dermatis, M. japonica, M. obtusa, M. restricta, M. yamatoensis*) and *Malassezia* occurring both in humans and other animals *(M. furfur, M. globosa, M. slooffiae, M. sympodialis* and *M. pachydermatis*) [3, 4, 14].

In humans, *Malassezia* yeasts inhabit sebum-rich areas of the skin, including the trunk and the head region (face and scalp). The skin ecosystem is complex and its balance depends on several factors. Sebaceous gland activity in children is small, but increases with age according to androgen production.

The aim of this study was to determine the presence of *Malassezia* yeasts from clinically normal skin of healthy randomly selected human individuals of different ages.

MATERIALS AND METHODS

The survey was carried out on 42 clinical healthy persons with no skin diseases and without any known underlying disease. Six children (4 boys and 2 girls) aged 4 to 11 years old and 36 adults (9 men and 27 women) aged 22 to 62 years were examined. From each person, two samples originating from different anatomical sites of the body that is the hair area of head and back (interscapular region) were collected by a standard swab method. A sterile cotton swab (Fungi-Quick, Dispolab) soaked with sterile saline was used to rub against the skin surface, with continuous rotation of the swab over at least 15 seconds, and immediately applied evenly onto three agar media. The samples were inoculated on specific media for the culturing of *Malassezia*: Sabouraud dextrose agar with chloramphenicol

Table 1. Sabouraud dextrose agar with chloramphenicol—composition

Substance	Quantity per 1 litre of medium
Enzymatic digest of casein	5 g
Enzymatic digest of animal tissue	5 g
Dextrose monohydrate	40 g
Chloramphenicol	0.05 g
Agar	15 g

Table 2. Modified Leeming & Notman agar (MLNA)—composition

Substance	Quantity per 1 litre of medium
Mycological peptone	10 g
Glucose	10 g
Yeast extract	2 g
Bovine bile	8 g
Glycerol	10 ml
Glycerol monostearate	0.5 g
Tween 60	5 ml
Olive oil	20 ml
Agar	15 g

Table 3. Modified Candida-Chrom agar—composition

Substance	Quantity per 1 litre of medium
HiCrome Candida differential agar	42.72 g
Mycological peptone	15 g
Yeast extract	4 g
Dipotassium phosphate	1 g
Chloramphenicol	0.5 g
Chromogenic mixture	7.22 g
Tween 40	10 ml
Agar	15 g

(Table 1), Modified Leeming and Notman agar medium (Table 2) and Modified Candida-Chrom agar with Tween 40 (Table 3). The yeasts were incubated on growth media at 32°C and were monitored every day for two weeks. The identification of yeasts was based both on macroscopic appearance of colonies and microscopic cell morphology. Each sample was stained by Gram staining and examined by a microscope for the presence of typical *Malassezia* yeast cells (Fig. 1). The identification of *Malassezia* genus was confirmed by their morphological and physiological properties according to K a n e k o et al. [11].

RESULTS

Thirty of the 42 persons scored positive for *Malassezia* spp. (71.4 %) from which 28 persons had a confirmed occurrence of *Malassezia* spp. on their back. Seventeen of the people had *Malassezia* yeasts on their head. Synchronously, *Malassezia* was confirmed in 13 samples from both the head and back (Table 4). A total of 45 positive samples (53.6 %) were obtained. No sample was identified as *M. pachydermatis*, since all isolated yeast species were lipid-dependent.

Out of the 6 children, 2 children had *Malassezia* present at both areas mentioned, while in another 2 children it was diagnosed only on their back. Of the 9 men, *Malassezia* has been confirmed in 4 persons both on the back and the head. In 7 cases, *Malassezia* yeasts were identified on the back and in 4 cases on the head. There were 22 women in the group, 15 of whom had *Malassezia* on the back and 9 on the head. Concurrently, 7 women had *Malassezia* on both body areas.

Table 4. Results of human samples

Number of individuals with confirmed *Malassezia* spp.	30 (71.4 %)
Number of *Malassezia* isolates from the back	28 (66.7 %)
Number of *Malassezia* isolates from the head	17 (40.5 %)
Number of *Malassezia* isolates from both sites	13 (31.0 %)
Total	**42**

DISCUSSION

Most of the available articles have focused on the incidence of *Malassezia* in humans in connection with skin diseases such as pityriasis versicolor, psoriasis, atopic and seborrheic dermatitis, dandruff and folicullitis. *Pityriasis versicolor* is the only cutaneous disease etiologically connected to *Malassezia* yeasts. In other dermatoses, such as

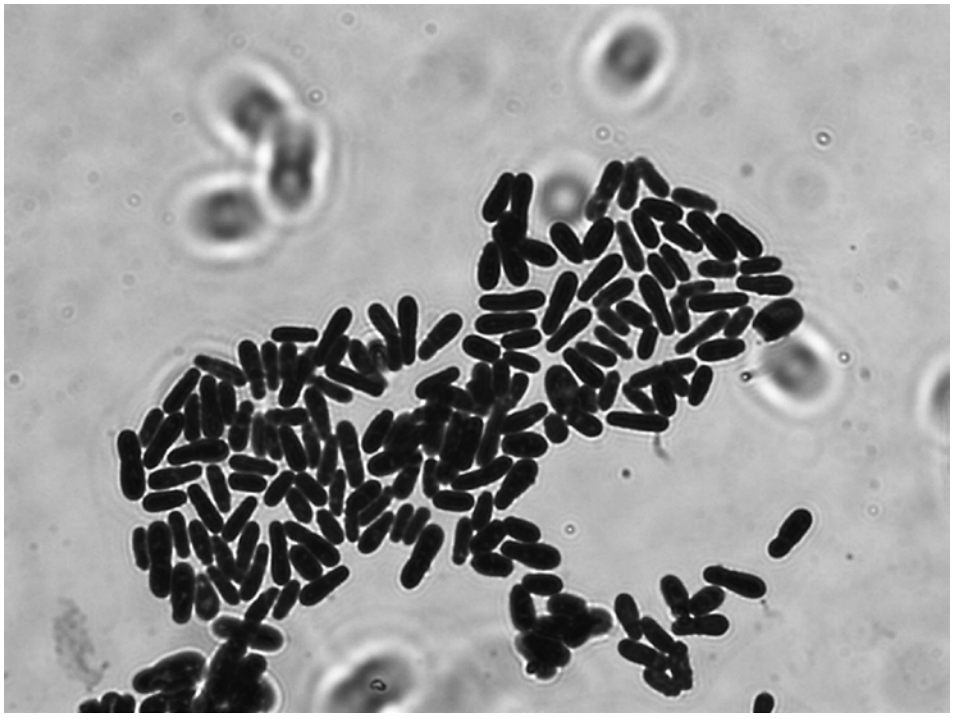

Fig. 1. Isolated *Malassezia* cells

Malassezia folliculitis, seborrheic dermatitis, atopic dermatitis, and psoriasis, these yeasts have been suggested to play pathogenic roles either as direct agents of infection or as trigger factors because there is no evidence that the organisms invade the skin [20]. Pityriasis versicolor is a superficial fungal infection of the skin caused by *Malassezia* species that induces a characteristic rash of well demarcated, thin, scaly plaques that can be hypopigmented, hyperpigmented, or erythematous [10].

Seborrheic dermatitis is a chronic relapsing inflammatory skin disorder clinically characterized by scaling and poorly defined erythematous patches. Although the cause of seborrheic dermatitis has yet to be understood, *Malassezia* yeasts, hormones (androgens), sebum levels and immune responses are known to play important roles in its development. Additional factors including drugs, winter temperatures and stress may exacerbate seborrheic dermatitis [2].

The pathophysiology of atopic dermatitis is complex and multifactorial, involving elements of barrier dysfunction, alterations in cell mediated immune responses, IgE mediated hypersensitivity, and environmental factors. One of several factors that complicate atopic dermatitis is *Malassezia* [7].

Malassezia folliculitis is a form of folliculitis caused by yeast infections. It is characterized by dome-shaped papules, pustules, nodules, and cysts in severe cases [21]. Formation of dandruff is a common but complex event which has been associated with numerous causal factors. An imbalance between physiological factors (pH, water content, or sebum secretion) can disturb the physiological equilibrium of the scalp that can lead to dandruff formation. However, the severity of the condition is strongly related to *Malassezia* yeasts [16]. Psoriasis is a chronic inflammatory skin disease, whose pathogenesis involves dysregulated interplay among immune cells, keratinocytes and environmental triggers, including microbiota, such as *Malassezia* spp, *Streptococcus* spp., *Micrococcus* spp., *Staphylococcus* spp.) [22].

Although *Malassezia* species is associated with various dermatological diseases, the exact pathological role of *Malassezia* yeasts remains obscure. *Malassezia* yeasts are commonly found on the skin of healthy people and do not usually cause any health problems. However, in some interplay of conditions (immune deficiency, genetic predisposition, systemic diseases, skin diseases, etc.), it can cause unpleasant skin disorders. A y h a n et al. [1] and N a g a t a et al. [17] describe the gradual settlement of baby skin by *Malassezia* immediately after birth. L e e et al. [12] found that the distribution of *Malassezia* on the body depends on the amount and type of lipids present on the skin. Even in adolescents during puberty, but generally also in adults, the incidence of *Malassezia* depends on the activity of the sebaceous glands.

In women the highest incidence of yeast in the population was recorded from the chest, upper back and forehead, while men yielded more yeasts on the lower back and thigh areas compared to women [13]. Some authors prefer that *Malassezia* microbiota of healthy skin is gender-dependent and changes with age. We assume that assessing the incidence of *Malassezia* yeasts in certain groups (age, gender) is of little importance, even if it has a certain informative value. The *Malassezia* occurrences should be assessed in a particular individual. In our group of people, we have found that *Malassezia* is fairly common on the skin of healthy people, whether children or adults. *M. pachydermatis* was not recorded from any of our samples either from the trunk or from the healthy scalp skin. This species is better adapted to animals, although it can be involved in some systemic human infections. The presence of this species on human skin is rare and transient, occurring possibly by transmission from pets or environmental sources [19].

In one group of people examined, we detected only lipid-dependent *Malassezia* yeasts, both in children and adults. Of the 42 subjects, up to 30 were positive for *Malassezia* (71.4 %), with the most common being detected on the back in up to 28 people (66.7 %). Of these, in 13 samples (31 %) *Malassezia* yeasts were present on both body areas concurrently. Other authors also consider these body areas as typical for the occurrence of *Malassezia* yeasts and this is explained by the higher number of sebaceous glands. Of the lipid-dependent species, *M. restricta* is most common on the head and *M. sympodialis* with *M. globosa* on the torso [5, 12, 18, 23, 24, 25].

CONCLUSIONS

Malassezia species have been associated with a variety of dermatological disorders. Certain environmental, genetic, and immunological factors can predispose to this pathogenic influence and contribute to the development

of the disease. Ten *Malassezia* species have been isolated in humans, namely *M. furfur, M. sympodialis, M. slooffiae, M. pachydermatis, M. globosa, M. obtusa, M. restricta, M. dermatis, M. japonica,* and *M. yamatoensis*. The most common site of occurrence is the scalp and the trunk. Up to 71.4 % of the person were positive for *Malassezia* yeasts and their highest incidence was confirmed on the back area (66.7 %). The results show that the *Malassezia* spp. are part of the normal skin microflora in healthy people and they usually do not cause any clinical problems.

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OPTIMAL CRITERIA FOR THE SELECTION OF PROBIOTICS, BASED ON THEIR MODE OF ACTION

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ABSTRACT

The objective of this review was to discuss some of the criteria which influence the selection of microorganisms with probiotic properties based on their mode of action. The most common bacteria that belong to the "group" probiotics are the *Lactobacillus* and *Bifidobacterium* species/strains. Probiotics have benefits and effects by their mechanism of action in different axial locations such as: producing substances, influencing immune function and response, modification as well as maintenance of a healthy population of microorganisms in the intestinal environment. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases Potential peripheral pathways that link probiotic ingestion in the brain function are focused on the role of the vagal afferent nerve signalling and changes in the cerebral levels of neuromodulators. The application of probiotic microorganisms represents a way to effectively influence the composition of the intestinal microbiome and the immune system of the host, as well as they can be considered as a suitable alternative to influence a healthy quality of life.

Key words: additives; immunity; intestinal tract; probiotic; properties; selection; technology

INTRODUCTION

The administration of live organisms is not without risk, particularly in certain populations. The important question is to determine if the health benefits of probiotics or even components/products of these agents can be successfully attained without the risks associated with the administration of a live organism to a host [31].

The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases, but the mechanisms responsible for these effects have not been fully characterized yet. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune

system to convey an advantage to the host [5]. By adhering to the alimentary tract, probiotic organisms may survive difficult conditions, and offer a beneficial effect on the stability and protection of the intestinal environment. They also influence the course of digestive and metabolic processes and the immunological response, leading to an improved health and an increased productivity of the animals [28].

Lactobacillus, Bifidobacterium, Enterococcus and several other microbial species are perceived to exert such effects by changing the composition of the gut microbiota [32]. The mucus layer of the intestinal tract plays an important role in forming the front line of innate host defence. The involvement of natural substances feeding on protection/prevention/promotion of mucus production in the intestinal environment is beneficial. The intestinal mucus forms enterocytes covered by transmembrane mucins and goblet cells secreting by the secreted gel-forming mucins (MUC2). The goblet cells continually produce mucins for the retention of the mucus barrier under physiological conditions, but different factors (e. g. microorganisms, microbial substances, viruses, cytokines, enzymes etc.) can have profound effects on the integrity of the intestinal epithelium covered by a protective mucus gel composed predominantly of mucin glycoproteins [59].

The microbiota, the intestinal epithelium, and the mucosal immune system constitute the gastrointestinal ecosystem. All three components are essential for the complete function and development of the system. Probiotics can influence immune function through a number of different pathways including effects on enterocytes, antigen presenting cells including both circulating monocytes and local dendritic cells (DC), regulatory T cells, and effector T and B cells. The mechanisms of action of probiotics involve modification of the microbial population, aggregation with pathogenic bacteria, competitive adhesion to epithelial receptors, competition for nutrients, modification of the structure and function of the intestinal epithelium and production of specific substances (e. g. bacteriocins, organic acids—lactic acid, hydrogen peroxide, biosurfactants, adhesion inhibitors, co-aggregation molecules) [63]. Certain probiotic microorganisms can enhance the function of the intestinal barrier through modulation of the phosphorylation of cytoskeletal and tight junction proteins and thereby influencing the intestinal mucosal cell to cell interactions and cellular "stability" [48].

Probiotics—definition and the mode of action

The definition of "Probioticum" was formulated in 1974, simultaneously with the use of living cultures in feed for various animals in order to substitute the application of nutritive antibiotics or chemotherapeutics [51]. The current defnition formulated in 2002 by FAO and WHO experts defines probiotics as "live strains of strictly selected microorganisms administered in adequate amounts and confer a health benefit on the host" [19]. The definition was in 2013 maintained by the International Scientific Association for Probiotics and Prebiotics (ISAPP). The term "probiotic' is reserved for formulas/products that keep some strictly defined criteria including: an appropriate count of viable cells, a beneficial effect on a host's health involving stimulation of growth, and a beneficial effect on the function of the gastrointestinal tract. Also the selection of bacterial strains with probiotic properties (probiotic cultures) and their application in a correct form as well as dose administered directly per os or as an additive to feed and premixes are highly important [43]. The selection of a suitable strain of a microorganism can be regarded as the primary requirement for the use as a probiotic. These cultures must be able to pass the stomach-duodenum barrier in a viable state and to multiply at the site of destination in the intestine. Additionally, they must be capable of producing antagonistic metabolites against a dominating saprophytic microflora resulting in a competitive growth. These abilities are common among lactic acid bacteria, e.g. lactobacilli bifidobacteria and enterococci. The special efficacy of probiotics must be strictly verified in animal nutrition, in pharmacy, and in food applications in accordance with law regulations. Safety aspects are considered very restrictively in feed applications, replace the presently reduced or even prohibited application of nutritive antibiotics or chemotherapeutics in animal nutrition [51]. Along with the intensive development of methods of livestock breeding, breeders' expectations are growing concerning feed additives that would guarantee such results as accelerating growth rate, protection of health from pathogenic infections and improvement of other production parameters such as: absorption of feed and quality of meat, milk and eggs. The main reason for their application would be to achieve some benefcial effects comparable to those of antibiotic based growth stimulators, banned in the European Union in 2006. High hopes are being associated with the use of probiotics, prebiotics and synbiotics as the alternative natural substances in animal nutrition [43].

In the USA, microorganisms used for consumption purposes should have the Generally Regarded As Safe (GRAS) status, regulated by the US Food and Drug Administration (USFDA). In Europe, the European Food Safety Authority (EFSA) introduced the term of Qualified Presumption of Safety (QPS), which involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics [21]. In 2006, the EFSA established the Nutrition and Health Claims regulation (Reg. 1924/2006) which was updated by QPS under EFSA's Panel on Biological Hazards during 2008 and 2009. The presence of transmissible antibiotic resistance markers in the evaluation of the strains has been established as the important health criterion. Following these rules, microbes claimed as probiotic in food/feed are supposed to be QPS probiotic (e. g. *Lactobacillus* sp. or *Bacillus* sp.) and non-QPS probiotic (e. g. *Enterococcus faecium*) [50]. According to this assessment, some *Enterococcus faecium* strains can be used for this purpose [33].

Basic properties of potentially probiotic organismS

The major properties of selected probiotic strains include their safety of human/animal origin, the isolation from healthy organism, the detection based on the phenotypization and genotypization, survival in dynamic variations of pH, viable cell counts, the absence of genes responsible for antibiotic resistance (also confirmed plasmid encoded antibiotic resistance) [2], the absence of the production of virulence factors (due to evaluation of genes encoding potential virulence factors e. g. cytolysin cylA, cylB, cylM; collagen-binding protein ace; gelatinize gelE; aggregation substance agg; cell wall anchored collagen adhesion acm; enterococcal surface protein esp) [47], bacterial adhesion to hydrophobic compounds, acid and bile salts tolerance, resistance to enzymes, stimulated gastrointestinal tract tolerance, cell adhesion/hydrophobic characteristics, the ability to colonize and survive in a beneficial dose and competitiveness in part of the intestinal tract, killer toxin productivity and antimicrobial activity against some clinical and food borne pathogens and survivability during simulated gastrointestinal transit. Subspecies were identified by partial 16S rRNA gene sequencing [55, 43, 68]. Probiotic use may help decrease the rate of development of antibiotic-resistant strains secondary to widespread antibiotic use. Given the emerging risk of spreading antibiotic resistance genes through probiotic strains, the qualified presumption of safety (QPS) is considered by many as the more applicable and flexible probiotics criteria [17, 18]. It is important that no other substances are used while probiotics are administered. An interval of 24—48 h between the end of antibiotic therapy or administration of any other antimicrobial agents and the beginning of the therapy with probiotic organisms presented in appropriate amounts 10^9 CFU.kg^{-1} of feed is very important [54]. Probiotics are gaining more interest as alternatives for antibiotics or anti-inflammatory drugs, modulate the host's immune system, affect other microorganisms or act on microbial products, host products or food components. What kind of effect(s) a certain probiotic executes depends on its metabolic properties, the molecules presented at its surface or on the components secreted. Even integral parts of the bacterial cell such as its DNA or peptidoglycan might be of importance for its probiotic effectiveness. The individual combination of properties in a certain probiotic strain determines its specific probiotic action and as a consequence its effective application for the prevention and/or treatment of a certain disease [49].

The resistance of probiotics toward technological processing

Probiotics are available commercially in many forms, including foods, dietary supplements, and clinical therapeutics with oral or non-oral delivery, e.g. lactic acid producing genera such as the bifidobacteria or lactobacilli or enterococci. To be a candidate for commercialization, a probiotic must retain its properties during large-scale industrial preparation and remain stable during storage and use. The probiotic should be able to survive in the intestinal ecosystem and the host animal should gain beneficially from its presence. Clearly, the organisms used should be generally regarded as safe due to USFDA as well as EFSA regulations or well documented in the literature [64].

Probiotic bacteria as probiotics used in technologies of pharmacological industry may be exposed to various environmental stresses during industrial production steps, including drying and storage, and during the digestion process. In accordance with their adaptation as well as survival to environmental conditions, they possess adaptation mechanisms, which can be induced by pre-treatments including the accumulation of compatible solutes and of energy storage compounds, which can be largely modu-

lated by the culture conditions. The regulation of energy production pathways, the modulation of the cell envelope, i.e. membrane, cell wall, surface layers, and exopolysaccharides leads to the overexpression of molecular chaperones and of stress-responsive proteases. Matrix components, such as proteins, carbohydrates and flavouring agents have been shown to alter probiotic efficacy and viability [20].

Many of the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. The probiotic paradox is that both live and dead cells in probiotic products can generate beneficial biological responses. Live probiotic cells influence both the gastrointestinal microflora and the immune response whilst the components of dead cells exert an anti-inflammatory response in the gastrointestinal tract. Heat-killed cells of *Enterococcus faecalis* stimulate the gastrointestinal immune system in chicks. Dead bifidobacteria induce significant increases in TNF-α production. Administration of heat-killed *E. faecalis* to healthy dogs increases neutrophil phagocytes [9].

The heat-killed, ultraviolet-inactivated, and even cell walls microencapsulated components of probiotics may be safer for the host. They finally lead to the overexpression of molecular chaperones and of stress-responsive proteases. Triggering these adaptive mechanisms can improve the resistance of beneficial bacteria toward technological and digestive stresses [22]. In fact, the microencapsulation of probiotics (probiotics in coated protected form) with specific materials is able to confer a significant resistance to gastric juice, thus protecting the probiotic cells during the gastric and duodenal transit and enhancing the probiotic efficacy [12]. Microencapsulation is a process by which individual particles or droplets of solid, liquid or gaseous material (the core; the intrinsic part) are surrounded or coated with a continuous film of material (the shell; the extrinsic part) to produce capsules in the micrometre to millimetre range, known as microcapsules (have a spherical or irregular shape). Compatibility of the core material with the shell is an important criterion for enhancing the efficiency of microencapsulation [23, 65]. There are some techniques which are used for microencapsulation, such as chemical (suspension, dispersion, emulsion, and polymerization); physicochemical (layer by layer assembly, sol gel encapsulation, supercritical CO_2 extraction); physico mechanical (spray drying, fluid bed coating, electrostatic encapsulation) [7]. Microencapsulation has been proven to be one of the most effective methods for maintaining high viability and stability of probiotic bacteria, as it protects probiotics both during food processing and storage as well as in gastric conditions [12, 52]. Chandramouli et al. [26] shoved that the coating of the calcium chloride on sodium alginate capsules containing *L. acidophilus* increased tolerance of the bacteria against harsh acidic (pH 2) and bile (1 %) conditions. The microencapsulation techniques using an alginate microparticulate system potentiality of various coating polymers such as chitosan and polylysine improved the stability of microencapsulation [12, 27].

In conclusion, probiotic cultures added to feed should be resistant to temperatures and pressures used in the process of pelleting, and to humidity and the effect of adverse substances during feed handling and storage, such as heavy metals or mycotoxins. The period of high activity of probiotics in feed and premixes must not be shorter than 4 months [46].

Immunity, intestinal mucosa and probiotics

The mucosal immune system must constantly monitor the environment and maintain a balance between tolerance to the normal microbiota and immunity to microbial pathogens while the systemic immune system is designed to vigorously react to any foreign antigen or microbe [11]. Development of 16S ribosomal RNA (rRNA) gene-sequence-based metagenomic methods has led to major advances in defining the total microbial population of the gut. This technique has been used to show that 90% of the bacteria belong to two phyla, the Bacteroidetes and Firmicutes [64]. The presence of beneficially acting bacteria in the intestine can influence the host and bacterial microenvironment to protect the homeostasis and effective immune response. IgA antibodies belong to the most important humoral immune factors present on mucosal surfaces.

Different defence mechanisms are involved in the permanent and effective surveillance of mucosal surfaces. Bacterial behaviours depend not only on the bacterial species, but also on the host. Commensal bacteria have been directly associated with the proper development of gut-associated lymphoid tissues. Mucosal antibodies inhibit the adherence of microorganisms and protect the host against absorption of antigens from mucosal surfaces [25]. Mucosal surfaces comprise various lymphoid structures collectively referred to as mucosa-associated lymphoid tissue (MALT) [40]. This secondary lymphoid organ can be further divided into functionally connected subregions, including

the gut-associated lymphoid tissue (GALT). A key component of this interface is the mucosal epithelium, which blocks invasion by pathogenic and commensal bacteria by forming multiple layers of immune protection [1] as well as maintaining the host–microbiota relationship in a dynamic homeostasis [53].

Enterocytes have a role not only in the digestion by ensuring the uptake of ions, water, nutrients, vitamins and absorption of unconjugated bile salts, but also in the induction of immunological tolerance to ingested proteins [45]. The epithelial barrier protects the internal medium from food antigens as well as from bacteria. The distal small intestine, caecum and colon have higher bacterial colonization levels than the proximal part. The small intestine contains lower numbers of commensal bacteria and contains higher levels of nutrients available for absorption. The small intestine has higher numbers of intraepithelial T cells; it also harbours lymphoid structures such as Peyer's patches and Paneth cells producing anti-microbial peptides [45]. The intestinal mucus layer is a balance of mucin secretion and degradation. This mucin layer creates an obstacle to proinflammatory compounds and uptake of antigens.

The intestinal lumen consisting of gastric acid, digestive enzymes and IgA constitutes the first line of defence and is lethal to invading and ingested pathogenic bacteria. The indigenous microbes degrade intraluminal antigens and inhibit the pathogenic microbes from adherence and colonization. They are also necessary for the induction of regulatory T cells [64]. The barrier function of the enterocytes is completed by anti-microbial peptides and mucin proteins production [45]. The administered probiotics stimulate the mucosal immune system (MIS) of the intestinal tract and induce signals mediated by the bacteria or their cell wall structure. Consumed probiotic bacteria interact with the intestinal epithelial cells (IEC) or immune cells associated with the lamina propria, through pattern recognition receptors such as Toll-like receptors (induce the production of different cytokines or chemokines) and nucleotide binding oligomerization domain-containing protein-like receptors, which modulate key signalling pathways, such as nuclear factor-κB and mitogen-activated protein kinase [5, 64]. The Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors play a key role in pathogen recognition and in the induction of innate effectors and inflammation. Pattern recognition receptors signalling in the IEC serve to maintain the barrier functions of the epithelium, including the translocation from the lamina propria in the intestinal lumen and the production of secretory IgA (sIgA). The IEC play a role in the immunosuppressive effect of the mucosa by inhibition of an overreaction against innocuous luminal antigens (due to the regulation of dendritic cells, macrophage and lymphocyte functions by epithelial secreted cytokines) [45, 67].

Macrophage chemoattractant protein 1 (produced by the IEC) sends signals to other immune cells leading to the activation of the MIS, characterized by an increase in immunoglobulin A+ cells of the intestine, and the activation of T cells (specifically activation regulatory T cells that release interleukin IL-10) [41]. Secretory sIgA antibodies at mucosal surfaces serve as the first line of defence against microorganisms through a mechanism called immune exclusion, fight pathogens without the damage of epithelial cells and improve the immune balance of the epithelial barrier through selective adhesion to M cells in intestinal Peyer's patches. In Peyer's patches, sIgA-based immune complexes are internalized by underlying antigen-presenting cells, leaving the antigen with masked epitopes, which translates into the onset of mucosal and systemic responses associated with the production of anti-inflammatory cytokines [10].

In conclusion, probiotics reinforce the intestinal barrier by an increase of the mucins, the tight junction proteins and the Goblet and Paneth cells, modulate intestinal microbiota by maintaining the balance and suppressing the growth of potential pathogenic bacteria in the gut [41].

The effect of the oral administration of probiotic bacteria cell walls as a new oral adjuvant in the stimulation of the immune system in healthy mice on IEC which are essential for coordinating an adequate mucosal immune response and on the functionality of macrophages was evaluated. The cell walls were able to stimulate the IEC exhibiting an important activation and cytokine releases as well as promoted macrophage activation from peritoneum and spleen, improving the functionality of the macrophages and increased IgA-producing cells in the gut lamina propria [37].

Some commensals are able to stimulate local immune response as shown in the case of the application of *Enterococcus faecium* AL41 to chickens infected with *Salmonella Enteritidis*. Immunohistochemical analysis revealed an increased number of IgA+ cells in the caecum after 7 days [6]. Also the effect of probiotic *Enterococcus faecium* AL41

(an environmental isolate) [42] on TGF-ß4 and IL-17 expression and on immunocompetent cell distribution after *Campylobacter jejuni* infection in broiler chickens was observed. The expression of selected cytokines (upregulation of TGF-ß4 but downregulation of IL-17 relative expression), and activation of IgA-producing cells in the caeca of chicks infected with *C. jejuni* CCM6191 was recorded [30, 38]. The immunomodulation effect on inflammatory response was revealed after the exposure of Intestinal porcine epithelial cells with Lactobacillus reuteri B6/1 under in vitro conditions presented by mRNA expression levels analysis of inflammatory cytokines (IL-8, IL-18) and transcriptional factors (MyD88 and NF-κβ) [60].

Probiotics are able to confer health benefits to the host, including specific gastrointestinal effects such as: reduction of the number of pathogens, secretion of enzymes and bacteriocins, improvement of immunomodulation, affection of proliferative activity of intestinal mucosa in various animal ecosystems [35, 36, 39, 56, 57, 58, 61]. The antimicrobial effect was evaluated in the pilot experiment with the application of enterocin M-producing strain *Enterococcus faecium* CCM8558 to infected chickens with *Campylobacter jejuni* CCM6191, while a significant increase in phagocytic activity was also noted in experimentally infected groups treated with the probiotic strain mentioned above [34].

Research has demonstrated that the administration of probiotics to the normal gut microbiota by stimulating the gastrointestinal immune response (antibody production and increasing phagocytic activity) can support the animal's defence systems against invading pathogens [6, 34].

THE role of probiotics in altering THE brain function

The mechanism whereby probiotic ingestion leads to changes in brain function and behaviour involves changes in gut permeability, and shifts in systemic immunity with decreased production of proinflammatory cytokines, including TNF-α [13]. These pathways traditionally have included signalling via neural pathways (mainly vagal nerve afferents) and immune signalling (mainly via circulating cytokines, which either enter the brain directly or activate cerebral endothelium) [8].

A novel peripheral signalling pathway was described occurring in the condition of liver inflammation, which involves increased peripheral TNF-α production driving increased microglial activation, followed by monocyte recruitment into brain vasculature and brain parenchyma, which in turn drives the development of sickness behaviours [14]. The potential peripheral pathways that link probiotic ingestion to changes in the brain function have primarily focused on the role of the changes in cerebral levels of neuromodulators such as brain-derived neurotrophic factor [3, 4].

Probiotic consumption has also been shown to alter brain function and behaviour in healthy organism. Specifically, probiotic ingestion can have beneficial effects on mood and cognition [44] and has also been associated with changes in neural activity in brain regions involved in emotional processing [62]. Changes in cross-talk among the intestinal epithelium, the intestinal immune system, and gut microbes has increasingly been recognized for its capacity to: modulate systemic immunity and prevent peripheral inflammation associated with increases in circulating TNF-α levels, cerebral microglial activation, and recruitment of activated monocytes into the brain. The probiotic therapy may have a therapeutic role in regulating peripheral inflammation-associated brain dysfunction and behavioural alterations [16, 24]. D' M e l l o et al. [15] defined a novel pathway of probiotic mixture VSL#3 (containing eight live, freeze-dried bacterial species: *Streptococcus salivarius* subsp. *thermophilus, Bifidobacterium breve, B. infanti, B. longum, Lactobacillus acidophilus, L. planarum, L. casei,* and *L. delbrueeki* subsp. *bulgaricus*) ingestion that prevented peripheral inflammation. Therefore, probiotic therapy may have a therapeutic role in regulating peripheral inflammation-associated brain dysfunction and behavioural alterations which may affect the the patient's quality of life.

Lactobacillus supplementation is beneficial to the barrier function of the intestinal physical barrier in piglets, e.g. the effects of dietary supplementation with *L. acidophilus* on the performance, intestinal physical barrier functioning, and NOD-like receptors (NLRs) were expressed in weaned piglets. As a result, dietary *L. acidophilus* supplementation was found to increase the average daily gain and reduce the serum diamine oxidase activity. These results demonstrated that *L. acidophilus* supplementation improved the growth performance, enhanced the intestinal physical barrier function, and inhibited the expression of NOD1 and NLR family pyrin domain containing 3 (NLRP3) signaling-pathway-related genes in the jejunum and ileum tissues, enhances the intestinal physical barrier functioning by inhibiting interleukin IL-1β and IL-18 proinflammatory cytokines via the NOD1/NLRP3 signalling pathway in weaned piglets [66].

The gut-brain-microbiota axis is increasingly recognized as an important regulator of intestinal physiology. Exposure to psychological stress causes activation of the hypothalamic-pituitary-adrenal (HPA) axis and causes altered intestinal barrier function, intestinal dysbiosis, and behavioural changes. The effects of psychological stress on intestinal physiology and behaviour, including anxiety and memory were investigated in mice. Both local (intestinal physiology and microbiota) and central (behavioural and hippocampal decreased c-Fos expression) changes were normalized by pre-treatment with probiotics, indicating an overall benefit on health conferred by changes in the microbiota. These findings indicate and show that probiotics can overcome this immune-mediated deficit in the gut-brain-microbiota axis [55]. Joseph and Law [29] conducted a cross-species examination of single- and multi-strain combinations of established probiotics while 58 non-human (twenty-five rat, twenty-seven mouse, five zebrafish, one quail) investigations satisfied the criteria. For the non-human studies, single- (60.5 %) and multi-strain (45.0 %) combinations modified stress, anxiety, or depression behaviours in addition to altering social or cognitive performance (single-strain 57.9 %; multi-strain 85.0 %).

The application of probiotic microorganisms can be considered as a suitable alternative to antibiotics as well as representing a way to effectively influence the composition of the intestinal microbiome and the immune system of the host. On the other hand, the other possibility of using probiotics is the influencing of the connection between the intestine and the brain through the gut-brain axis. The further studies of the presented problem related to alternative use of probiotics is needed.

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COMBINATION OF BETA GLUCAN, HONEY AND CHLORHEXIDINE IN THE WOUND MANAGEMENT IN A CAT A CASE REPORT

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ABSTRACT

Wound management is one of the oldest and one of the most frequent therapeutic activities in medicine. Over the centuries there has been described and tested many therapeutic substances for the treatment of wounds with various effects. Due to the discovery of antibiotics, a wound management regime used to be limited only to a local application. Over years, it has been shown, that comprehensive therapy which uses only antibacterial preparations, also may contain some negative points (resistance of aggressive pathogens, toxicity, allergic reactions, etc.). According to studies, the best solution to this problem is a local application, using preparations that ensure the sterility of the affected parts of the skin, and the utilization of agents that are able to accelerate the granulation and lead to the healing process of the wound. Products that contain beta glucan combined with a gentle but effective antiseptics (chlorhexidine digluconate), or natural substance with antiseptic and also nourishment effect (honey), are very beneficial in wound management. A good penetration of active agents, the ability to moisturize defective tissues, and to make a protective film that hinder the intersection of impurities and decrease secondary contamination, are the benefits of a gel formulation, that is the most appropriate external form of application in veterinary practice that can improve and accelerate a successful healing process of wounds in animals.

Key words: beta glucan; chlorhexidine digluconate; honey; wounds

INTRODUCTION

Beta glucan

Beta glucans belongs to prominent immunomodulators and activators of white blood cells, especially macrophages which are able to significantly accelerate the wound healing process and its positive effect is manifested in all phases of healing. Glucans are long-chain polysaccharides. They are isolated from Saccharomyces and have an ability to improve and promote immunity which has become compro-

mised mainly in the case of long term diseases [2]. In addition to the immuno-stimulatory effect, they also have anti-infective activity and positive effects on cancer and increase the body's resistance to chemical and carcinogenic agents [11, 28, 31]. Beta glucan (BG) is mainly present in yeast and other fungal organisms, and it is composed of glucose polymers that have a positive effect on: the growth of bacterial probiotic strains in the intestinal tract, improve the blood lipid profile, and stimulate cellular immunity by the limiting of various secondary immuno-deficiencies developments due to the limitation of immune cell activation [25, 37]. The application of beta-(1,3/1,6)-D-glucan activates macrophages which are able to remove disintegrated parts of cells, foreign particles and pathogenic microorganisms, that significantly stimulate the natural immune response [34]. The main mechanisms of action are: binding to the BG receptor which is found on the surface of macrophages, neutrophils, monocytes and natural killer (NK) cells, and stimulation of the natural immune response [30]. These activated macrophages have: the ability to release large amounts of active substances (lysozyme, complement components, etc.), have a bactericidal effect, involve immunomodulatory processes through the secretion of cytokines (granulocyte macrophage colony stimulating factor (GM-CSF), stimulates the growth of hematopoietic cells, interleukin-1 that is a major cytokine for T lymphocytes activation), participates in antigen presentation processes, and interleukin-2 production. Interleukin-12 promotes NK-cell activity and is involved in the anti-proliferative, immunoregulatory and antiviral action of macrophages [4, 5, 20, 29]. Other mechanisms of action of BG are including its binding ability to the complement receptor, which acts as an adhesive molecule. In addition, the effects of some β-glucans have also been anti-infective and have antibacterial activity against a broad spectrum of Gram-negative and Gram-positive bacteria [4]. Beta-(1, 3/1,6)-D-glucan has very beneficial effects in the healing of ulcerations and wounds [17, 18]. Its binding areas are also found on dermal fibroblasts, keratinocytes and have the ability to functionally modulate these cells by stimulation of macrophages that prevent many disorders. The results of studies and the practical use of BG, confirm an increased protective effect against bacterial, viral, parasitic and fungal infections in all animal categories [30]. Its use in practice generally increases the activity of the immune response, alleviating clinical signs of disease reducing the process of healing and secondary infections, improving the efficacy of causal therapy and the prognosis of healing. Beta glucan also has an adjuvant effect in enhancing the effectiveness of therapy with other drugs such as antibiotics (ATB) and increase the effectiveness of the immune response after vaccination [9], and it has also been safe and non-toxic for animals [35].

The effects of beta-(1,3/1,6)-D-glucan include:

- Increase immunity system against infections (viral, bacterial, parasitic and fungal),
- Adjuvant effect –- promotes the effectiveness of vaccination and antibiotic therapy,
- Stimulation of other cells of the immune system (cytotoxic T-lymphocytes and NK-cells) and subsequent anticancer action,
- Support of regeneration and repair processes through fibroblast and keratinocyte activation promoting wound healing and reducing ulceration,
- Shortens recovery period,
- Radio-protective effect due to inactivation of free radicals,
- Promotion of haematopoesis and bone marrow activity.

Honey

Honey is a saturated solution of sugars, composed mainly of fructose, glucose, water, minerals, vitamins, enzymes, free amino acids, polyphenols, and around 0.17–1.17 % of organic acids. Pure bee honey has antibacterial effects and the ability to moisturize the wound. Its high viscosity helps to create a protective barrier that protects against infection. Its immunomodulation and antimicrobial activity is due to the enzymatic production of hydrogen peroxide, low pH and high osmolality that can sufficiently prevent the growth of microorganisms [12, 14, 27, 38]. The antimicrobial activity of honey has been described "in vivo" conditions, by the direct application of honey to the wound, which in 3-6 days was marked by its ability to inhibit microbial growth, because sugar molecules build up water molecules and cause bacteria to have insufficient water for their growth [1]. Honey effectively delivers hydrogen peroxide which is slowly released, because the enzyme that produces it becomes active only when honey is diluted and continues to its stable production during at least 24 hours. If honey is diluted with a buffer in the same proportion, the hydrogen peroxide concentration collected over 1 hour is about 1000 times less, than the concentration in the solu-

tion of 3 % hydrogen peroxide, which is commonly used as an antiseptic. Honey also contains a relatively large amount of antioxidants that protect wound tissues from oxygen radicals that could be produced by hydrogen peroxide [7].

Chlorhexidine digluconate

Chlorhexidine is a broad-spectrum biocidal antiseptic that is effective against fungi, gram-positive and gram-negative bacteria. It inhibits bacterial growth and also acts as a bactericidal agent. It can kill nearly 100 % of the gram-positive and gram-negative bacteria within 30 seconds. This effective skin antiseptic is beneficial in the prevention of various infections, injuries, and surgical procedures. Microorganisms that colonize the skin are found on the top of skin surface, in hair follicles and also in deeper layers of the skin. Many antimicrobial agents are not able to penetrate into the deeper layers of the skin and hair follicles where there could be a presence of coagulase-negative staphylococci [8, 16, 26]. Chlorhexidine binds to proteins, releases slowly, and has longer lasting effects against microorganisms [38]. Its antimicrobial activity is maintained on the skin for at least 48 hours [10]. The efficacy has not been affected by the presence of body fluids such as blood [19]. Also it is able to inhibit the adherence of microorganisms on the surface, and blocks the creation and growth of the biofilm [23].

MATERIALS AND METHODS

According to studies focused on the use of beta glucan, pure bee honey and chlorhexidine digluconate individually in the wound management and the positive and beneficial effects of each substance, we created a gel, where all three substances together were represented and we observed in post-traumatic wound in the cat, if the treatment and time duration of the complete healing would be effective, faster and without any side effects. The gel was applied once daily throughout the process of complete wound recovery. We administered also oral suspension of beta 1,3/1,6 D-glucan in combination with ascorbic acid and zinc (*Imulergan Compositum* susp. a. u. v.) that was administered during all treatment periods.

CASE PRESENTATION

Anamnesis

A nine-year-old cat "Lisa" had a free movement between the outside and inside of her home. During her stay outside her owners assumed that the cat was caught in a knocker and hurt. Her skin on the right front limb was totally peeled off and she was pulling the distal part of her front limb behind her. The cat was immediately brought to a veterinary clinic where she was treated. The torn joint was fixed and the remaining skin was sewed. Antibiotics were immediately administrated to the cat (Cefalexin a. u. v. inj., 50 ml) for seven days, and Metacam 0.5 $mg.ml^{-1}$ given once daily to reduce the pain and to relieve the inflammation. Later it was necessary to perform a plastic skin surgery to the patient. We provided an artificial skin replacement but the wound was damp and drippy.

Diagnosis

Post trauma necrosis of the skin and subcutaneous structures.

Treatment

After plastic skin surgery, the wound healing was very difficult. The process of healing was protracted, because the wound had to be covered and protected. According to the status of the animal, we began with an oral administration of 2 ml of beta glucan oral suspension (Imulergan Compositum susp. a. u. v.), that consisted of beta 1,3/1,6 D-glucan, and ascorbic acid and zinc, which potentiate the effect of BG. This peroral application of beta glucan was served throughout the treatment period. Also given was a topical application of gel containing beta glucan, chlorhexidine digluconate and pure bee honey to the wound once daily (Fig. 1), where a significant improvement after every application was observed. The enhancement was recorded after five (Fig. 2), ten (Fig. 3), and fifteen (Fig. 4) days of gel application. After 2 weeks of treatment we examined the blood for all relevant blood parameters. The blood test parameters were in the normal range, so additional administration of antibiotics was unnecessary. The wound did not have to be covered anymore after 4 weeks of treatment. The topical application of gel continued for an additional two weeks and after 6 weeks of therapy we terminated the topical application of beta glucan containing gel because the wound was healed. Treatment was maintained

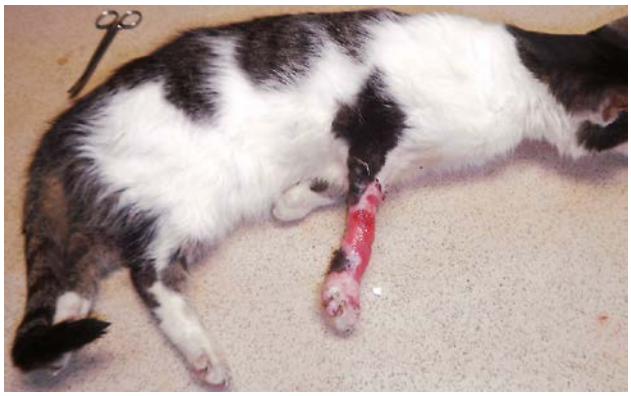

Fig. 1. First day of the product application

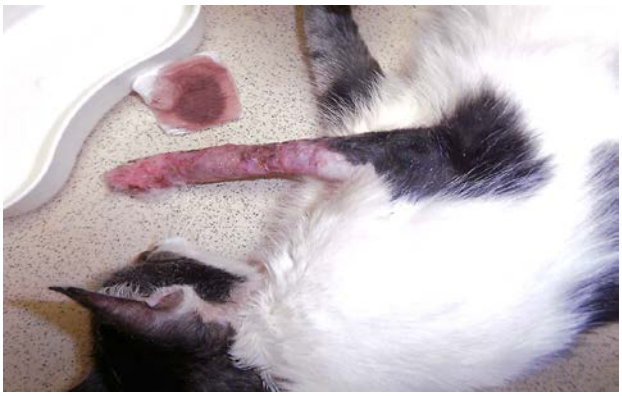

Fig. 2. Five days after first application

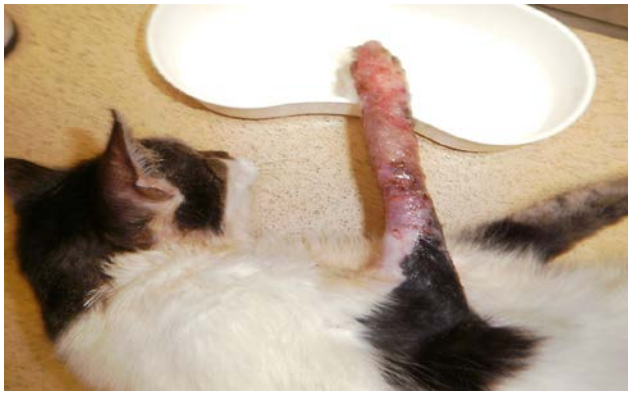

Fig. 3. Ten days after first application

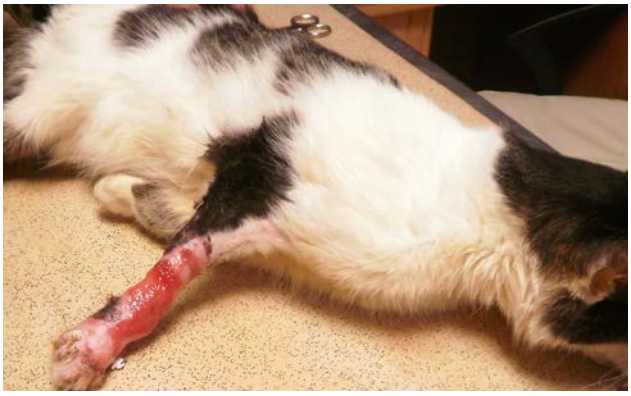

Fig. 4. Fifteen days after first application

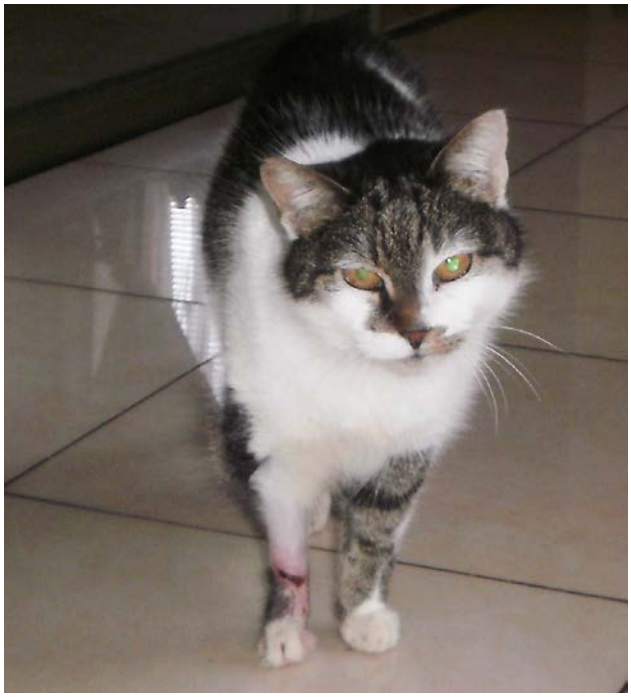

Fig. 5. After 35 days of product application

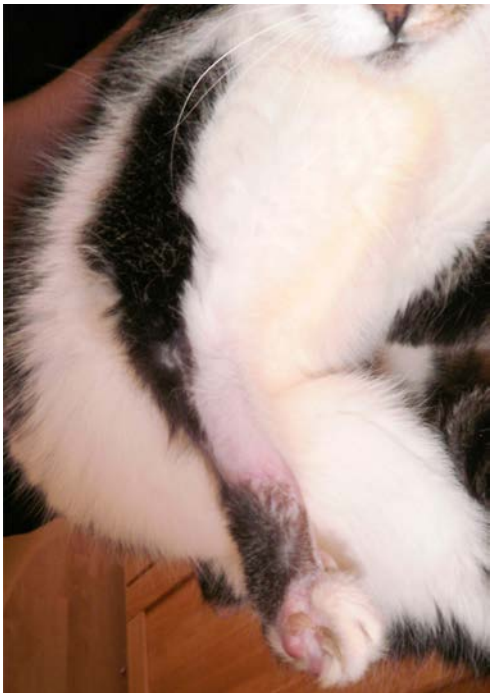

Fig. 6. The cat after 10 weeks of therapy

only with the oral administration of beta glucan during an additional 4 weeks. After 35 days of treatment (Fig. 5) the condition of the animal was significantly improved, and after 10 weeks of therapy complete healing occurred and her hair began to grow again (Fig. 6).

DISCUSSION

The topical use of beta glucan, pure honey or chlorhexidine digluconate in wound treatments have been proven in a number of various studies around the world independently [1, 10, 39]. All results of these studies concentrated on the usage of BG and confirmed its ability to stimulate cell-mediated and partial humoral immunity, mechanisms of non-specific immunity and haematopoiesis [36]. The oral administration of beta glucan demonstrated positive effects on the altered immune parameters. Their improvement was correlated with the clinical picture of the investigated dogs, which in the case of pyoderma and Malassezia showed skin improvement. The positive effects of BG have also been demonstrated in the case of immunosuppressed parvovirus dogs after their vaccination. After the first day following an administration of BG, there was observed: an increased phagocytic activity, the stimulation index increased significantly, and the antibody titre was higher in the observed group of patients [9]. In addition to antibiotic therapy, BG was administered to patients with impaired health. There was a significant clinical improvement after BG was applied to patients with atopic allergy, folliculitis and non-specific upper respiratory tract inflammation. All clinical observation studies have confirmed that BG preparations had no adverse side effects and were well tolerated [12, 14].

Z y k o v a et al. [39] studied a topical use of BG in patients with diabetic foot and leg ulcers and have confirmed that in skin infections associated with various degrees of immunosuppression, it is necessary to apply an immunomodulatory therapy. The immunostimulatory effect of BG on the functional activity of lymphocytes and phagocytes were also evaluated in skin diseases in dogs affected by dermatomycosis, demodicosis and pyoderma [22]. The clinical study of the topical application of BG under laboratory conditions was conducted by the Biotec company and was focused on patients to whom the formulation was applied twice daily for coverage of the primary wound in diabetic ulcers, pressure ulcers, leg ulcers, postoperative wounds, first and second degree burns, abrasions and skin wounds. The formulation was administrated directly to the open wounds, and every other day to wounds covered with a primary dressing until complete wound healing during 12 weeks [39]. Its application with antibiotic therapies was also very beneficial and its effect reduced any possibility of the formation of mutated bacterial strains resistant to ATB. Glucan is able to enhance an intensity of immune response and accelerates an onset of specific antibody production upon vaccination. By local application, it can positively influence wound healing, optimize surgical wound regeneration and contribute to the elimination of secondary infections after surgery [29]. Beta glucan application increases the cell immunity reaction and capacity of immune system, is safe, can be used as a supportive therapy, and can combine with other remedies and chemical substances that act synergistically. Beta glucan in prophylaxis allows the body to activate defence mechanisms which helps to relieve stress and prevent diseases [6]. It has the same mechanism of action in humans and other animals, so it can be used in pets (dogs, cats, parrots), cattle, pigs, horses, farmed freshwater and sea fish as well as other sea animals (shrimps, lobsters, crabs). Nowadays, glucan is regarded as useful due to its beneficial effects to the organism; a part of feed and nutritional supplements for improving the health of all types of domestic animals and fish [31].

Honey has been used in the treatment of many types of wounds, including burns, traumatic, surgical, necrotizing or ulcerative injuries. In the case of burns, the treatment with honey has been more effective than other products. Some studies have also shown that the effects of honey are as effective in the treatment of burns as silver sulfadiazine. When comparing honey and silver sulfadiazine, it was found that in acute, partial burns, the application of honey provided an early reduction of acute inflammatory changes, better infection control, faster healing process, decreased irritation and exudation, pain relief, reduced hypertrophic scar and post burn contractures [32, 33]. Studies also confirm the total inhibition of MRSA (Methicillin-Resistant *Staphylococcus aureus*), full inhibition of 58 coagulase-positive *Staphylococcus aureus* strains, isolated from infected wounds and complete inhibition from 20 *Pseudomonas* strains, also isolated from infected wounds [12, 14, 27, 38]. In a comparative study between honey and hydrogen peroxide, as a single antiseptic in wound therapy, the single

hydrogen peroxide had been less beneficial, although its presence in honey was used in a different way. This possibility is due to a sufficiently high concentration of hydrogen peroxide that cause the breakdown of proteins and cells in the tissues by increasing oxygen radicals, which limited the concentration of hydrogen peroxide as an antiseptic [24].

Compared to other antimicrobial agents, chlorhexidine has been antibacterial in the case of protozoa and bacterial spores covered by the virus [3, 21]. According to several studies it was confirmed that chlorhexidine has had an ability to inhibit an adherence of microorganisms to the surface and is able to prevent formation and growth of a biofilm [36].

CONCLUSIONS

Beta glucan has immunomodulatory and granulation effects, chlorhexidine is an effective disinfectant and antiseptic even in low concentrations, and honey nourishes the wound and creates a hyperosmotic environment that prevents bacterial growth. According to other studies and our experience we can confirm that:

1. Topical administration of beta glucan in a complex formulation in combination with other agents that potentiate its effect, makes the therapy easier and more effective (instead of a multi-stage wound treatment application the only one preparation).
2. A disinfectant such as chlorhexidine digluconate, and pure bee honey act synergistically with beta glucan and complement its effect.
3. Gel formulation seems to be the most suitable vehicle for animals if compared to other forms:
4. Solutions: short effect, worse penetration,
5. ointment, cream: worse application possibilities,
6. powder: dries a wound, limited indication.
7. Beta glucan in this form has been shown to be safe and hypoallergenic, since we used it in the formulation BG isolated from Pleurotus ostreatus (Oyster mushroom) so there were no reactions or side effects observed during its administration.
8. The formulation has also hydration effects so there was no irritation or any secondary complications caused by pruritus.
9. The healing process was shortened by approximately 30 %.

The objective of this case report was to remind others, that the use of beta glucan, which has often been studied and monitored, has been a very beneficial, safe and inexpensive substance with a strong immunomodulatory and adjuvant effect providing regeneration and repair processes. BG in combination with other components (chlorhexidine digluconate and pure bee honey) leads to significant shortening of the healing process without any application risks and unnecessary pharmacotherapeutic costs. Of course there is a need for further studies to confirm exactly the time duration and effectiveness of the treating process in other animals and case studies.

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