# FOLIA

# VETERINARIA

4

LIX • 2015

SLOVAKIA
Košice

## EDITORIAL BOARD

# FOLIA VETERINARIA, 59, 4, 2015

## CONTENTS

# DECREASED ALKALINE PHOSPHATASE ACTIVITY IN JEJUNAL ENTEROCYTES OF 28-DAY-OLD GROWTH-RETARDED PIGLETS: A HISTOCHEMICAL STUDY

**Tolnaiová, E.[1], Lorinčák, Ľ.[2], Dancáková, Ľ.[1], Gál, P.[1, 3], Lenhardt, Ľ.[1]**

[1]University of Veterinary Medicine and Pharmacy, Department of Pathological Anatomy
Komenského 73, 041 81 Košice
[2]District Veterinary and Food Administration of Trnava, Trnava
[3]East-Slovak Institute of Cardiovascular Diseases, Department for Biomedical Research, Košice
The Slovak Republic

ludovit.lenhardt@uvlf.sk

## ABSTRACT

Changes of alkaline phosphatase activity (AP) in the jejunal enterocytes were investigated in growth-retarded piglets. Twenty-eight-day-old clinically healthy piglets with normal growth (n = 6, body weight $10.70 \pm 0.49$ kg) and growth-retarded animals (n = 6, body weight $8.60 \pm 0.50$ kg) were used in this study. A histochemical technique and densitometric analysis were employed to evaluate the changes in the AP activity. A significant decrease in the density of AP activity was observed in the growth-retarded piglets compared to the normal piglets (P < 0.001). Our results point to a relationship between the AP density in the microvillous zone of enterocytes and the functional state of those enterocytes. Thus, these data can be found useful in the early diagnosis of growth disturbances in food-producing animals.

Key words: alkaline phosphatase; enterocytes; histochemistry; piglets

## INTRODUCTION

In pig farming, the mortality among very young pigs due to microbial infections (in particular coli infections) is often a serious problem. During the first 4 to 6 days after birth, the piglets are usually fed exclusively by suckling. At this age, they do not receive any food, liquid or solid, other than the sow's milk. The natural immune system of piglets against microbial infections during these first few days has not yet been fully developed, so that they are highly sensitive to various infections [21]. Infections usually leads to animal dehydration and weakness which is clinically manifested by violent diarrhoea. Consequently, mortalities occur. However, in the case that piglets do not succumb with the diarrhoea, the pathological changes lead to retarded growth. Due to both the mortality and the retarded growth, the stockman sustains appreciable economic losses.

C i g á n k o v á et al. [2] showed that enzyme histochemistry is a useful tool in the determination of enzyme localisation and activity in tissues, cells, and cellular organelles.

Densitometric analysis is usually used to quantify enzyme presence and activity by semi-quantitative and quantitative methods. These methods have frequently been used in different research and/or clinical studies [1, 3, 6, 10, 12, 14, 18]. Moreover, it has been shown that alkaline phosphatase (AP) is a hydrolytic enzyme involved in the transport of selected nutrients from the lumen of the bowel into the enterocytes [1]. From this point of view, it can be suggested that histochemistry is a suitable method to evaluate AP activity in the enterocytes of the small intestine. Further, the determination of alkaline phosphatase activity might reflect the functional state of the enterocytes.

Accordingly, the aim of this investigation was to determine whether the brush-border-bound jejunal AP activity differs in growth-retarded and normal healthy piglets, in an attempt to find an early histochemical marker of growth disturbance in food-producing animals.

## MATERIALS AND METHODS

### Animals

A total of 12 suckling piglets (Slovak White × Landrace crossbreeds) of both sexes were used in the experiment. All animals showed no clinical signs of sickness and had negative bacteriological findings for pathogenic bacteria causing diarrhoea. During the first 14 days, the experimental animals were not supplemented with commercial creep-feed. After 28 days, the animals were divided into two groups depending on their body weight. Control group (n = 6) consisted of clinically healthy piglets with normal growth (mean body weight 10.70 ± 0.49 kg); growth-retarded piglets (mean body weight 8.60 ± 0.25 kg) were included into the second group (n = 6). All animals were sacrificed by jugular incision and samples of the jejunum were removed for histological examination.

### Histochemistry

The samples were frozen in cold petroleum ether at –20 °C. Sections, 8 μm thick, were cut on a cryostat (Cryocut 27 000) in a cabinet temperature of –21 °C. Sections were picked onto clean glass slides (0.96 to 1.06 mm of thickness) and stored in the cryostat cabinet until used. The cryostat sections were allowed to dry for 5 min at 37 °C and incubated with AP. From each tissue segment, six sections were cut for the enzyme assays.

The AP activity analysis was performed using a modified simultaneous azocoupling method [11]. The incubation medium contained 2.0 mM naphthol AS-BI phosphate (Sigma, Deisenhofen, Germany), 0.8 mM hexazotized new fuchsin (Serva, Heidelberg, Germany), 20 mM N, Ndimethylformamide (solvent of naphtol AS-BI phosphate), and 0.05 M veronal acetate buffer. The sections were incubated at 37 °C for 10 min at a pH of 8.9 [13].

### Section analysis

The histochemically stained slides were visualized at low magnification (objective 4×). In order to define the standard density values of enzyme activities at the wavelength of 520 nm [4] required for calibration, a Vickers M 85 microdensitometer was used. A special semi-interactive algorithm was used to find the relevant pixels along the villus length whose density was measured. The quantification of the enzyme activity was carried out along the villus length in a whole section of at least six jejunal slides and the mean values recorded were referred to one animal.

### Statistical analysis

Statistical analyses were carried out using the statistical package Statistica 6.1 (StatSoft CR, Prague, Czech Republic). The data were expressed as the mean ± SEM and the statistical significance was accepted at the $P < 0.05$ level. Statistical evaluations of the developmental characteristics between both animal groups were carried out by the unpaired Student's $t$-test.

## RESULTS

Intestinal activities of AP in the growth-retarded pigs and control group are shown in Table 1. The presence of AP was detected in both groups in the brush border of the microvillous zone of the enterocytes. Our investigation revealed that the AP activity in the segments of the jejunal enterocytes was significantly lower in growth-retarded piglets when compared to their normal-growing controls (3.5 ± 0.31 vs. 6.2 ± 0.24; $P < 0.001$).

## DISCUSSION

The brush border of enterocytes contains many hydrolytic enzymes, e.g. disaccharidases, peptidases, and phosphatases [5]. AP is a brush border representative enzyme involved in the active uptake of nutrients [1, 8, 19]. The AP activity in the small intestine displays circadian fluctuations closely related to food intake. It markedly decreases after food deprivation as well as when food-deprived rats are given an increased food ratio [17]. Thompson (1984) observed that the malabsorption syndrome usually results from short atrophic villi with flattened epithelium at the surface. Those epithelial changes may, however, be caused by many etiological factors.

The results obtained in our experiments pointed to a high activity of alkaline phosphatase in normal piglets which is in accordance with previously obtained results by Lenhardt and Dudrikova [7], where a decrease of alkaline phosphatase activity was recorded in 3-months-old piglets with retarded growth. In addition, those results revealed a similar pattern of intestinal AP activity when compared to broilers with retarded growth [9] as well as to sheep with supermethrin intoxication [3]. Nevertheless, the exact mechanism which induced these changes has not yet been fully clarified. It is, however, well known that a decrease in energy intake led to adaptational changes of jejunal enzymes, i.e. lowered AP activity and increased maltase and sucrase levels [16].

On the other hand, a similar phenomenon was observed in birds after limited food intake, as well as in broilers suffering from the stunting syndrome. It was found that in underfed animals, after switching to normal energy intake, the intestinal morphometrically and densitometrically detected disturbances gradually disappeared and the functional state of the small intestine returned to the normal state [15, 16, 22]. This observation is in agreement with our results in the case that a total villi atrophy does not develop. From this point of view, it may be speculated that the level of villi damage is crucial for the development and compensation of the malabsorbtion syndrome.

In conclusion, disturbance of the functional state of the digestive tract, accompanied by malabsorption and body weight decrease, occurs during a reduction of AP activity in the microvillous zone of enterocytes. This decrease of AP activity in our experiments strongly suggests a similar degree of decreased functional conditions in the digestive tract in animals with retarded growth. Hence, our results point to a relationship between the amount of AP enzyme (found in the microvillous zone of enterocytes), and the functional state of enterocytes. Thus, these data can be found useful in the early diagnosis of growth disturbances in food-producing animals.

## ACKNOWLEDGEMENT

## REFERENCES

**1. Bernard, A., Caselli, C., Blond, J. P., Carlier, H., 1992:** Dietary fatty acid composition, age and rat jejunal microvillous enzyme activity. *Comp. Biochem. Physiol. A.*, 101, 607—612.

**2. Cigánková, V., Neuschl, J., Bugarský, A., Takáčová, D., 2002:** Densitometric analysis of alkaline phosphatase in the duodenal and jejunal enterocytes of 8-week-old piglets with retarded growth. *Bull. Vet. Inst. Pulawy*, 46, 105—109.

**3. Dudriková, E., Lenhardt, Ľ., Poráčová, J., 1992:** Biochemical aspects of the effect of supermethrin intoxication and histochemistry of alkaline phosphatase, acid phosphatase and non-specific esterase under the conditions of subchronic intoxication of sheep. *Vet. Med.* (Prague), 37, 613—622.

**4. Frederiks, W. M., Marx, F., Van Noorden, C. J., 1987:** Quantitative histochemical assessment of the heterogeneity of glycogen phosphorylase activity in liver parenchyma of fasted rats using the semipermeable membrane technique and the PAS reaction. *Histochem. J.*, 19, 150—156.

**5. Jones, T. C., Hunt, R. D., 1983:** Malabsorption syndrome. In **Lea and Febiger (Ed.):** *Veterinary Pathology*, 5th edn., Philadelphia, 1392—1394.

**6. Lenhardt, Ľ., Švický, E., Dudriková, E., 1997:** Histochemistry in veterinary pathology. *Slov. Vet. Čas* (Slovak Veterinary Journal), 22, 327—329.

**7. Lenhardt, Ľ., Dudriková, E., 1998:** A densitometric analysis of alkaline phosphatase and aminopeptidase M in the jejunum enterocytes of the piglets with retarded growth. *Vet. Med.-Czech.*, 43, 75—79.

**8. Lenhardt, Ľ., Cigánková, V., Zibrín, M., Kočišová, J., Tomková, I., Sabo, V. et al., 2001:** Functional development of small intestine of Japanese quails hatched on Mir orbital station. *Acta Vet. Brno*, 70, 127—131.

9. **Lenhardt, Ľ., Mozeš, Š., 2003:** Morphological and functional changes of the small intestine in growth-stunted broilers. *Acta Vet. Brno*, 72, 353—358.

10. **Levkut, M., Marcin, A., Lenhardt, Ľ., Porvaz, P., Revajova, V., Soltysova, B. et al., 2010:** Effect of sage extract on alkaline phosphatase, enterocyte proliferative activity and growth performance in chickens. *Acta Vet. Brno.*, 79, 177—183.

11. **Lojda, Z., Gossrau, R., Schiebler, T. N., 1979:** *Enzyme Histochemistry. A Laboratory Manual.* Springer Verlag, Berlin, Heilderberg, New York, 399 pp.

12. **Longauer, F., Bilčík, P., 1993:** Enzymes in tumours of the breast. Histochemical study of the stroma in breast carcinomas. *Bratisl. Lek. Listy* (Bratislava Medical Letters), 94, 192—200.

13. **Mozeš, Š., Lenhardt, Ľ., Martinková, A., 1998:** A quantitative histochemical study of alkaline phosphatase activity in isolated rat duodenal epithelial cells. *Histochemical Journal*, 30, 583—589.

14. **Mozeš, Š., Bujňáková, D., Šefčíková, Z., Kmeť, V., 2008:** Developmental changes of gut microflora and enzyme activity in rat pups exposed to fat-rich diet. *Obesity* (Silver Spring), 16, 2610—2615.

15. **Palo, P. E., Sell, J. L., Piquer, F. J., Soto-Salanova, M. F., Vilaseca, L., 1995a:** Effect of early nutrient restriction on broilers chickens. 1. Performance and development of the digestive tract. *Poult. Sci.*, 74, 88—101.

16. **Palo, P. E., Sell, J. L., Piquer, F. J., Vilaseca, L., Soto-Salanova, M. F., 1995b:** Effect of early nutrient restriction on broilers chickens. 2. Performance and digestive enzyme activities. *Poult. Sci.*, 74, 1470—1483.

17. **Raček, Ľ., Lenhardt, Ľ., Mozeš, Š., 2001:** Effect of fasting and refeeding on duodenal alkaline phosphatase activity in monosodium glutamate obese rats. *Physiol. Res.*, 50, 365—372.

18. **Seven, P. T., Yilmaz, S. Seven, I., Cerci, I. H., Azman, M. A., 2009:** Effects of propolis on selected blood indicators and antioxidant enzyme activities in broilers under heat stress. *Acta Vet. Brno*, 78, 75—83.

19. **Takase, S., Goda, T., 1990:** Effects of medium–chain triglycerides on brush border membrane-bound enzyme activity in rat small intestine. *J. Nutr.*, 120, 969—976.

20. **Thompson, R. G., 1984:** Atrophy. In **Saunders, W. B. (Ed.):** *General Veterinary Pathology*, 2nd edn., Comp., W. B. Saunders, Philadelphia, 283—287.

21. **Ybema, J., 1987:** Method of Promoting the Health and the Growth of Piglets. *United States Patent 4857338.*

22. **Uni, Z., Ganot, Z., Sklan, D., 1998:** Post-hatch development of mucosal function in the broiler small intestine. *Poult. Sci.*, 77, 75—82.

# MODIFICATION AND OPTIMIZATION OF METHODS FOR THE IDENTIFICATION OF PROTOZOAL AND FUNGAL PARASITES IN WATER SAMPLES

## Danišová, O. Valenčáková, A., Kalinová, J., Hatalová, E.

Department of Biology and Genetics, Institute of Biology, Zoology and Radiobiology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

olga.danisova@uvlf.sk

## ABSTRACT

Intestinal parasitosis constitutes a major public health problem, where inadequate sanitary conditions and the lack of information results in the contamination of food and water sources with the consequences of perpetuation of the parasite cycles. Thus, there is a need for a rapid and accurate method of identifying the species of protozoal and fungal parasites. In our modified method, we streamlined the process of pre-isolation and we increased the yield and purity of the isolated DNA for the use of a molecular methods, where only the DNA is needed, not whole cysts, oocysts or spores. In artificially contaminated water with positive cattle and mice faecal contamination, we have identified the species of *Cryptoporidium parvum* and *Enterocytozoon bieneusi* by this modified method. The unmodified methodology of processing water samples used only for molecular analyses are time-consuming and the use of filters, buffer solutions and chemicals needed to purify the samples are economically disadvantageous and time consuming.

## INTRODUCTION

Wastewater of humans, livestock and wild animals can contribute to the contamination of surface water with protozoal and fungal parasites. In countries where a substantial proportion of drinking water is produced from surface water, this constitutes a constant threat to drinking water safety. The protozoan parasites *Cryptosporidium*, *Giardia*, *Blastocystis* and the fungal parasite *Microsporidia*, are worldwide and considered as important causes of gastrointestinal disease in human and other animals. Furthermore, potential human pathogenic species increase the risk of zoonotic transmission, not only for immunosuppressed patients, but also for immunocompetent asymptomatic patients [4, 6].

Gastrointestinal infections are due to the oral ingestion of *Cryptosporidium* oocysts, *Giardia* cysts, *Blastocystis* or *Microsporidian* spores. The high number of (oo)cysts ex-

creted shortly after infection, together with the low infectious dose, facilitate the spread of infections [16]. Since the excreted (oo)cysts remain infectious for several weeks in the water, both treated tap water and recreational water are considered as excellent vehicles for infections, especially as *Cryptosporidium* and *Microsporidium*, in particular, are highly resistant to chlorine disinfection [3].

The existing techniques commonly used for the identification of *Cryptosporidium*, *Giardia*, *Blastocystis* and *Microsporidia* in water, such as method 1623 [2, 17], provide a quantitative assessment of the number of parasites present within a water sample. This method does not identify the species or genotypes present in the water and therefore does not provide an accurate assessment of the human health risk posed by these parasites when they are detected in a water sample. Although large numbers of *Cryptosporidium* oocysts, *Giardia* cysts, *Blastocystis* and *Microsporidian* spores could be detected in a water sample, the risk to human health may be low because the species and genotypes present may not be capable of infecting humans. Conversely, low numbers of human-adapted parasites (i.e., *C. hominis*, *G. duodenalis*, *Ent. bieneusi*) present in the water may represent a significant human health threat. The molecular analysis carried out with *Cryptosporidium* oocysts recovered from water samples demonstrates that a diverse range of species and genotypes may be present in raw water samples [7, 8, 15, 19, 21]. The effective risk assessment requires the identification of the species and/or genotypes that may be present in a water sample.

## MATERIALS AND METHODS

### Commonly used methods for the filtration of water and the detection of pathogens

The most common techniques used for the identification of *Cryptosporidium* and *Giardia* in water is the method 1623 [17].

In Slovakia, the methodology used for the detection of; *Giardia*, *Blastocystis* and *Microsporidia* in water samples, have been described by V e l i c k á et al. [18] and M i n a r o v i č o v á et al. [12].

## MODIFICATION OF THE METHODS USED FOR THE FILTRATION OF WATER AND THE DETECTION OF PATHOGENS

### Filtration

For filtration we used a membrane microfilter made of a mixture of cellulose acetate and cellulose nitrate (diameter, 47 mm; pore size, 3.0 μm; Advantec, Tokyo, Japan) [12], due to the interception of oocysts and cysts (oocysts *Cryptosporisdium* 4.5 to 7.0 μl; *Giardia* cysts 8.0 to 12.0 μl, *Blastocystis* cysts 4.0 to 15.0 μl) and we repeatedly filtered the water using a small filter with a pore size of 0.45 μl (diameter, 47 mm; pore size, 0.45 μl; MS MCE Membrane Filter Membrane Solution, Tokyo, Japan), due to the interception of *Microsporidian* spores (*Encephalitozoon* and *Enterocytozoon* spores 1.0 to 5.0 μl). Water artificially contaminated with cattle faecal samples positive for the species *Cryptoporidium parvum* and mice faecal samples positive for the species *Enterocytozoon bieneusi* (0.5 g feces.l$^{-1}$ water) was passed through a filter by the application of a vacuum [1, 5] according to the protocol of V e l i c k a et al. [18].

### DNA isolation

After filtration, the filters were not eluted and centrifugation was not applied. We did not use any buffer solutions or other solutions. The filters were shredded into smaller pieces, transferred to microtubes, a lysis solution was added, and the isolation performed as usual.

The genomic DNA was extracted from 100 mg of the stool samples using the DNA-Sorb-B Nucleic acid Extraction kit or 100 mg of the tissue samples using the DNA-Sorb-AM Nucleic acid Extraction kit according to the manufacturer's instructions (AmpliSence, Russia). Before extraction, the shredded filters with 500 μl lysis solution of DNA-Sorb-B Nucleic acid Extraction kit were homogenized and disrupted by entrapping oocysts/cysts/spores at 6500 rpm for 90 seconds with the addition of 0.5-mm-glass beads, 1.0-mm-zircon beads in a homogenizer Precellys 24 (Bertin technologies).

After homogenization of the samples, we continued with the isolation and purification of the DNA according to the kit manufacturer's instructions (AmpliSence, Russia).

Purified DNA was stored at −20 °C until use in the molecular analysis.

## MOLECULAR ANALYSIS

### Nested PCR for the detection *Cryptosporidium spp.*

For the nested PCR, we used a modified protocol [10, 20] with genus-specific primers Xiao F2/ Xiao R2 (819—825 bp) and VKSS F1/VKSS R2 (345—355 bp) for amplification of the SSU region of the *Cryptosporidium* species DNA. Secondary PCR products were analysed by electrophoresis in 1.5 % agarose gel and visualized by UV light at a wavelength of 312 nm [22].

### PCR reaction mixtures for Nested PCR

The volumes of the PCR reaction mixtures were, in both cases, 50 µl, from which the DNA sample was 5 µl. In these reactions, we used primers with a concentration of 0.2 µM and 5 U Taq DNA polymerases (FIREPol).

The PCRs were run in a thermo cycler (XP Thermal Cycler Blocks) with an initial denaturation of 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 61/57 °C for 1 min, and 72 °C for 2 min. A final elongation step of 72 °C for 7 min was included to ensure the complete extension of the amplified products [D a n i š o v á, unpublished].

### Real-Time PCR for detection of the *Microsporidium* spp.

For real-time SYBR Green amplification, we used the procedure of M a l č e k o v á et al. [11] with the use of specific primer pair PMP1/PMP2 with an annealing temperature of 60 ºC.

The volumes of the Real-Time PCR reaction mixtures were 25 µl, from which the DNA sample was 7 µl. In these reactions, we used primers with a concentration of 0.5 µl (30 pmol.µl⁻¹) and 12.5 µl FastStart Universal SYBR Green Master (Roche).

After an initial step at 50 °C for 2 min following the initial denaturation of the DNA at 95 °C for 10 min, and 40 amplification cycles were run: denaturation 95 °C for 15 sec and hybridisation 60 °C for 1 min. The sample was recorded as positive when the melting temperature was the same or ±0.5 °C in comparison to a positive control [11].

The PCR products were directly sequenced in both directions. Sequences were aligned and completed using ChromasPro, Bioedit and Clustal X, and compared with known sequences in the National Centre for Biotechnology Information GenBank database. The sequences generated in this study have been deposited in the GenBank database under accession numbers, which are described in the results.

## RESULTS AND DISCUSION

Generally, diarrhoeal pathogens (viruses, bacteria and parasites) constitute the most significant causes of human morbidity and mortality, exceeding nearly all other forms of infectious diseases. Intestinal parasitoses are a major public health problem, especially in specific groups of people in the countries where inadequate sanitary conditions and the lack of information result in the contamination of food and water sources with a consequent perpetuation of the parasite cycles [14]. According to the World Health Organization (WHO), various types of water-borne intestinal diseases, that affect mainly children in developing countries, cause about 1.8 million deaths annually. WHO attributes 88 % of these deaths to the vulnerability to contaminated water sources and poor hygiene [9]. While the parasites can spread in several different ways, water (drinking water and recreational water) is the most common method of transmission. *Cryptosporidium* and *Giardia* belong to the most frequent causes of waterborne diseases among humans, for example in the United States. Regarding the possibility of intentional pollution of water sources, *Cryptosporidium* spp. is classified according to the CDC (Centres for Disease Control) and the NIH (National Institutes of Health) as a category B bioterrorism pathogen [13].

On the basis of water-borne infections, it is important to filter the water, disinfect and monitor pathogens with zoonotic potential. There is a need for a rapid, accurate detection method used for the identification of the fungal and protozoal parasites. For the detection of *Cryptosporidium* oocysts, *Giardia* cysts, *Blastocystis* cysts and *Microsporidian* spores in Slovakia, there is used a well-established USEPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA [2, 17]; and also methods according to the procedures of V e l i c k á et al. [18] and M i n a r o v i č o v á et al. [12].

The above methods are used to capture oocysts, spores and cysts from water samples by filtration, followed by DNA purification. The oocysts, cysts and spores are identified primarily by microscopy, which requires intact oocysts, cysts and spores for the most accurate identification.

Subsequently, the samples can be processed by molecular analysis using specific primers to determine the type /genotype of the pathogens.

We identified the species of *Cryptosporidium parvum* and *Enterocytozoon bieneusi* in a positive sample of water artificially contaminated with cattle and mice faeces. The water was filtered according to a modified protocol of V e l i c k a et al. [18]. We did not use elution after filtration, therefore centrifugation was not needed. We did not use any buffer solutions, or other solutions. Filters were cut up into smaller pieces and after adding a lysis solution, the isolation continued as usual. Purified DNA was used in the molecular analysis. To detect *Cryptosporidium* spp., we used nested the PCR and for the detection of *Microsporidia*, we used the Real-Time PCR. After sequencing, we identified the species *Cryptosporidium parvum* and *Enterocytozoon bieneusi*, which were previously added to the water during the artificial infection.

In our modified method, we streamlined the process of pre-isolation which allowed us to increase the yield and purity of the isolated DNA for the molecular methods, where only DNA is needed, not whole cysts, oocysts or spores.

The methodology of processing of the water samples used only for molecular analysis are time-consuming and the use of filters, buffer solutions and chemicals needed to purify the samples are economically disadvantageous.

## CONCLUSIONS

The concern regarding the detection of protozoal and fungal infections has markedly increased mostly due to their frequent occurrence in relatively healthy populations of susceptible hosts. *Cryptosporidium*, *Giardia*, *Blastocystis* and *Microsporidia* are water-borne pathogens, which can be an important source of zoonotic infections and may affect the public health of human populations adversely. For these reasons, it is essential to monitor the prevalence of these pathogens based on direct evidence and molecular identification. An accurate, rapid, economically advantageous and time-undemanding modified method is described in this paper.

## REFERENCES

**1. Danišová, O., Valenčáková, A., Stanko, M., Luptáková, L., Hasajová, A., 2015:** First report of *Enterocytozoon bieneusi* and *Encephalitozoon* intestinalis infection of wild mice in Slovakia. *AAEM,* 22, 251–252. DOI: 10.5604/12321966.1152075.

**2. Environmental Protection Agency, 2006:** National primary drinking water regulations: Long term 2 enhanced surface water treatment rule; final rule. *Fed. Register.*, 71, 654—786.

**3. Fayer, R., 2004:** *Cryptosporidium*: a water-borne zoonotic parasite. *Vet. Parasitol.*, 126, 37—56.

**4. Geurden, T., Levecke, B., Caccio, S.M., Visser, A., De Groote, G., Casaert, S. et al., 2009:** Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. *Parasitol.*, 136, 1161—1168.

**5. Hasajová, A., Valenčáková, A., Malčeková, B., Danišová, O., Halán, M., Goldová, M. et al., 2014:** Significantly higher occurrence of *Cryptosporidium* infection in Roma children compared with non Roma children in Slovakia. *Eur. J. Clin. Microbiol. Infect. Dis.*, DOI 10.1007/s 10096-014-2082-2.

**6. Izquierdo, F., Castro-Hermida, J.A., Fenoy, S., Mezo, M., Gonzalez-Warleta, M., 2011:** Detection of *microsporidia* in drinking water, wastewater and recreational rivers. *Water. Res.*, 45, 4837—4843.

**7. Jellison, K.L., Hemond, H.F., Schauer, D.B., 2002:** Sources and species of *Cryptosporidium* oocysts in the Wachusett Reservoir watershed. *Appl. Environ. Microbiol.*, 68, 569—575.

**8. Jiang, J., Alderisio, K.A, Xiao, L., 2005:** Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. *Appl. Environ. Microbiol.*, 71, 4446—4454.

**9. Koppová, K., Bobáľová, P., 2009:** *Professional Qualification for Carrying out Epidemiologically Important Activities in Water Treatment Plants and During Operation of Water-supply Equipment* (In Slovak). Banská Bystrica, RÚVZ, 1—17.

**10. Leetz, A.S., Sotiriadou, I., Karanis, P., 2006:** Design of a new specific *Cryptosporidium* primers and optimization of PCR conditions for oocyst detection. In *Proceedings of ICOPA XI, 11th International Congress of Parasitology*. Glasgow, Scotland, UK, 653 pp.

**11. Malčeková, B., Valenčáková, A., Molnár, L., Kočišová, A., 2013:** First detection and genotyping of human — associated *microsporidia* in wild waterfowl of Slovakia. *Acta. Parasitol.*, 58, 13—17.

**12. Minarovičová, J., Lopašovská, J., Valík, Ľ., Kuchta, T., 2010:** Methods for the detection of *Cryptosporidium parvum* oocyts in milk, based on microfiltration and Real-Time Polymerase Chain Reaction. *Food. Anal. Methods*, DOI 10.1007/s12161-010-9141-9.

**13. Rotz, L. D., Khan, A. S., Lillibridge, S. R., Ostroff, S. M., Hughes, J. M., 2002:** Public health assessment of potential biological terrorism agents. *Emerg. Infect. Dis.*, 8, 225—30.

**14. Rudohradská, P., Halánová, M., Ravaszová, P., Goldová, M., Valenčáková, A., Halán, M. et al., 2012:** Prevalence of intestinal parasites in children from minority group with low hygienic standards in Slovakia. *Helminthology*, 2, 63—66.

**15. Ruecker, N. J., Braithwaite, S. L., Topp, E., Edge, T., Lapen, D. R., Wilkes, G. et al., 2007:** Tracking host sources of *Cryptosporidium* spp. in raw water for improved health risk assessment. *Appl. Environ. Microbiol.*, 73, 3945–3957. Epub 2007 May 4.

**16. Smith, H. V., Caccio, S. M., Tait, A., McLauchlin, J., Thompson, R. C., 2006a:** Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. *Trends in Parasitology*, 22, 160—167.

**17. U.S. Environmental Protection Agency, 2003:** Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA 815-R-03-XXX. Office of Ground Water and Drinking Water Technical Support Center, U.S. *Environmental Protection Agency*, Washington, D.C. 47. U.S.

**18. Velická, Z., Tóthová, L., Mogoňová, E., 2007:** Observation of the occurrence of *Cryptosporidium parvum*, *Giardia lamblia* and *Clostridium perfringens* in selected reservoirs and public water supplies in Slovakia (In Slovak). *Acta Environmentalica Universitatis Comenianae* (Bratislava), 15, 190—199.

**19. Ward, P. I. P., Deplazes, W. Regli, H. Rinder, Mathis, A., 2002:** Detection of eight *Cryptosporidium* genotypes in surface and waste waters in Europe. *Parasitol.*, 124, 359—368.

**20. Xiao, L., Morgan, U. M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R. C., Fayer, R., Lal, A. A., 1999:** Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Appl. Environ. Microbiol.*, 65, 3386—3391.

**21. Xiao, L. K. Alderisio, J., Limor, M., Royer, Lal, A. A., 2000:** Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. *Appl. Environ. Microbiol.*, 66, 5492–5498.

**22. Xiao, L., Fayer, R., Ryan, U., Upton, S. J., 2004:** *Cryptosporidium* taxonomy recent advances and implications for public health. *Clin. Microb. Rev.*, 17, 72—97.

# WATER CONTAMINATION CAUSED BY PROTOZOAN PATHOGENS
## (A REVIEW)

**Kalinová, J., Valenčáková, A., Danišová, O.**

University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
The Slovak Republic

jana.kalinova@student.uvlf.sk

## ABSTRACT

Protozoan pathogens are among the major risks of waterborne infection. The prevalence of such infections caused by these parasites constitutes an increasing trend worldwide in both immunosuppressed and immunocompetent people. By the year 2010, more than 524 parasitic outbreaks caused by contaminated water have been reported. Almost 93 % of these outbreaks occurred in North America and Europe, in which 30 % of the cases occurred in Europe alone. The majority of these epidemic outbreaks were caused by two species of protozoan parasites, i.e. *Giardia duodenalis* (40.6 %) and *Cryptosporidium parvum* (50.8 %). Other common waterborne protozoa include; *Entamoeba histolytica*, *Naegleria fowleri*, *Toxoplasma gondii*, *Balantidium coli*, *Isospora belli*, *Acanthamoeba* spp. and *Blastocystis* spp., that were detected in 2.8—0.6 % of the cases, which surely does not diminish the importance of these pathogens.

Key words: prevalence; protozoa; water; waterborne epidemic

## INTRODUCTION

Protozoan pathogens (domain *Eukaryota*) consist of species, the life cycle of which are associated with various environments, such as water and soil environments or the gastrointestinal tract of animals. Protozoa are basically, usually single-cell eukaryotic organisms. There are more than 65,000 species of protozoa, and approximately 10,000 of them are pathogenic. Water can be the major transmission factor for diseases, especially if protozoa are excreted into the water, in which they can remain viable and infectious [40].

Pathogenic protozoa that cause diseases in humans and other animals are usually unable to multiply in water, but they are able to survive there for days, months or years. Important for the beginning of a disease, is a specific infectious dose, where this dose depends on: the species of the microorganism; its ability to infect an individual; and from the competence of the immune system of the individual.

A large number of parasitic protozoan species are capable of causing waterborne infections. The most common pathogens are; *Cryptosporidium* spp., *Toxoplasma* spp.,

*Giardia* spp., *Cyclospora* spp., *Blastocystis* spp., *Balantidium* spp., *Cystoisospora* spp., and *Entamoeba* spp. [35].

According to the World Health Organisation (WHO), different species of waterborne intestinal diseases that infects mainly children in developing countries, cause yearly around 1.8 million deaths. WHO attributes as much as 88 % of these deaths to poor hygiene and insufficient protection from infected water sources [37].

## *ENTAMOEBA HISTOLYTICA*

The genus *Entamoeba* belongs to the phylum *Apicomplexa* and its most important species is *Entamoeba histolytica*. This species is divided into different genotypes, where genotype *Entamoeba histolytica* HM-1:IMSS belongs to the most virulent, while genotype *Entamoeba histolytica Rahman* is avirulent [56].

Infections begin after the consumption of contaminated food or water, or directly by faecal-oral route. Waterborne transmission is common in developing countries with insufficient drinking water treatment or after contamination of water caused by animal faeces [11].

In Africa, *Entamoeba histolytica* belongs to the most common cause of death due to parasitic infection after malaria [3]. A high prevalence was also detected in Mexico, India, eastern and southern parts of America. The occurrence in immigrants and tourists returning from these countries is also high [32]. The prevalence of *Entamoeba histolytica* infections in developing countries is 18—48 % [29]. In developed countries, the prevalence of this infection is 0.2—10.8 %, but these data may not be reliable because amoebiasis can also occur in an asymptomatic form [43].

*Entamoeba histolytica* is occasionally responsible for some waterborne epidemics. In 1986, this parasite caused simultaneous epidemic with *Giardia* in the Swedish town of Sälen, where more than 1,400 people became infected in a ski resort after penetration of waste water into the potable water supply system [4]. Two outbreaks occurred in 1961 and 1970 in the USA, where at least 25 people became infected after the contamination of the public water supply with cysts of this parasite [66]. In 1993, in Taichung City in Taiwan, an epidemic broke out in a private school, where 730 students became infected. The cause of the outbreak was the contamination of a well with waste water in an old water supply system [16]. In 1998, 117 people became infected in Tbilisi (Georgia) following faecal contamination and insufficient filtration of water [38]. In 2006, in Jiangshan City, in China, 31 people were infected by *Entamoeba histolytica*; the cause of the infection was unknown [45].

## OTHER AMOEBAS — *ACANTHAMOEBA* SPP. AND *NAEGLERIA FOWLERI*

Among other amoebas, important waterborne threats pose free-living amoebas from the genera *Acanthamoeba* and *Naegleria*. The most significant representative from the genus *Naegleria* is thermophilic *Naegleria fowleri*. This amoeba can be found in the soil and water, especially in hot springs and swimming pools, but also in rivers, lakes and ponds. It can survive in water with temperatures higher than 45 °C [59].

*Naegleria fowleri* was the cause of 16 infections and subsequent deaths in all cases during the years 1962—1965 in the Czech town of Ústí nad Labem, after the contamination of a swimming pool and insufficient treatment of the water of this pool [34].

Amoebas from the genus *Acanthamoeba* belong to the most common free-living parasites, which can be detected in fresh, and salt water, soil or plants [46]. They are the causative agents of meningoencephalitis and keratitis in humans and these infections begin after skin penetration or via the respiratory system. From there, the *Acanthamoeba* spp. are carried by the blood to organs, such as the brain, where they create inflammatory foci.

Epidemics caused by parasites from the genus *Acanthamoeba* has occurred in the years 1993—1994 in Iowa (USA), where floods caused the contamination of the water source and more than 43 people were infected [47]. In the years 2003—2005, forty people were infected in Chicago (USA). The source of the infection was a contaminated water supply. In the years 2005—2007 there were 138 people infected in Puerto Rico [33].

The diagnosis of amoeba infections is possible by various methods. Serological methods for antibody detection like indirect haemagglutination, immunoelectrophoresis, immunofluorescence test and ELISA are used [26].

Light and fluorescent microscopy is used for the examination of histological samples from biopsies. For staining of these samples, trichrome stain and hematoxylin are

used. Cultivation in cell lines is another possible diagnostic method. Transmission electron microscopy (TEM) is used for the observation of parasites in the tissues obtained from biopsies, samples from body fluids (urine, bile, cerebrospinal fluid), or faecal samples. Some amoebas can be identified on the genus or species level, based on their morphological characteristics [26].

Molecular methods are highly specific methods, from which PCR methods are commonly used. PCR methods used for the identification of amoebas are based on the detection of various gene areas, such as SSU and LSU rRNA and intergenic sections. This allows us to diagnose and differentiate species of parasites. In the database of gene bank (GenBank) there are available, various gene sequences of SSU rRNA obtained from amoebas and other protozoan pathogens. Amoebas can be identified with real-time PCR, which allows not only the detection of the pathogen, but also its quantification. Pathogenic *Entamoeba histolytica* can be differentiated from apathogenic *Entamoeba* dispar with multiplex PCR, which allows simultaneous identification and comparison of species [26].

## BALANTIDIUM COLI

Other important waterborne protozoan pathogens include *Balantidium coli* (genus *Balantidium*, phylum *Ciliophora*). In Europe, this infection is rare, while it is commonly detected in tropical and subtropical countries [58]. In 1971 an epidemic broke out in Truk (USA), where 110 people were infected. The cause of this infection was the contamination of a water source caused by a typhoon [70].

*Balantidium coli* can be detected by a microscope, from either native or stained sample, where for the staining of the sample, hematoxylin-eosin or Lugol's iodine are used. Also, transmission electron microscopy or cultivation is used, but the PCR methods are more common. PCR methods for the identification of the ITS gene regions are used as well. Serological tests are not regularly used for the diagnosis of *Balantidium coli* [58].

## BLASTOCYSTIS SPP.

The genus *Blastocystis* belongs to the single-cell parasites and is able to infect a wide range of vertebrates and invertebrates. Seventeen subtypes of *Blastocystis* spp. are known, where subtypes ST1–ST4 infect people [2].

*Blastocystis* spp. was detected in many countries, where the prevalence in the human population ranges from 0.5 to 62 %. The prevalence in Europe is 30 % [57]. It has been estimated that 1—2 millions of people worldwide are infected by this parasite [63].

In 1985, in Kathmandu (Nepal) 247 immigrants were infected by *Blastocystis* spp. and the aetiology was never determined [6]. In 1988, five cases of the infection were identified in the Italian city of Siena, where the infection was probably imported from a foreign country [28]. The biggest epidemic of *Blastocystis* was detected in 1996, in the Chinese city of Hengshui, where 1,122 people became infected from contaminated water [71]. Among the last documented outbreak of this infection were 102 cases from 2013 in Malaysia, where the cause of the infection was the inadequate treatment of drinking water [5].

The genus *Blastocystis* can be diagnosed with the light microscopy, where staining can be done with safranin-methylene blue stain and with the modified Ziehl-Nielsen stain. From the serological methods for detection of this pathogen, the ELISA test was used successfully, and from molecular methods, common is real-time PCR with primers specific SSU rRNA region of gene [36].

## CYCLOSPORA CAYETANENSIS

*Cyclospora cayetanensis* is a parasite from the phylum *Apicomplexa*, an intracellular parasite capable of causing intestinal infections, which start after faecal-oral ingestion, where the source of the infection is the faecal contamination of the food or water [8].

*Cyclospora cayetanensis* is spread worldwide. In some countries (for example in Nepal, Haiti, Peru and Guatemala) cyclosporiasis is endemic [51]. Immunodeficient patients and children belong to the group most commonly infected by this disease. Infections can be also found in tourists which includes cyclosporiasis in the group of the so-called traveller's diseases [14]. The prevalence is identified mainly in endemic and third-world countries, so it is unknown in Europe and Slovakia.

In the years 1989—1990, there were two simultaneous epidemics in Kathmandu (Nepal). In the first epidemic, 55 and in the second, 85 foreigners were infected, while the

aetiology remained unknown [53]. In 1990, in Chicago (USA) 21 people were infected in a hospital after contamination of the water supply [31]. In the USA, 115 outbreaks of cyclosporiasis occurred during the years 1995—2000 and more than 3,200 people were infected after consumption of raspberries that were irrigated with contaminated water [49]. In 2005 in the Turkish town of Izmir, 191 people were infected after drinking contaminated water [1].

*Cyclospora cayetanensis* can be detected by a microscope after an examination of a stained stool. This parasite can be stained with safranin or with modified acid-fast staining. Flow cytometry is used for the identification of this parasite with good results. Serological tests for antibody detection are used rarely, but an immunofluorescent test and the Western Blot have both been used experimentally. Molecular methods, such as RFLP PCR and real-time PCR have been successfully used for the amplification of ITS gene region, which allows us to differentiate the species of the parasites [62].

## ISOSPORA BELLI

*Isospora belli* belongs to the phylum *Apicomplexa*. It is a gastrointestinal parasite that is common in tropic and subtropical countries. A higher prevalence of this parasite has also been described in less developed countries. Its prevalence in Haiti has been recorded as 15 % in HIV infected patients, while in the USA, the prevalence of *Isospora belli* was reported as 0.2 % [39].

There was only one outbreak caused by this parasite which occurred in Chile (Antofagasta City) in the year 1977, where 90 people became infected after the consumption of vegetables that were irrigated with water that contained oocysts of *Isospora belli* [55].

The diagnosis can be accomplished by microscopic examination of stool samples. For staining of these samples, a modified acid-fast technique can be used. Cultivation, transmission electron microscopy and serological methods have been used exceptionally. The PCR methods, with primers specific for SSU rRNA and ITS regions are commonly used [39].

## GIARDIA DUODENALIS

*Giardia duodenalis* (also known as *Giardia intestinalis* and *Giardia lamblia*) is a bilaterally symmetric protozoa from the genus *Giardia*, which infects a wide range of vertebrates, including humans. *Giardia duodenalis* is divided into assemblages, where assemblages A and B have the widest range of hosts, including humans [27]. Assemblage C that is specific for dogs, was detected in humans in Slovakia [64].

Infection begins after consumption of contaminated food or water through the oral-faecal route, human-to-human transmission, or by zoonotic transmission [13].

The prevalence of giardiasis in 2010 was 5.68 cases in Europe per 100,000 people, where most cases occurred in Bulgaria and in a group of children under 4 years of age [24]. In the USA, in 2010 the prevalence was 2.96 cases per 100,000 people. The most common source of infection was water, either untreated surface water or water used for recreational purposes [13]. In Slovakia, 169 cases occurred in the year 2010, which represents 3.21 cases for 100,000 people [24]. In 2012, the number of cases in Slovakia rose to 243; most cases occurred in the Žilina region [54].

Giardiasis has been involved in important waterborne epidemics. The first outbreak occurred in years 1954—1955 in Portland (USA), where the source of the infection was found in inadequately treated drinking water [69]. In the years 1974—1975, in Roma (USA), more than 5,000 people were infected after consumption of insufficiently filtrated and disinfected water [60]. In 1977, two simultaneous epidemics broke out in Berlin (USA), where the cause was the presence of *Giardia* cysts in the drinking water. In 1978, in Vail (USA) 5,000 people become infected after a contamination of the water supply with sewage. One year later, in Bradford (USA), 3,500 were infected after contamination of a water source by beaver faeces [12]. On Christmas in 1986 in a ski resort in the Swedish town of Sälen, 1,400 people got infected by giardiasis. The simultaneous epidemics of giardiasis and amoebiasis was caused by the penetration of sewage into the water supply system [4]. In the year 1995, in New York (USA), 1449 people got infected by cysts of *Giardia duodenalis* after inadequate treatment of lake water [41]. In Bergen (Norway) outbreak occurred, 1 300 people got infected. Source of infection was contamination of the water supply by *Giardia* cysts [50].

The laboratory standards for the diagnosis of this parasite are as follows [23]: (A) Evidence of *Giardia duodena-*

*lis* cysts or trophozoites in the stool, duodenal fluid or in samples from a biopsy of the small intestine; (B) Evidence of *Giardia duodenalis* antigen in the stool.

The microscopic detection of *Giardia* in stool samples can be done from native or stained samples. For staining, trichrome stain is usually used. From serological methods, ELISA, immunoelectrophoresis and immunochromatographic tests can be used. Molecular methods are more precise and various PCR modifications are used, such as nested PCR. Most are commonly applied primers specific for SSU rRNA and TPI gene region [15].

## CRYPTOSPORIDIUM SPP.

Parasites from the genus *Cryptosporidium* (phylum *Apicomplexa*) are small coccidian protozoa that infect epithelial cells of the intestinal microvilli in the digestive tract of vertebrates. More than 30 species from this genus are known, with *Cryptosporidium parvum* and *Cryptosporidium hominis* being the most important ones. Other species that can cause infections are: *Cryptosporidium meleagridis*, *Cryptosporidium canis*, *Cryptosporidium felis*, *Cryptosporidium suis*, *Cryptosporidium ubiquitum* and *Cryptosporidium muris* [17].

The route of transmission is usually the oral-faecal, mostly after consumption of contaminated food or water. The possible transmissions also occurs by human-to-human transmission and zoonotic transmission [25].

The incidence of cryptosporidiosis in 2012 in Europe was 2.3 cases per 100,000 people, where a total of 6,605 cases were confirmed from 21 countries. The highest number of infected was described in Great Britain [24]. In 2012, the number of cases in Europe rose to 3.15 per 100,000 people, with 9,591 confirmed infections of cryptosporidiosis. Most of the cases occurred in Ireland and in the age group of children under 5 years. In 2012, only one case was detected in Slovakia, while in 2013 twelve cases were confirmed [54, 67]. In 2014 in Slovakia, only one case of *Cryptosporidium* infection was described [68].

Among the first outbreaks of cryptosporidiosis was the epidemic from the year 1987, in Carrollton (USA) where 13,000 people became infected. The cause of this infection was the contamination of river water by a species from the genus *Cryptosporidium* [30]. The largest and most well-known epidemic occurred in Milwaukee (USA) in 1993,

when after a contamination of lake water by *Cryptosporidium* oocysts, more than 400,000 people became infected and 112 died [42]. The first outbreak in Europe was described in 1995 in Italy, where 294 cases occurred in a rehabilitation facility in Emilia Romagna. Seven HIV positive patients died as a result of the infection. The cause of the infection was the contamination of covered water tanks [52]. In 1996, 4,000 people were infected in Kelowna (Canada). In the same year an outbreak occurred in Cranbrook, Canada, where 2,000 people became infected. In both epidemics, the cause of the infection was the contaminated water in the lake and tank [18]. In 1996 epidemics broke out in Saitama (Japan), where the drinking water was contaminated by oocysts and 8,705 people got infected [61]. In 2001, in Dracy le Fort County (France), 563 people became infected with oocysts of *Cryptosporidium parvum* from the public water supply that was contaminated by sewage [19]. In the Swedish town of Östersund more than 12,700 cases of *Cryptosporidium* infection were detected in 2010. The source of the infection was drinking water that became contaminated by sewage [24].

The laboratory standards for the diagnosis of cryptosporidiosis are [23]: (A) Evidence of *Cryptosporidium* oocysts in the stool; (B) Confirmation of *Cryptosporidium* presence in intestinal fluids or in biopsy samples from the small intestine; (C) Detection of *Cryptosporidium* nuclear acid in the stool; and (D) Evidence of *Cryptosporidium* antigen presence in the stool.

*Cryptosporidium* oocysts can be detected by light microscopy, by staining the samples with: Kinyoun staining, Ziehl-Nielsen staining, Miláček-Vítovec staining, dimetyl-sulfoxid-carbol-fuchsin staining and safranin-methylen blue [44]. Commonly used serological methods are: ELISA, enzyme immunoassay (EIA), and the direct fluorescent antibody test (DFA). Molecular methods, like Nested PCR, Real-time PCR, RFLP-PCR and DGGE are commonly used for the amplification of specific loci of *Cryptosporidium* DNA, like SSU rRNA or other regions [20].

## TOXOPLASMA GONDII

The genus *Toxoplasma* belongs to the phylum *Apicomplexa* and the only species and causative agent of toxoplasmosis is *Toxoplasma gondii*, which induce parasitic diseases of people and homeothermic animals [21]. There are three

main genotypes, I, II, and III, and all of them can infect humans [9].

*Toxoplasma* infects a wide range of hosts, while the definitive host is only the cat. Humans and other homeothermic animals can get infected horizontally or vertically. The horizontal route is possible by the ingestion of oocysts or tissue cysts. Oocysts can occur in the contaminated water and food and tissue cysts can be found in raw or inadequately cooked meat. The vertical transmission is possible by the transplacental route [22].

In 2012 in Slovakia, 103 cases of toxoplasmosis were detected; the highest number of cases was in the Žilina and Nitra regions. Most infected people were in 10 to 14 year olds [54]. In Europe, in 2010, 279 cases of congenital toxoplasmosis occurred; 87 % of the infections were in France [24].

One of the first cases of waterborne toxoplasmosis was detected in 1979, in Panama, where 32 were infected after the consumption of lake water. During 1994 and 1995, in Victoria (Canada) an epidemic occurred where 7,718 people became infected after the contamination of water with faeces from pumas and wild cats [10]. In Santa Isabel do Ivai (Brazil), 290 people became infected in 2001. The source of the infection was the contamination of the water supply with *Toxoplasma gondii* oocysts [65]. During 2004 and 2005, in Coimbatore City (India), 249 people became infected with ocular toxoplasmosis. The cause of the infection was the contamination of the water source after a heavy rainfall [7].

The laboratory standards for the diagnosis of *Toxoplasma gondii* are as follows [23]: (A) Confirmation of *Toxoplasma gondii* presence in the tissues or body fluids; (B) Evidence of *Toxoplasma gondii* nuclear acid in clinical samples; (C) Specific antibody response (IgM, IgG, IgA) in newborns; and (D) Permanent titre of *Toxoplasma gondii* in children younger than 12 months of age.

If *Toxoplasma* is detected by the microscope, it can be stained with Giemsa, Dif-Quick or Hemacolor. For diagnosis by serological methods; ELISA, indirect immunofluorescent reaction or the complement fixation reaction can be used. Molecular methods like real-time PCR are often used for the detection of this parasite [48].

## CONCLUSIONS

Protozoan parasites are present all around us in the environment, especially in the water. Untreated water, water from rivers, ponds and lakes should not be used under any circumstances for drinking because it can be the source of infections. In many cases, waterborne infections can be caused by the contamination of drinking water which presents a permanent threat when drinking water supplies are insufficiently controlled and treated. As mentioned previously, these parasites can cause asymptomatic forms of infections and stay undiagnosed, therefore their prevalence remains unknown. It is assumed that many potential epidemic outbreaks remain unreported. But after the failure of the immune system, these infections can cause severe clinical symptoms and even death. Toxoplasmosis is especially extremely dangerous, because it can be transmitted by the transplacental route to newborns. Further education of the population, detailed diagnosis and constant control of water sources is therefore highly recommended.

## ACKNOWLEDGEMENT

## REFERENCES

**1. Aksoy, U., Akisu, C., Sahin, S., Usluca, S., Yalcin, G., Kuralay, F. et al., 2007:** First reported waterborne outbreak of cryptosporidiosis with *Cyclospora* co-infection in Turkey. *European Surveillance*, 12, 31—42.

**2. Alfellani, M. A., Taner-Mulla, D., Jacob, A. S., Imeede, C. A., Yoshikawa, H., Stensvold, C. R. et al., 2013:** Genetic diversity of *Blastocystis* in livestock and zoo animals. *Protist*, 164, 497—509.

**3. Ali, I. K., Haque, R., Alam, F., Kabir, M., Siddique, A., Petri, W. A. Jr., 2012:** Evidence for a link between locus R-R sequence type and outcome of infection with *Entamoeba histolytica*. *Clin. Microbiol. Infect.*, 18, 235—237.

**4. Andersson, Y., de Yong, B., 1989:** An outbreak of giardiasis and amoebiasis at a ski resort in Sweden. *Wat. Sci. Technol.*, 21, 143—146.

5. **Anuar, T. S., Ghani, M. K., Azreen, S. N., Salleh, F. M., Moktar, N., 2013:** *Blastocystis* infection in Malaysia: evidence of waterborne and human-to-human transmission among Proto-Malay, Negrito and Senoi tribes of Orang Asli. *Parasit. Vectors*, 6, 21—32.

6. **Babcock, D., 1985:** *Blastocystis hominis* in Kathmandu, Nepal. *N. Engl. J. Med.*, 313, 1419.

7. **Balasundaram, M. B., Andavar, R., Palaniswamy, M., Venkatapathy, N., 2010:** Outbreak of acquired ocular toxoplasmosis involving 248 patients. *Archiv. Ophthalmol.*, 128, 28–32.

8. **Bern, C., Hernandez, B., Lopez, M. B., Arrowood, M. J, de Mejia, M. A., de Merida, A. M. et al., 1999:** Epidemiologic studies of *Cyclospora cayetanensis* in Guatemala. *Emerg. Infect. Dis.*, 5, 766—774.

9. **Boothroyd, J. C., Grigg, M. E., 2002:** Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? *Curr. Opin. Microbiol.*, 5, 438—442.

10. **Bowie, W. R., King, A. S., Werker, D. H., Isaac-Renton, J. L., Bell, A., Eng, S. B. et al., 1997:** Outbreak of toxoplasmosis associated with municipal drinking water. The BC *Toxoplasma* investigation team. *Lancet*, 150, 173—177.

11. **Bruckner, D. A., 1992:** Amebiasis. *Clin. Microbiol. Rev.*, 5, 356—369.

12. **Centres for Disease Control and Prevention, 1980:** Waterborne giardiasis: California, Colorado, Oregon, Pennsylvania. *MMWR*, 29, 155.

13. **Centres for Disease Control and Prevention, 2012:** Cryptosporidiosis Surveillance — United States, 2009—2010 and Giardiasis Surveillance — United States, 2009—2010. MMWR, 61, 1—28.

14. **Chacín-Bonilla, L., 2010:** Epidemiology of *Cyclospora cayetanensis*: A review focusing in endemic areas. *Acta Trop.*, 115, 181—193.

15. **Chakarova, B., 2010:** Comparative evaluation of the diagnostic methods for detection of *Giardia intestinalis* in human faecal samples. *Trakia J. Sci.*, 8, 174—179.

16. **Chen, K. T., Chen, C. J., Chiu, J. P., 2001:** A school waterborne outbreak involving both Shigella sonnei and *Entamoeba histolytica*. *J. Environ. Health*, 64, 9—13.

17. **Cho, E. J, Yang, J. Y., Lee, E. S., Kim, S. C., Cha, S. Y., Kim, S. T. et al., 2013:** A waterborne outbreak and detection of *Cryptosporidium* oocysts in drinking water of an older high-rise apartment complex in Seoul. *Korean J. Parasitol.*, 51, 461—466.

18. **Craun, G. F., Hubbs, S. A., Frost, R., Calderon, R. L., Via, S. H., 1998:** Waterborne outbreaks of cryptosporidiosis. *J. Am. Water Wks. Assoc.*, 90, 81—91.

19. **Dalle, F., Roz, P., Dautin, G., Di-Palma, M., Kohli, E., Sire-Bidault, C. et al., 2003:** Molecular characterization of izolates of waterborne *Cryptosporidium* spp. collected during an outbreak of gastroenteritis in South Burgundy, France. *J. Clin. Microbiol.*, 41, 2690—2693.

20. **Danišová, O., Valenčáková, A., Hasajová, A., 2015:** Diagnostic methods used for detection of *Cryptosporidium* spp. in Slovakia (In Slovak). *Slovenský Lekár*, 32, 14—19.

21. **Dubey, J. P., Beattie, C. P., 1988:** *Toxoplasmosis of Animals and Man*. Boca Raton: CRC Press, 220 pp.

22. **Dubey, J. P., Lindsay, D. S., Speer, C. A., 1998:** Structure of *Toxoplasma gondii* tachyzoites, bradyzoites and sporozoites, and biology and development of tissue cysts. *Clin. Microbiol. Rev.*, 11, 267—299.

23. **Epidemiological information system:** Standard definitions of transmissible diseases (In Slovak). Available on internet: <http://www.epis.sk/getdoc/8c3f6910-210c-408e-a2f1-30cfe6832e7e/ EU_standard_definicie.aspx> [cit. 2015.09.09.].

24. **European Centre for Disease Prevention and Control, 2013:** Annual epidemiological report 2012. Stockholm: ECDC, 72–74. Available on internet: <http://www.ecdc.europa.eu/en/ publications/Publications/Annual-Epidemiological-Report-2012.pdf> [cit. 2013.06.10.].

25. **Fayer, R., Xiao, L., 2008**: *Cryptosporidium and Cryptosporidiosis*. Boca Raton: CRC Press, 560 pp.

26. **Fotedar, R., 2007:** Laboratory diagnostic techniques for *Entamoeba* species. *Clin. Microbiol. Rev.*, 20, 511—532.

27. **Gomez-Puerta, L. A., Lopez-Urbina, M. T., Alarcon, V., Cama, V., Gonzales, A. E., Xiao, L., 2014:** Occurrence of *Giardia duodenalis* assemblages in alpacas in the Andean region. *Parasitol. Int.*, 63, 31—34.

28. **Guglielmetti, P., 1989:** Family outbreak of *Blastocystis hominis* associated gastroenteritis. *Lancet*, 2, 1394.

29. **Haque, R., Mondal, D., Duggal, P., Kabir, M., Roy, S., Farr, B. M., 2006:** *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infect. Immun.*, 74, 904—909.

30. **Hayes, E. B., Matte, T., O'Brien, T. R., McKinley, T. W., Logsdon, G. S., Rose, J. B. et al., 1989:** Large community outbreak of cryptosporidiosis due to contamination of a filtered public water supply. *N. Eng. J. Med.*, 320, 1372—1376.

31. **Huang, P., 1995:** The first reported outbreak of diarrheal illness associated with *Cyclospora* in the United States. *Ann. Intern. Med.*, 123, 409—414.

32. **Hughes, M., Petri, W., 2000:** Amoebic liver abscess. *Infect. Dis. Clin. North Am.*, 14, 565—582.

**33. Joslin, C., Tu, Y. E., McMahon, T. T., Passaro, D. J., Stayner, L. T., Sugar, J. et al., 2006:** Epidemiological characteristics of a Chicago-area *Acanthamoeba* keratitis outbreak. *Am. J. Ophthalmol.*, 142, 212—217.

**34. Kadlec, V., Skvárová, J., Cerva, L., Nebáznivá, D., 1980:** Virulent *Naegleria fowleri* in indoor swimming pool. *Folia Parasitol.* (Prague), 27, 11—17.

**35. Karanis, P., Kourneti, C., Smith, H., 2007:** Waterborne transmission of protozoan parasites: A worldwide outbreaks and lessons learned. *J. Water Health.*, 5, 1—38.

**36. Khalifa, A. M., 1999:** Diagnosis of *Blastocystis hominis* by different staining techniques. *J. Egypt. Soc. Parasitol.*, 29, 157—165.

**37. Koppová, K., Bobáľová, P., 2009:** *Professional Competence for Carrying out Epidemiologically Important Activities in Water Treatment Plants and in the Water Supply Facilities* (In Slovak), Banská Bystrica: RÚVZ 1—17.

**38. Kreidl, P., Imnadze, P., Baidoshvili, L., Greco, D., 1999:** Investigation of an outbreak of amoebiasis in Georgia. *Eurosurveill. Monthly*, 4, 103—104.

**39. Legua, P., Seas, C., 2013:** *Cystoisospora* and *Cyclospora*. *Curr. Opin. Infect. Dis.*, 26, 479—483.

**40. Lešník, F., Danko, J., Bálent, P., 2005:** *Medical Lymphology* (In Slovak). Bratislava, H&H, 253—264.

**41. Levy, D. A., Bens, M. S., Craun, G. F., Calderon, R. L., Herwaldt, B. L., 1998:** Surveillance for waterborne disease outbreaks: United States 1995—1996. *MMWR*, 47, 1—34.

**42. MacKenzie, W. R., Hoxie, N. J., Proctor, M. E., Gradus, M. S., Blair, K. A., Peterson D. E. et al., 1995:** A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *Epidemiol. Infect.*, 115, 545—553.

**43. Mackey-Lawrence, N. M., Petri, W. A. Jr., 2010:** Amoebic dysentery. *Clin. Evid.,* 1, 1—17.

**44. Magi, B., 2006:** *Cryptosporidium* infection: diagnostic techniques. *Parasitol. Res.*, 98, 150—152.

**45. Mao, L. X., Zheng, J. J., Wang, X. M., Yao, L. N., Cheng, H. L., Zhu, Q. G., 2009:** Epidemiological investigation on an outbreak of amoebic dysentery in Jiangshan City of Zhejiang Province. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 27, 182—183.

**46. Martinez, A. J., Visvesvara, G. S., 1997:** Free-living amphizoic and opportunistic amoebas. *Brain Pathol.*, 7, 583—598.

**47. Meier, P. A., Mathers, W. D., Sutphin, J. E., Folberg, R., Hwang, T., Wenzel, R. P., 1998:** An epidemic of presumed *Acanthamoeba* keratitis that followed regional flooding. Results of a case-control investigation. *Arch. Ophthalmol.*, 116, 1090—1094.

**48. Montoya, J. G., 2002:** Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. *J. Infect. Dis.*, 185, 73—82.

**49. Murrow, L. B., Blake, P., Kreckman, L., 2002:** Outbreak of cyclosporiasis in Fulton County, Georgia. *Georgia Epidemiol. Rep.*, 18, 1.

**50. Nygård, K., Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N. et al., 2006:** A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. *BMC Public Health*, 6, 141.

**51. Ortega, Y. R., Sanchez, R., 2010:** Update on *Cyclospora cayetanensis*, a food-borne and waterborne parasite. *Clin. Microbiol. Rev.*, 23, 218—234.

**52. Pozio, E., Rezza, G., Boschini, A., Pezzotti, P., Tamburrini, A., Rossi, P. et al., 1997:** Clinical cryptosporidiosis and Human Immunodeficiency Virus (HIV)-induced immunosuppression: Findings from a longitudinal study of HIV-positive and HIV-negative former injection drug users. *J. Infect Dis.*, 176, 969—975.

**53. Rabold, J. G., Hoge, C. W., Shlim, D. R., Kefford, C., Rajah, R., Echeverria, P., 1994:** *Cyclospora* outbreak associated with chlorinated drinking water. *Lancet*, 344, 1360—1361.

**54. Regional Office for Public Health, 2012:** Analysis of epidemiologic situation and activity of epidemiologic division in Slovakia in 2012 (In Slovak). Banská Bystrica, RÚVZ, Available on internet: <http://www.epis.sk/InformacnaCast/ Publikacie/VyrocneSpravy/Files/VS_SR_2012.aspx> [cit. 2013.08.11.].

**55. Sagua, H., Soto, J., Délano, B., Fuentes, A., Becker, P., 1978:** An isosporosis outbreak caused by *Isospora belli* at Antofagasta City (Chile). Considerations of 90 cases diagnosed within a period of 3 months. *Bol. Chil. Parasitol.*, 33, 8—12.

**56. Sargeaunt, P. G., Williams, J. E., Neal, R. A., 1980:** A comparative study of *Entamoeba histolytica* (NIH:200, HK9,etc.) "*E. histolytica*-like" and other morphologically identical amoebae using isoenzyme electrophoresis. *Trans. R. Soc. Trop. Med. Hyg.*, 74, 469—474.

**57. Scanlan, P. D., Stensvold, C. R., 2013:** *Blastocystis*: getting to grips with our guileful guest. *Trends Parasitol.*, 29, 523—529.

**58. Schuster, F. L., Ramirez-Avila, L., 2008:** Current world status of *Balantidium coli. Clin. Microbiol. Rev.*, 21, 626–638.

**59. Schuster, F. L., Visvesvara, G. S., 2004:** Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int. J. Parasitol.*, 34, 1001—1027.

**60. Shaw, P. K., Brodsky, R. E., Lyman, D. O., Wood, B. T., Hibler, C. P., Gealy, G. R. et al., 1977:** A community wide of outbreak of giardiasis with evidence of transmission by municipal water supply. *Ann. Intern. Med.*, 87, 426—432.

**61. Smith, H. V., Rose, J. B., 1998:** Waterborne cryptosporidiosis: current status. *Parasitology Today*, 14, 14—22.

**62. Soave, R., 1996:** *Cyclospora*: An overview. *Clin. Infect. Dis.*, 23, 436—437.

**63. Stensvold, C. R., Smith, H. V., Nagel, R., Olsen, K. E., Traub, R. J., 2010:** Eradication of *Blastocystis* carriage with antimicrobials: reality or delusion? *J. Clin. Gastroenterol.*, 44, 85—90.

**64. Štrkolcová, G., Maďar, M., Hinney, B., Goldová, M., Mojžišová, J., Halánová, M., 2015:** Dog's genotype of *Giardia duodenalis* in human: first evidence in Europe. *Acta Parasitol.*, 60, 796—799.

**65. Taverne, J., 2002:** Toxoplasmosis in Brasil. *Trends Parasitol.*, 18, 203—204.

**66. Taylor, A. Jr., Craun, G. F., Faich, G. A., McCabe, L. J., Gangarosa, E. J., 1972:** Outbreaks of waterborne diseases in the United States, 1961—1970. *J. Infect. Dis.*, 125, 329—331.

**67. Úrad Verejného Zdravotníctva (Public Health Office), 2013:** Annual report about activity of Regional office of public health care in Slovakia in the year 2013 (In Slovak): Available on internet: <http://www.uvzsr.sk/docs/vs/vyrocna_sprava_SR_2013.pdf> [cit. 2015.07.29.].

**68. Úrad Verejného Zdravotníctva (Public Health Office), 2015:** Annual report about activity of offices of public health care in Slovakia according to the different departments of public health (In Slovak). Available on internet: < http://www.uvzsr.sk / docs/vs/vyrocna_sprava_SR_2014.pdf> [cit. 2015.07.27.].

**69. Veazie, L., 1969:** Epidemic giardiasis. *N. Engl. J. Med.*, 281, 853.

**70. Walzer, P. D., Judson, F. N., Murphy, K. B., Healy, G. R., English, D. K., Schultz, M. G., 1973:** Balantidiasis outbreak in Truk. *Am. J. Trop. Med. Hyg.*, 22, 33—41.

**71. Wu, G., 2005:** Investigation of an epidemic outbreak of blastocystiasis. *Chin. J. Parasitol. Dis. Control.*, 13, 25.

# FEEDING WITH LUPIN CAN AFFECT SHEEP OVARIAN CELL CYCLE BUT NOT APOPTOSIS

Vlčková, R.[1], Sirotkin, A. V.[2, 3], Kádasi, A.[3]
Sopková, D.[1], Andrejčáková, Z.[1], Valocký, I.[4]

[1]Department of Anatomy, Histology, and Physiology, Institute of Physiology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
[2]Constantine the Philosopher University, Trieda A. Hlinku 1, 949 74, Nitra
[3]Animal Production Centre, Hlohovecká 2, 951 41 Lužianky near Nitra
[4]Equine Clinic, University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

radkavl@seznam.cz

## ABSTRACT

The stimulatory effect of lupin feeding on sheep ovarian folliculogenesis and fecundity is known, but the mechanisms of this effect remain to be elucidated. We hypothesised that the lupin-induced stimulation of ovarian follicular growth could be due to changes in ovarian proliferation or apoptosis. To validate this hypothesis we compared accumulation of cell cycle- and apoptosis-related peptides in ovarian granulosa cells of sheep fed and not fed with lupin groats. Percentages of cells containing proliferation markers — proliferating cell nuclear antigen (PCNA), cyclin B1, mitogen-activated protein kinase (MAPK/ERK1, 2), as well as apoptosis markers – bax, caspase 3 and terminal deoxynucleotidyl transferase (TdT) in smears of ovarian granulosa cells aspirated from ovarian follicles were evaluated using immunocytochemistry and TUNEL. It was found that lupin consumption increased the proportion of cells containing MAPK/ERK1, 2 and decreased accumulation of PCNA.

There was no effect of lupin feeding on accumulation of cyclin B1, bax, caspase 3 or TdT. These observations suggest that lupin can promote the cell cycle at the G1 phase, or suppress it at the S-phase without influencing the G2 phase, and without affecting either cytoplasmic or nuclear apoptosis in ovarian granulosa cells. Lupin can therefore promote sheep ovarian follicular growth affecting the G1 and S phases of the cell cycle and the subsequent proliferation/apoptosis rate.

Key words: apoptosis (TUNEL, bax, caspase 3); lupin; ovarian granulosa cell; proliferation (PCNA, cyclin B1, MAPK/ERK1, 2); sheep

## INTRODUCTION

High protein diets such as lupin seeds seem to be a very promising source of plant protein for farm animal nutrition [23]. Besides metabolic effects, lupin can influence farm

animal reproduction processes. Lupin supplementation positively influences the ovulation rate in ewes during the breeding season [5, 11, 15, 19] especially with short-term feeding (4–6 days) between days 9 to 14 of the oestrus cycle [2, 8, 19]. The reproductive effects of lupin can be mediated by changes in either metabolic and/or reproductive hormones. Feeding with lupin increases levels of insulin through hyperglycaemia, insulin-like growth factor I (IGF-I) and leptin [1, 5, 11, 19] and decreases levels of growth hormone (GH) [5] in blood plasma and in both cyclic and acyclic sheep. Lupin-induced stimulation of sheep ovarian folliculogenesis is associated with suppression of oestradiol production by follicles [19, 22], but some authors [24, 25] have found increases in oestradiol concentrations using a different experimental sheep model. It is supposed that lupin consumption reduces negative feedback within the hypothalamus-pituitary-ovarian axis, resulting in high levels of follicle stimulating hormone (FSH) in the blood, which in turn leads to stimulation of ovarian folliculogenesis [19, 22]. It increases the number of small follicles to be selected for ovulation [6, 22] and prevents atresia of large follicles [6, 21]. The mechanisms of this effect remain to be elucidated, because some authors have ascertained that there is no change in FSH concentration and that metabolic hormones are included in this mechanism [24, 25]. We hypothesised that lupin-induced stimulation of ovarian follicular growth could be due to changes in the ovarian proliferation/cell cycle or apoptosis. To validate this hypothesis, we compared accumulation of cell cycle- and apoptosis-related peptides in ovarian granulosa cells of sheep fed and not fed with lupin groats. To understand what can be influenced by lupin supplementation in the cell-cycle phase, we compared the accumulation of markers/regulators of different phases of mitosis – proliferating cell nuclear antigen (PCNA, marker and promoter of S-phase of the cell cycle) [14], cyclin B1 (marker of G2 phase of mitosis) [13] and mitogen-activated protein kinase (MAPK/ERK1,2, marker and promoter of G1 phase of mitosis and in some cases of the cell cycle arrest and apoptosis) [3, 4, 12, 17]. To examine whether lupin supplementation can influence either cytoplasmic or nuclear apoptosis, we analysed the influence of lupin feeding on the accumulation of bax and caspase 3 (markers and promoters of cytoplasmic apoptosis) [7] and TdT (marker of nuclear apoptosis and DNA fragmentation) [10].

## MATERIAL AND METHODS

Merino anoestrous ewes (May to June) of approximate age 4 to 6 years were transported from a nearby farm (approx. 1 hour transfer) and kept under standard conditions in the Experimental Station of the University of Veterinary Medicine and Pharmacy in Košice, the Slovak Republic, were used in this experiment. For the reason that we wanted to obtain metabolic parameters (not included in this study) to know the influence of lupin supplementation after short transport stress, the animals were not adapted to the new conditions of the experimental station for 2 weeks. All procedures were approved by the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2371/08-221). The ewes weighed approximately 41.5 kg each, and their condition score was approximately 2.5. One day after transport (Day 0 of the experiment) the ewes were divided into 2 groups: the diet of the control group (C; n = 7) consisted of trefoil silage and hay, while the diet of the experimental group (L; n = 7) was supplemented with lupin groats (Lupinus angustifolius, var. SONET; 500 g per animal per day). All ewes were induced for oestrus and synchronized with intramuscular injection of lecirelinum (LHRH super analogue; 12.5 μg per head; Supergestran inj. a.u.v. Nordic Pharma, Jesenice, Czech Republic) on Day 6 and with prostaglandin analogue (cloprostenolum D 37.5 μg per head; Remophan inj. a.u.v. Bioveta, Ivanovice na Hané, Czech Republic) five days later (Day 10). Lupin groats were introduced into the diet of experimental ewes on the day of lecirelinum injection (Day 6) and maintained for another 8 days until the third day after prostaglandin analogue application (Day 13). On the last day of the experiment (Day 15) the ewes were euthanized by intravenous application of T61 inj. a.u.v. (4–6 ml per head; Intervet international B.V., Boxmeer, The Netherlands) and laparotomy was used for the ovary collection. Granulosa cells from all follicles visible on the ovary surface (follicles 2 to 4 mm in diameter) were aspired together with follicular fluid and separated by centrifugation at ×200 g. The granulosa cells were smeared on slides, dried, fixed with 4% formalin, dried again and stored at +4 °C until ready for staining for markers of proliferation and apoptosis.

Markers of cell cycle and cytoplasmic apoptosis in granulosa cells were detected by means of immunocytochemistry [16] using primary monoclonal antibodies against MAPK/ERK1, 2, PCNA, cyclin B1, bax and caspase 3 (all

from Santa Cruz Biotechnology, Inc.; dilution 1:100) cross-reacted with corresponding human, rat, rabbit, porcine, bovine, sheep and yeast proteins and secondary goat IgG labelled with horseradish peroxidase and DAB (both from Millipore Corp., Bellerica, MA, USA). Cells treated with secondary antibody and DAB but omitting the primary antibody were used as negative controls. The presence of specific immunoreactivity in granulosa cells and the percentage of cells containing MAPK/ERK1, 2, PCNA, cyclin B1, bax and caspase 3 were determined using light microscopy.

The chamber slides were subjected to TUNEL (TdT-mediated dUTP nick end labelling) assay using the In Situ Cell Death Detection Kit (Boehringer Mannheim, GmbH, Mannheim, Germany) according to the producer's instructions. Cells containing intensive TdT-positive staining in the nuclei were considered apoptotic. Fixed and permeabilized cells incubated without TdT but with secondary FITC-conjugated antibody were used as negative controls. Permeabilized cells incubated with bovine pancreatic DNAase 1 (Boehringer Mannheim, GmbH, Mannheim, Germany; 0.01 mg/ml; 10 min at room temperature) before TdT treatment to induce DNA fragmentation, were used as positive controls. The percentage of TdT-positive cells in each culture was determined by counting TUNEL-positive and TUNEL-negative cell numbers using fluorescent microscopy.

Six smears of granulosa cells were obtained from each ovary. Each experimental group was represented by 42 smears of granulosa cells collected from 7 animals. The data presented are means of values obtained in two separate experiments performed on separate days using separate pools of ovaries obtained from separate animals. The proportion of cells containing each analyzed substance was calculated following immunocytochemical analysis by counting at least 1000 cells per smear. Significant differences between the animals and experiments were evaluated using two-way ANOVA. When the effects of treatments were revealed, data from the experimental and control groups were compared with Duncan's test using Sigma Plot 11.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences from controls were considered significant at $P < 0.05$.

## RESULTS

Our analyses demonstrated the presence of proliferation-associated antigens such as MAPK/ERK1, 2, PCNA and cyclin B1, as well as apoptosis-associated antigens such as bax, caspase 3 and TdT within sheep ovarian granulosa cells. The panel of positive signals of these markers is shown in Figure 2.

Quantitative immunocytochemical analysis demonstrated that lupin supplementation significantly increased the proportion of granulosa cells containing MAPK/ERK1, 2 ($P < 0.05$) but decreased the proportion of granulosa cells

Fig. 2. The panel of positive signals of cell-cycle markers — MAPK/ERK1, 2 (A), PCNA (B), cyclin B1 (C), cytoplasmic apoptosis — caspase 3 (D), bax (E), and nuclear apoptosis — TdT (F) in smears of ovarian granulosa from sheep fed and not fed with lupin groats.
Images A—E were observed using light microscopy, and image F using fluorescent microscopy. Magn. ×400
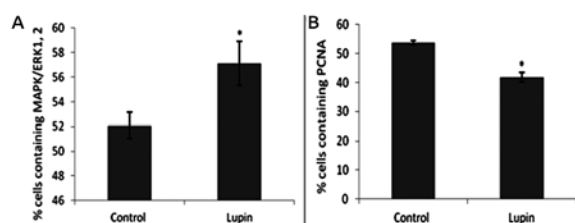
Fig. 1. The percentage of granulosa cells containing cell-cycle markers – MAPK/ERK1, 2 (A), PCNA (B) in a smear of ovarian granulosa of sheep supplemented and not supplemented with lupin, based on immunocytochemical staining and light microscopy.
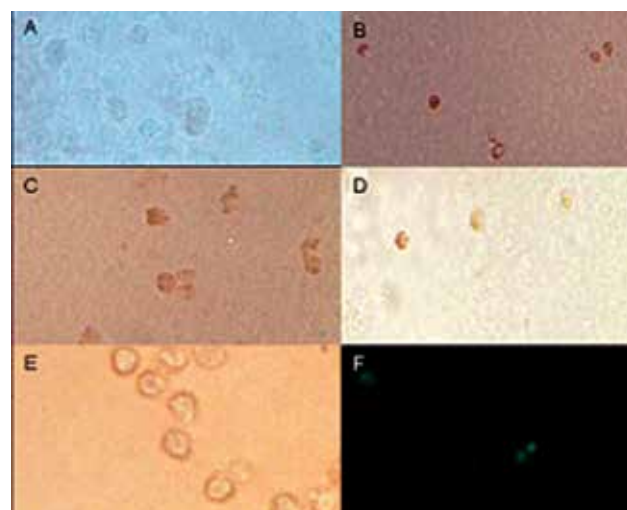Values represent means ± S. E. M. *— P < 0.05 lupin-fed group versus control group (sheep not fed with lupin)

containing PCNA (P < 0.05) compared to controls. The percentages of granulosa cells containing the cell-cycle related antigens (MAPK/ERK1, 2 and PCNA) are presented in Figure 1. The percentage of cells containing cyclin B1 did not change after lupin consumption (Table 1).

No effect of lupin feeding was observed on the proportion of granulosa cells containing bax and caspase 3. The percentages of granulosa cells containing these markers of cytoplasmic apoptosis are presented in the Table 1.

TUNEL did not reveal any significant lupin-induced changes in accumulation of TdT, the marker of nuclear apoptosis (Table 1).

**Table 1. The percentage of granulosa cells containing markers of cytoplasmic apoptosis (bax, caspase 3), nuclear apoptosis (TdT) and proliferation (cyclin B1) in the ovaries of sheep fed and not fed with lupin. Values are means ±S.E.M. All values represent the % of cells containing particular antigens**

| Supplement | Bax | Caspase 3 | TdT | Cyclin B1 |
|---|---|---|---|---|
| **None** (control) | 38.95 ± 1.91 | 49.54 ± 1.31 | 61.34 ± 1.74 | 44.36 ± 1.47 |
| **Lupin** | 38.86 ± 1.51 | 52.09 ± 2.04 | 59.81 ± 2.03 | 43.29 ± 1.45 |

## DISCUSSION

Our analysis demonstrated the presence of cell-cycle markers MAPK/ERK1, 2, PCNA, and cyclin B1 in granulosa smears. This suggests that the ovarian cells were viable, and part of the cells were proliferating (containing markers of the three main phases of mitosis — G1, S and G2). On the other hand, the presence of apoptosis markers caspase 3, bax and TdT in granulosa cells was revealed too. This indicates that part of the cells had undergone both cytoplasmic and nuclear apoptosis. Our observations confirmed the presence of these markers of cell cycle and apoptosis in sheep ovarian granulosa cells, as reported previously [9, 18].

We are the first who have demonstrated the influence of lupin supplementation on the expression of cell cycle- and apoptosis-related proteins. In our experiments, lupin consumption was associated with increased proportion of cells containing MAPK/ERK1, 2 (marker and promoter of the G1 phase of mitosis, and in some cases of cell-cycle arrest and apoptosis) [3, 4, 12, 17], and decreased accumulation of PCNA (marker and promoter of S-phase of the cell cycle) [14]. The percentage of cells containing cyclin B1 (marker of G2 phase of mitosis) [13] did not changed after lupin consumption. These observations suggest that lupin feeding can promote the cell cycle at the G1 phase, but suppress (or shorten) it at the S-phase without influencing the G2 phase of the cell cycle. The acceleration of the cell cycle at the G1 phase could be a cause of increased cell proliferation, which in turn induced promotion of ovarian follicular growth and fecundity after feeding sheep with lupin, as reported previously [2, 5, 8, 11, 15, 19].

It has been demonstrated that MAPK/ERK1, 2 can be involved not only in promotion of cell proliferation, but also in cell-cycle arrest and development of apoptotic changes in some pathological conditions [4, 12]. It is more probable that in our experiments MAPK/ERK1, 2 was rather the promoter of cell proliferation than of apoptosis, because we did not detect any effect of lupin feeding on accumulation of markers of either cytoplasmic (bax, caspase 3) or nuclear (TdT) apoptosis. The fate of ovarian follicles depends on the equilibration of proliferation and apoptosis of their cellular components. Since no changes in apoptosis was observed in our experiments, it might be hypothesised that the ability of lupin to prevent atresia of ovarian follicles reported previously [6, 21] can be explained by its ability not to change apoptosis and apoptosis-induced atresia, but to promote ovarian cell proliferation, turnover and remodelling, and therefore to increase the proliferation/apoptosis rate in the follicular cell population.

The mechanisms of lupin effect on ovarian cell proliferation remain somewhat unclear. It might be proposed that this effect could be mediated by FSH, IGF-I, insulin, leptin and œstradiol, the known hormonal promoters of ovarian cell proliferation [20], which increase in ovine blood after feeding with lupin, as reported previously [1, 5, 11, 19]. Due to the positive effect of lupin supplementation on sheep production and reproduction, understanding the mechanisms of lupin action is important from both scientific and practical viewpoints. Our observations are the first evidence that the stimulatory effect of lupin feeding on ovine reproduction can be due to its effect on the ovarian cell cycle, but not on either cytoplasmic or nuclear apoptosis.

## REFERENCES

**1. Armstrong, D. G., Gong, J. G., Webb, R., 2003:** Interactions between nutrition and ovarian activity in cattle: Physiological, cellular and molecular mechanisms. *Reproduction Suppl.*, 61, 403—414.

**2. Brien, F. D., Cumming, I. A., Baxter, R. V., 1977:** Effect of feeding a lupin grain supplement on reproductive performance of maiden and mature ewes. *J. Agr. Sci.*, 89, 437—443.

**3. Cagnol, S., Chambard, J. C., 2010:** ERK and cell death: mechanisms of ERK-induced cell death — apoptosis, autophagy and senescence. *FEBS J.*, 277, 2—21.

**4. Chang, F., Steelman, L. S., Shelton, J. G., Lee, J. T., Navolanic, P. M., Blalock, W. L. et al., 2003:** Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). *Int. J. Oncol.*, 22, 469—480.

**5. Downing, J. A., Joss, J., Connel, P., Scaramuzzi, R. J., 1995:** Ovulation rate and the concentrations of gonadotrophic and metabolic hormones in ewes fed lupin grain. *J. Reprod. Fertil.*, 103, 137—145.

**6. Haresign, W. 1981:** The influence of nutrition on reproduction in the ewe. 1. Effects on ovulationrate, follicle development and luteinizing hormone release. *Anim. Prod.*, 32, 197—202.

**7. Hockenbery, D. M. 2010:** Targeting mitochondria for cancer therapy. E*nviron. Mol. Mutagen.*, 51, 476—489.

**8. King, B. J., Robertson, S. M., Wilkins, J. F., Friend, M. A. 2010:** Short-term grazing of lucerne and chicory increases ovulation rate in synchronised Merino ewes. *Anim. Reprod. Sci.*, 121: 242—248.

**9. Lea, R. G., Andrade, L. P., Rae, M. T., Hannah, L. T., Kyle, C. E., Murray, J. F., Rhind, S. M., Miller, D. W., 2006:** Effects of maternal undernutrition during early pregnancy on apoptosis regulators in the ovine foetal ovary. *Reproduction*, 131, 113—124.

**10. Loo, D. T., 2011:** In situ detection of apoptosis by the TUNEL assay: an overview of techniques. *Method Mol. Cell. Biol.*, 682, 3—13.

**11. Marton, A., Faigl, V., Kerestes, M., Kulcsar, M., Nagy, S., Febel, H. et al., 2009:** Milk progesterone profiles, blood metabolites, metabolic hormones and pregnancy rates in Awassi ewes treated by gestagen + eCG at the early breeding season. *Vet. Med.-Czech*, 54, 507—516.

**12. Meloche, S., Pouyssegur, J., 2007:** The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1-to S-phase transition. *Oncogene*, 26, 3227—3239.

**13. Miyazaki, T., Arai, S., 2007:** Two distinct controls of mitotic cdk1/cyclin B1 activity requisite for cell growth prior to cell division. *Cell Cycle*, 6, 1419—1425.

**14. Naryzhny, S. N., Lee, H., 2001:** Protein profiles of the Chinese hamster ovary cells in the resting and proliferating stages. *Electrophoresis*, 22, 1764—1775.

**15. Oldham, C. M., Lindsay, D. R., 1984:** The minimum period of intake of lupin grain required by ewes to increase their ovulation rate when grazing dry summer pasture. In **Lindsay, D. R., Pearce, D. T.:** *Reproduction in Sheep*. Australian wool corporation technical publication, Cambridge University Press, 274—276.

**16. Osborn, M., Isenberg, S., 1994:** Immunocytochemistry of frozen and of paraffin tissue sections. **Celis, J. E.:** *Cell Biology: A Laboratory Handbook*. Academic Press, New York/London, 361—367.

**17. Roskoski, R. Jr., 2012:** ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol. Res.*, 66, 105—143.

**18. Salvetti N. R., Ortega H. H., Veiga-Lopez A., Padmanabhan V., 2012:** Developmental programming: impact of prenatal testosterone excess on ovarian cell proliferation and apoptotic factors in sheep. *Biol. Reprod.*, 87, 1—10.

**19. Scaramuzzi, R. J., Campbell, B. K., Downing, J. A., Kendall, N. R., Khalid, M., Munoz-Gutiérrez M. et al., 2006:** A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. *Reprod. Nutr. Dev.*, 46, 339—354.

**20. Sirotkin, A. V., 2011:** *Regulators of Ovarian Functions*. Nova Science Publishers, Inc., New York, 193 pp.

**21. Somchit, A., Campbell, B. K., Khalid, M., Kendall, N. R., Scaramuzzi, R. J., 2007:** The effect of short-term nutritional supplementation of ewes with lupin grain (*Lupinus luteus*), during the luteal phase of the estrous cycle on the number of ovarian follicles and the concentrations of hormones and glucose in plasma and follicular fluid. *Theriogenology*, 68, 1037—1046.

**22. Somchit-Assavacheep, A., 2011.** Influence of nutritional management on folliculogenesis in ewes. *Thai J. Vet. Med. Suppl.*, 41, 25—29.

**23. Straková, E., Suchý, P., Večerek, V., Šerman, V., Mas, N., Jůzl, M., 2006:** Nutritional composition of seeds of the genus Lupinus. *Acta Vet. Brno*, 75, 489—493.

**24. Viñoles, C., Paganoni, B. L., Glover, K. M., Milton, J. T., Blaache, D., Blackberry, M. A. et al., 2010:** The use of a 'first-wave' model to study the effect of nutrition on ovarian follicular dynamics and ovulation rate in the sheep. *Reproduction*, 140, 865—874.

**25. Viñoles, C., Paganoni, B. L., McNatty, K. P., Heath, D. A., Thompson, A. N., Glover, K. M. et al., 2014:** Follicle development, endocrine profiles and ovulation rate in adult Merino ewes: effects of early nutrition (pre- and post-natal) and supplementation with lupin grain. *Reproduction*, 147, 101—110.

# EXPRESSION OF CYTOKERATIN 18 IN THE TASTE BUDS OF THE DOG TONGUE

## Marettová, E., Maretta, M.

University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

elena.marettova@uvlf.sk

## ABSTRACT

Cytokeratin 18 filaments in the taste buds of the dog tongue was identified immunohistochemically. The distribution of cytokeratin 18 filaments was restricted to taste cells and progenitor cells of the vallate papillae and acinic cells of the salivary Ebner glands and cells of their ducts. The immunoreaction in the taste cells was distributed in the cytoplasm around the nucleus. Variation in the intensity of the reaction of the taste cells and progenitor cells for cytokeratin have been displayed. The results provide a basis for studies on the biological events during development and pathological processes.

Key words: cytokeratin 18; dog; taste buds; tongue

## INTRODUCTION

The taste buds are composed mainly of clusters of sensory cells located in the stratified squamous epithelium of the fungiform, vallate and foliate papillae of the tongue, or other areas of the mouth and throat. Canine taste buds were divided into five types. The Type I cell was supportive in nature, located between other cell types and enveloping nerve fibres. This cell apically secretes dense mucous substances. The Type II cell was in broad contact with nerve fibres and consistently contained a subsurface cistern beneath them. In the dog, this cell was characterized by a large supranuclear Golgi apparatus. The Type III cell was, as in other animals, regarded as the gustatory cell, since it made synaptic contacts with nerves and contained synaptic vesicles. The Type IV cell was the immature basal cell which has been established in other species; mitosis could be seen in this cell. Another type, a slender, immature-looking cell located at the outermost layer, was identified as the peripheral cell known in the rat [9].

Additional functions have been attributed to taste buds. The taste bud is generally classified as a chemoreceptor for the sense of taste. An important early event in mammalian gustatory transduction with respect to sodium chloride has been found to be the passage of sodium ions through specific transport pathways in the apical region of the taste bud [5]. Immunostaining studies have shown a distribution of

CD36 along the apical side of the circumvallate taste bud cells [10]. Expression, physiological action, and coexpression patterns of neuropeptide Y in rat taste-bud cells have been detected [23]. To date, two peptides, cholecystokinin and vasoactive intestinal peptide, have been localized in subsets of taste-bud cells and one, cholecystokinin, has been demonstrated to produce excitatory physiological actions [23]. GABA and glutamate have been detected in nerve fibres that innervate the taste buds, and, to a substantially lesser extent, in fine, varicose axons that penetrated the surrounding non-taste epithelium [7]. Noradrenaline has been found in the connective tissue underlying the taste buds, whereas serotonin has been located in the basal area of the gustatory epithelium but not inside the taste buds [2].

Cytokeratins (CK) are the intermediate filament proteins of the epithelial cells. They form a complex family of 20 different polypeptides in human tissues, with molecular weights ranging from 40 to 68 kD [3, 11, 13, 14]. Different sets of CK molecules are expressed in various epithelial tissue and those at various stages of differentiation [1]. In the adult human tongue, changes of the distribution of the cytokeratin filaments CK 8, 18, 19 and 20 ("gastrointestinal" type), cytokeratin filaments CK 7 ("ductal" type), and CK 13 has been reported [15, 19]. The patterns of keratin expression of taste buds in the murine oral mucosa were examined using a panel of antibodies with various specificities for cytokeratins [21]. The differentiation of taste bud cells, by describing the expression profiles of cytokeratins 8 and 14 [1] and cytokeratin 19-like immunoreactivity in gustatory epithelia has been evaluated [20]. With the exception of CK 13, which remains negative, all cytokeratins tested have been present in taste cells. The patterns of keratin expression of taste buds in murine oral mucosa were examined using a panel of antibodies with various specificities for cytokeratins [18]. The patterns for taste buds differed markedly from those of the surrounding epithelium but no regional differences in the staining patterns of the taste buds themselves have been detected. In the caudal third of the rat's tongue, keratin 20 immunoreactivity was restricted to taste buds, whereas keratins 7, 8, 18, and 19 were expressed in vallate and foliate taste buds and in the cells of salivary ducts that merge with these taste epithelia [22]. The antibodies against keratins 7, 8, 18, and 19 were considered to be useful markers for intragemmal cells in the studies of taste bud development, degeneration, regen-

eration, turnover and tissue culture. Several histochemical studies made on the lingual papillae in various animal species demonstrated the expression of keratins and other bioactive substances in specific cell types of taste cells for the analysis of cell lineage of the taste buds [1, 3, 5, 7, 23]. However, the cell types in the taste bud differ from species to species. The aim of the study was immunohistochemically to localize CK18 in the lingual taste buds of the dog tongue.

## MATERIALS AND METHODS

The samples of the tongue with vallate papillae from five dogs were obtained and placed in 0,1 mol phosphate buffered 10% formalin for 24 hours at room temperature, dehydrated and embedded in paraffin. The 5 µm thick sections were deparaffinised and rehydrated. For immunostaining, an avidin-biotin-peroxidase complex (ABC) method was used [6]. Sections were pretreated with 3 % $H_2O_2$ in methanol for 30 min to reduce endogenous activity and preincubated with 2 % goat serum to mask nonspecific binding sites. Afterwards, the sections were incubated at 4 °C overnight with monoclonal anti-keratin 18 antibody (Sigma), dilution 1:50. The sections were washed twice in a phosphate-balanced salt solution (PBS) and then incubated with biotinylated polyvalent secondary antibody for 45 min, washed in PBS, and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame Calif., USA). After washing with PBS, the peroxidase activity was visualized with 0.05 % 3´.3´-diaminobenzidine (DAB) and 0.03 % v/v H2O2. Some sections were counterstained with Mayer's haematoxylin. For negative controls, the first antibody was substituted by PBS.

## RESULTS

The taste buds were ellipsoid clusters of sensory taste cells embedded in the stratified squamous epithelium (Fig. 1). Histologically, taste buds contained specialized epithelial cells (taste cells, gustatory cell), sustentacular cells and proliferative cells (progenitor cells). The progenitor cells were located at the basal portion of the taste buds.

Immunohistochemically, in the tongue, a positive reaction to cytokeratin18 was restricted to taste cells of the vallate papilla. Staining within the taste buds was not homo-

**Fig. 1. Lateral sides of the vallate papilla with gustatory furrow (F). Taste buds (TB) in the epithelium (Azan)**
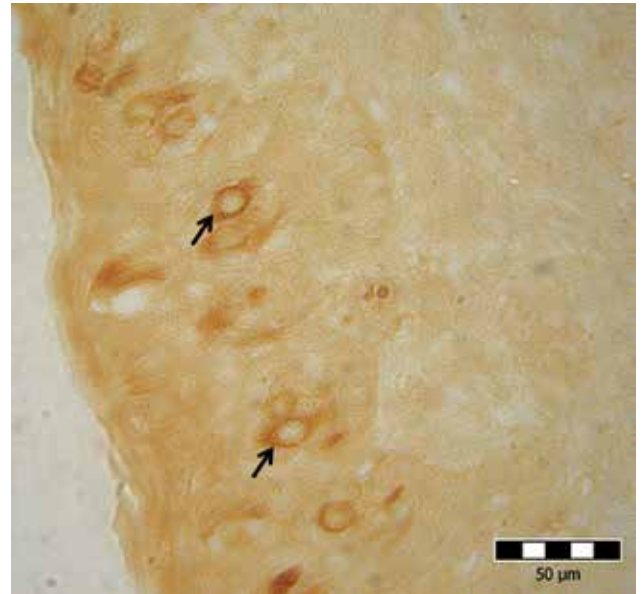


**Fig. 2. Lateral sides of the vallate papilla. The positive reaction to CK 18 is confined to the TB (arrow)**
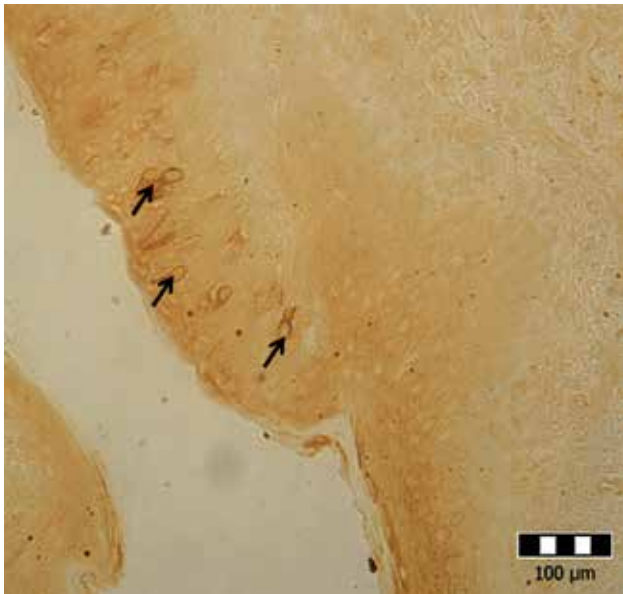


**Fig. 3. Lateral side of the vallate papilla. The positive reaction is mainly seen in the perinuclear region of the cytoplasm of the taste cells (arrow)**
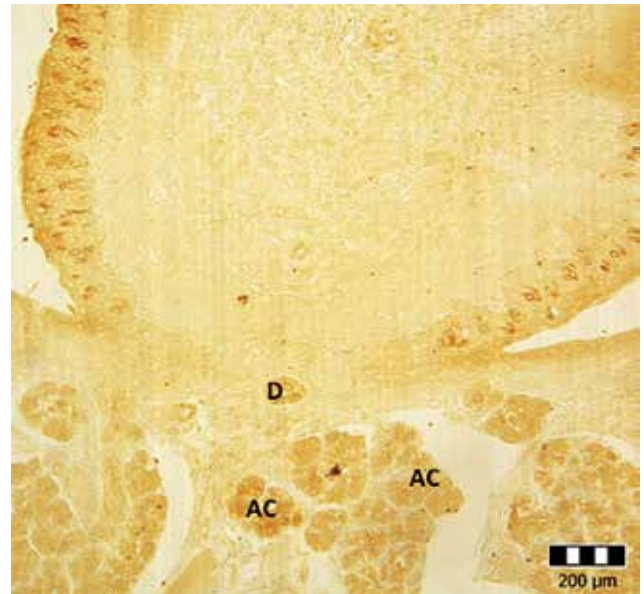


**Fig. 4. Von Ebner's glands. Positive reaction is in the acinary cells (AC) and the duct (D)**

geneous but no clear differences of keratin staining could be directly related to the subtypes of cells constituting them. Cytokeratin 18 was preferentially detected in taste cells located at the central position of the papilla. There was a marked increase in the number of TB cells on the external surfaces of the papillae. In these taste buds, taste cells revealed a high positive reaction. Inside the taste cells, a positive reaction was confined to the perinuclear region of the cytoplasm (Fig. 2). Variations in the intensity of taste cells and progenitor cells in the taste buds for cytokeratin were displayed. The epithelial reaction to anti-CK 18 was greatly reduced or even negative in the lining and papillary epithelia. The interpapillary epithelia were weakly reactive for cytokeratin 18 (Fig. 3). As for the reaction to CK 18 in glandular tissue, this was detected in the minor salivary glands (von Ebner glands). A positive reaction of acinar cells was less strong than in the taste cells of the taste buds. A stronger reaction was found in the salivary ducts than in the acinar cells (Fig. 4).

## DISCUSSION

Morphologically, the taste buds are islets of simple epithelium embedded in a stratified squamous epithelium. The patterns for taste buds differed markedly from those of the surrounding epithelium. Immunocytochemical studies indicate that keratin-18-like immunoreactivity was present in the taste cells. Similarly, in the dog tongue distribution of cytokeratin 18, it was restricted to the taste cells of the taste buds. Cytokeratin tested present in taste buds displayed various reactions in the intensity of the taste cells. Apart from taste cells, no other epithelial cells of the surface of the vallate papilla and in the tongue were immunoreactive for this keratin in other animal species. Zhang et al. [21] reported that in the rat, that the taste buds were strongly stained by monoclonal antibodies against cytokeratins 8, 18 and 19; those typically expressed by simple epithelia. The authors observed that the staining within the taste buds was not homogeneous, but no clear differences of keratin staining could be directly related to the subtypes of cells constituting them [21]. The markedly differing patterns of keratin expression between the taste buds and the adjacent epithelium raise questions about the type of inductive signals that produce and maintain these patterns [18].

The CK immunoreactivity we observed in the taste cells

in the dog was intensive. Like in the rats [21], also in the dog, CK 18 was totally absent from the stratified squamous epithelia. Zhang and Oakley [22] observed in the caudal third of the rat's tongue, keratins 7, 8, 18, and 19 were expressed in the intragemmal cells of the taste buds in the vallate and foliate taste buds and in the cells that merge with these taste epithelia. In the human embryonal development of the tongue, from the 14th week, CK 18 is present only in the taste buds, making this polypeptide a reliable marker for this sensory organ [16]. In our study, taste cells positive for CK 18 displayed variations in the intensity of the reaction among the taste cells. We suppose that the different reactivity of taste cells may be related with the type of the taste cells and with differences in the metabolic activity or different stage of their life. Taste buds cells exhibit heterogeneous life spans ranging from 3 days to at least 3 weeks. Therefore, cells with different ages are assembled into a single taste bud [6].

In the rat, in each of six different epithelial sites in the rat oral cavity, intragemmal cells of taste buds were immunoreactive for keratin polypeptides 8, 18, and 19, as well as for keratin 7. Keratin-18-like immunoreactivity was present in fewer than half of the intragemmal cells, whereas all intragemmal cells were immunopositive for keratins 7, 8, and 19. No other epithelial cells in the tongue were immunoreactive for any of these four keratins [21]. Morphological and immunocytochemical profiles indicate that taste buds are islets of simple epithelium embedded in an expanse of stratified squamous epithelium. These simple epithelial cells and their keratins are nerve-dependent. Close contact of the nerve fibres with the taste cells [11] and the influence of innervation on the levels of noradrenaline and serotonin in the circumvallate papilla of the rat has been confirmed [2]. Denervation eliminated four keratins (CK 7, 8, 18, 19) and replaced elongated taste cells of the vallate papilla with stratified squamous epithelium [8]. Type II cells sense taste stimuli and type III cells transmit taste signals to sensory afferent nerve fibres [17]. In contrast to the remarkable progress in the understanding of the molecular basis for taste reception, the mechanisms of taste bud maintenance have remained a major area of inquiry [12].

The taste buds and the ducts of von Ebner's glands of the mice were strongly stained by monoclonal antibodies against cytokeratins 8, 18 and 19, those typically expressed by simple epithelia [18]. The findings of Kanazawa et al. [9] supported a hypothesis that the transmitters may be re-

leased from the base of the gustatory cell and possibly exert paracrine and endocrine (hemocrine) effects. The salivary gland of Ebner was suggested as one such target. In contrast to mice, in the dog the immunoreaction was weaker and resemble the reaction observed in other types of serous salivary glands.

A taste bud contains both short-lived and long-lived cells: the short-lived cells are eliminated in a time course similar to the surrounding epithelial cells, and the long-lived cells, including taste receptor cells, have a lifespan longer than the previous estimation [4]. Mammalian taste bud cells arise from progenitor cells common to the surrounding epithelial cells and undergo continual turnover through life [12]. Taste bud cells undergo continual turnover even in adulthood, and their average lifespan has been estimated as approximately 10 days. H a m a m i c h i et al. [4] described the age and life cycle of taste bud cells in rat circumvallate papillae, and indicate that the lifespan is heterogeneous, ranging from 2 days to over 3 weeks. The differences in the intensity of immunoreaction may be related to the different days of their lifespan.

In conclusion, the distribution of cytokeratin filaments 18 was restricted to taste cells and progenitor cells of the vallate papillae and acinic cells of salivary Ebner glands and cells of their ducts. Variations in intensity of the reaction of the taste cells and progenitor cells for cytokeratin have been displayed.

## ACKNOWLEDGEMENTS

## REFERENCES

**1. Asano-Miyoshi, M., Hamamichi, R., Emori, Y., 2007:** Cytokeratin 14 is expressed in immature cells in rat taste buds. *J. Mol. Histol.*, 39, 193—199.

**2. Cano, J., Lobera, B., Rodriguez-Echandia, E. L., Machado, A., 1982:** Influence of innervation on the levels of noradrenaline and serotonin in the circumvallate papilla of the rat. *J. Neurobiol.*, 13, 1—7.

**3. Franke, W. W., Schiller, E. L., Moll, R., Winters, S., Schmid, E., Engelbrecht, I., 1981:** Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J. Mol. Biol.*, 153, 933—959.

**4. Hamamichi, R., Asano-Miyoshi, M., Emori Y., 2006:** Taste bud contains both short-lived and long-lived cell populations, *Neuroscience*, 141, 2129—2138.

**5. Hecket, G. L., Mierson, S., DeSimone, J. A., 1984:** Salt taste transduction occurs through an amiloride-sensitive sodium transport pathway. *Science*, 27, 403—405.

**6. Hsu, S. M., Raine, L., Fanger, H., 1981:** The use of avidin-biotin peroxidase complex in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29, 577—580.

**7. Jain, S., Roper, S. D., 1991:** Immunocytochemistry of gamma-aminobutyric acid, glutamate, serotonin, and histamine in Necturus taste buds. *J. Comp. Neurol.,* 22, 675—682.

**8. Knapp, L., Lawton, A., Oakley, B., Wong, L., Zhang, Ch., 1995:** Keratins as markers of differentiated taste cells of the rat. *Differentiation*, 58, 341—349.

**9. Kanazawa, H., 1993:** Fine structure of the canine taste bud with special reference to gustatory cell functions. *Arch. Histol. Cytol.*, 56, 533—548.

**10. Laugerette, F., Passilly-Degrace, P., Patris, B., Niot, I., Febbraio, M., Montmayeur, J. P., Besnard, P. J., 2005:** CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. *Clin. Invest.*, 115, 3177—3184.

**11. Marettová, E., Maretta, M., 2012:** Innervation of the circumvallate papilla of the cat tongue. *Anat. Histol. Embryol.*, 41, 300—305.

**12. Miura, H., Kusakabe, Y., Harada, S., 2006:** Cell lineage and differentiation in taste buds. *Arch. Histol. Cytol.*, 69, 209—225.

**13. Moll, R., Schiller, D. L., Franke, W. W., 1990:** Identification of proteins IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns. *J. Cell Biol.*, 111, 567—580.

**14. Quinlan, R. A., Schiller, D. L., Hatzfeld, M., Achtstatter, T., Moll, R., Jorcano, J. L., Magin, T. M., Franke, W. W., 1985:** Patterns of expression and organization of cytokeratin intermediate filaments. *Ann. NY Acad. Sci.,* 455, 282—306.

**15. Sawaf, M. H., Ouha youn, J. P., Shabana, A. H., Forest, N., 1990:** Cytokeratin expression in human tongue epithelium. *Am. J. Anat.*, 9, 155—166.

**16. Sawaf, M. H., Shabana, A. H., Pelissier, A., Forest, N., Ouhayoun, J. P., 1991:** Characterization of cytokeratin patterns in the developing human tongue. *Int. J. Dev. Biol.*, 35, 91—100.

**17. Suzuki, T., 2007:** Cellular mechanisms in taste buds. *Bull. Tokyo Dent. Coll.*, 48, 151—161.

**18. Toh, H., Rittman, G., Mackenzie, I. C., 1993:** Keratin expression in taste bud cells of the circumvallate and foliate papillae of adult mice. *Epithelial Cell Biol.*, 2, 126—133.

**19. Witt, M., Kasper, M., 1999:** Distribution of cytokeratin filaments and vimentin in developing human taste buds. *Anatomy and Embryology*, 199, 291—299.

**20. Wong, L. B., Oakley, A., Lawton, Y., Shiba, Y., 1994:** Keratin 19-like immunoreactivity in receptor cells of mammalian taste buds. *Chem. Senses*, 19, 251—264.

**21. Zhang, Ch., Wong, L., Oakley, B., Lawton, A., Knapp, L., 1995:** Keratins as markers of differentiated taste cells of the rat. *Differentiation*, 58, 341—349.

**22. Zhang, Ch., Oakley, B., 1996:** The distribution and origin of keratin 20-containing taste buds in rat and human. *Differentiation*, 61, 121—127.

**23. Zhao, F. L., Shen, T., Kaya, N., Lu, S. G., Cao, Y., Herness, S., 2005:** Expression, physiological action, and coexpression patterns of neuropeptide Y in rat taste-bud cells. In *Proc. Natl. Acad. Sci., U. S. A.*, 2, 10211100-5.

# SURGICAL APPROACH IN THE TREATMENT OF SKIN DEFECTSIN SMALL ANIMALS
## (A Review)

### Kožár, M.

Small Animals Clinic, Section of surgery, orthopaedics, roentgenology and reproduction
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

martin.kozar@uvlf.sk

## ABSTRACT

Skin damages are frequently encountered in the clinical practice of small animals. Due to the large number of possible causes and diversified etiology, they are classified as multi-etiological diseases and that subsequently affects the selection of therapies. It is generally well-known that the best protection of a skin wound is its counterpart with available skin. This fact gives grounds for the main objective of the wound therapy by the soonest possible closure thereof. In principle, a wound therapy is based on the use of conservative or surgical methods. A surgical therapy plays a key role in a skin defect therapy. A therapy success depends also on the applied surgical method. The aim of this paper is to summarize and compare the methods applicable in skin defect therapy in small animals, depending on the skin damage type and extent.

Key words: primary skin closure; skin defects; skin flap; skin graft; surgical approach

## INTRODUCTION

Skin defects belong to one of the main groups of diseases occurring in a clinical practice with dogs, cats, and free-living animals, but also exotic birds. According to the definition, skin defects damage the normal skin barrier which is primarily the body's frontline of defence against microorganisms [20]. A multitude of causes, along with the lesions demonstrating variable damages, require, in the majority of cases, an individual approach to be taken with each patient.

The specific properties, as well as the anatomical specifics of animal skin, including their behaviour, can be helpful in the selection of the proper therapies and application of dressing techniques [12]. The general, and also the main objective of the management of wound therapy, is to accomplish the fastest possible wound closure [16]. Only the thorough knowledge of; the healing mechanisms, therapeutical methods, in conjunction with practical experience, brings success in animal skin defect therapy [7].

Wound therapy management techniques include: the prevention of further wound contamination, mechani-

cal and chemical debridement, removal of necrotic tissue parts, application of local and comprehensively acting antimicrobial preparations, protection of the newly grown granulation tissue, and wound closure management [11].

Prior to the therapy commencement and identification of the cause of skin defects, a thorough evaluation of a patient's health is required. A patient's health is a decisive factor for further diagnostics and therapy selection. In the majority of critical conditions, a patient is usually in shock, dehydrated, and in the case of long-lasting conditions, anorexia is present as well [8]. The initial stabilization of a patient should be followed further by surgical therapy of the skin defect. A therapy is chosen after a complex wound evaluation. Wound evaluation is focused on: the type, location, contamination, vascular integrity and wound extent [12].

## METHODS OF SURGICAL THERAPY OF SKIN DEFECTS

### Primary wound closure

The primary wound closure is the simplest surgical therapy of skin defects and should be carried out as soon as possible [13]. It should only be used in cases where no infection or gross contamination is present (Fig. 1a), or as a prevention against further complications in the wound healing process. The application of such a method is suitable for fresh clean wounds, such as lacerations within 8 hours of the initial injury [15]. The primary wound closure can be carried out using suture, staples, or cyanoacrylate adhesive (Fig. 1b).

Within the primary wound closure, it is necessary to close all damaged layers of the skin and subcutaneous tissue, while the main objective is to avoid the formation of dead space which might contribute to seroma formation [2].

In the case of wounds with contamination or infection, a surgical therapy must be delayed until this condition is corrected. The time between the initial debridement and the closure depends on the degree of contamination or infection of the affected location. The time of wound closure delay can range between three to five days [3].

Wound closure carried out more than five days after the initial wounding is regarded as a secondary wound closure, as the granulation tissue formation has begun to form in the wound before closure [19]. In some cases, it is appropriate to close a wound above a forming granulation bed [3].

### Vascularised skin flap

A vascularised skin flap is defined as a partially separated segment of the skin and subcutaneous tissue with a preserved base and blood supply during the transport to a new recipient bed [7]. It is most successful in the surgical therapy of an animal skin defect, which is facilitated by a simple application with the preserved blood supply. In avian patients, the vascularised skin flap is limited by the skin thinness on the majority of the body surface, which can hinder easy manipulation and thus damage the vascularity [11]. By using a skin flap in the skin defect therapy, we often avoid complications occurring in the open wound healing management, which is often accompanied with excessive scaring, contractures of skin or adjacent subcutaneous structures, or prolongation of the healing process [17].

The majority of simple skin flaps are based on non-specific, or the random supply of a subdermal plexus [14]. A skin flap which overlaps the defect, should include the presence of nerves, vessels, tendons, and other delicate structures inside the flap [17]. The factors affecting the flap formation, separation, and transportation to a recipient bed include the thickness of the cornified epidermis layer with a linear arrangement of collagen fibres of the dermis. In the case of birds, it is essential to assess the flexibility of a skin segment due to the thinner and reduced elasticity of avian skin, as compared to mammalian skin [10].

To form a live skin flap, surgeons must possess detailed knowledge of the animal skin anatomy and of the presence of the blood vessels, in order to preserve the arterial and venous circulation (Fig. 2) [7]. To cover the damaged location, a vascularised skin flap is required, with full blood supply, which provides a much higher chance of survival and attachment, as compared to a skin graft [9]. For small mammals and birds, no papers have been published so far describing the accurate procedures of skin flap application with regard to the blood supply of vessels within the respective skin segments.

To achieve sufficient skin defect coverage and ensure an ideal adaptation of a vascularised skin flap into the recipient bed in the defective zone, and to avoid formation of skin folds, we can combine the transportation technique and the formation of the releasing incisions. An advantage of releasing incisions is a larger coverage area with the creation of a large number of starting points for epithelisation, but it also serves as the drainage for the discharge of the wound secretions [17].

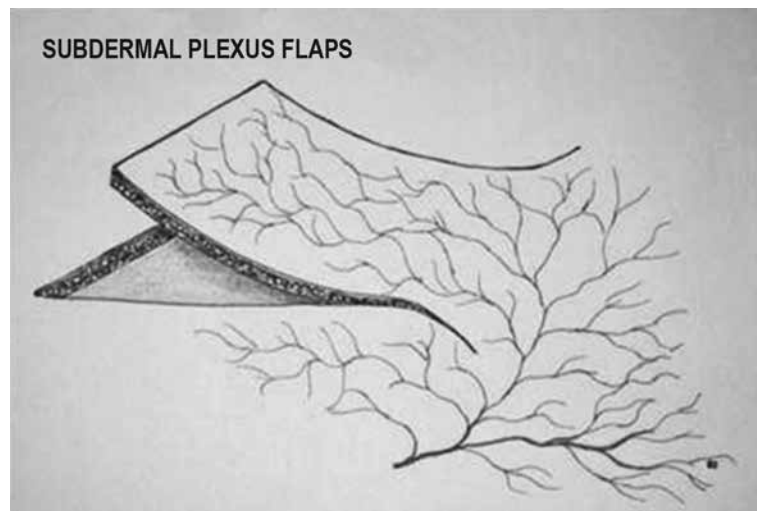**Fig. 1. a/b Primary wound closure**
(Source: Author)



**Fig. 2. Presence of skin vessels [1]**

**Fig. 3. Advancement skin flap – type Y**
(Source: Author)



**Fig. 4. Axial pedicle type of skin flap [1]**



**Fig. 5. Meshed skin graft [4]**

Many different methods have been used to classify flaps. Furthermore, these classification systems are often complex and varied in principle. To improve the understanding of flap classification, it was recently summarized that the most commonly used classification is divided into 3 simplified categories: (a) type of blood supply; (b) type of tissue to be transferred; and (c) location of the donor site.

**(a) type of blood supply**

Like any living tissue, flaps must receive adequate blood flow to survive. A flap can maintain its blood supply in 2 main ways: random (blood supply is not derived from a recognized artery but, rather, comes from many little unnamed vessels) and axial (blood supply comes from a recognized artery or group of arteries).

## (b) type of tissue to be transferred

Flaps may be composed of just one type of tissue or several different types of tissue. Flaps composed of one type of tissue include skin (cutaneous), fascia, muscle, bone, and visceral (e.g., colon, small intestine, omentum) flaps. Composite flaps include: fasciocutaneous (e.g., radial forearm flap), myocutaneous (e.g., transverse rectus abdominis muscle flap), osseocutaneous (e.g., fibula flap), tendocutaneous (e.g., dorsalis pedis flap), and sensory/innervated flaps (e.g., dorsalis pedis flap with deep peroneal nerve).

## (c) location of donor site

Based on types of location and donor sites, we divide flaps into: local flaps (tissue may be transferred from an area adjacent to the defect), pivotal flaps (rotation, transposition, and interpolation), advancement flaps (single pedicle, bipedicle, and V-Y flaps /Fig. 3/) and distant flap (a tissue transferred from a non-contiguous anatomic site from a different part of the body) [6].

During the skin flap preparation, it is necessary to assess: the wound size, condition of the skin around the defect necessary for a flap formation, and the flap type selection. The three most frequently used types of vascularised skin flaps, include a simple stretched skin flap, i.e. a stretched skin segment, transposition and rotation of a skin flap, and a pedicle graft. It is also important to consider elasticity, i.e. segment of skin being stretched, which is the highest in a simple type, moderate in a rotated type, and lowest in the skin flap transposition [17]. Skin flaps can further be divided depending upon blood supply, tissue selected for a flap formation, and a recipient bed localisation [5].

A simple vascularised skin flap is the type most frequently used in clinical practice. It is based on overlapping the skin defect with a simple stretched skin segment without lateral shift. A primary wound should be treated in a sterile environment with the application of the granulation supporting preparations. If excessive growth of granulation tissue is present after two weeks of the application, this flap must be removed [7].

Transposition, as the second type of flap, represents rotation of a skin flap overlapping the wound. A skin segment should be incised parallel to the pull line, corresponding to the size necessary for the cosmetic closure of the wound [7]. This type is used mostly at locations with lack of available skin around the defect and in the case of skin dam-

age located where there is a solid connection with the base and a small amount of subcutaneous tissue. The presence of a defect at such locations, more extensive skin structure damage, as well as fast onset of tissue fibrosation hinder a direct simple closure of a skin defect.

The third skin flap type used in practice is a pedicle graft (Fig. 4). It belongs to axial skin flap types and it remains attached to the skin artery and vein on its base. Upon the assessment of the procedure corresponding to the defect location and size, a skin segment is gently separated by incision, released from the subcutis and moved to the defect location and then used to cover this location and attached by sutures [7].

## Skin graft

Skin graft is a separated skin segment, either in its total or partial thickness, taken from one part of the body and moved to a wound on another part of the body [17]. Skin graft attachments can fail due to: presence of infection, movement against the granulation bed, fluid accumulation, or weak vascularisation frequently connected with an allergic reaction [11]. Therefore, acceptance of a new location depends on the absorption of tissue fluids as well as the creation of the new blood supply [7].

A skin graft differs from a vascularised skin flap, by not having a preserved blood supply that must be created after the transport to a new location, which is known as a recipient bed. Revascularization can be carried out by a direct connection (inoculation) of vessels located in the skin graft with those located in the recipient bed. The second method of blood supply creation is the growth of blood vessels located in the recipient bed and the adjacent skin into the skin graft along the wound edges [17].

## There are the following skin graft types:

## Autotransplant

It is a skin graft taken from a certain area on an animal body and moved to cover the damaged skin of the same animal. The use of this skin graft type minimises the acceptance risks. However, a great disadvantage for the skin graft formation is the lack of available skin on bodies of small animals, especially birds. In small individuals, a location of possible creation is limited only to places with freely moving skin, i.e. in the neck and groin areas [11].

### Allotransplant

This is a skin graft taken from a certain area on one animal body and moved to cover a skin defect of a different animal of the same species. Taking a skin graft from a different animal solves the problem of the lack of available skin, but it requires the surgical procedure to be mainly on a healthy animal, which is unethical and illegal. In a majority of cases, the recipient's allergic reaction is important as well [11].

### Xenotransplant

It is a skin graft taken from a certain area on an animal body and moved to cover a skin defect of a different animal of a different species. Multi-purpose tissue grafts (extracellular matrixes) include porcine small intestine submucosa (SIS) with low immunogenicity and with the content of plenty of extracellular factors and basement membrane factors which support the implantation success. The disadvantage is the calcification leading to poor tissue acceptance and mechanical failure [11], [17].

Skin grafts can have the form of a foil, stripe, or a mesh. Mesh grafts are used mainly for their flexibility, the ability to cover unevenness as well as more extensive skin defects (Fig. 5) [4], [17]. They are also used in the case of the lack of skin necessary for the creation of a skin flap, in the case of moving adjacent tissue, and at locations with increased pulling of the wound [18]. A great advantage is also the creation of plenty of epithelisation points and the possibility of the discharge of the secretion from the wound [17]. Granulation tissue growing in the openings supports graft immobilisation as well as the growing of skin vessels into the dermis with the restoration of revascularization [18]. Skin graft application is suitable at locations with good blood supply, due to better skin epithelisation and faster acceptance. In skin grafts, skin segments with total thickness are used [17].

### CONCLUSIONS

When assessing the use of the surgical therapy of skin defects in small animals, based on the available literature, it is necessary to underline faster healing efficiency characterised by covering the damaged locations with missing skin structures, more aesthetic wound closure, and lower patient's exposure to stress than observed in the conservative wound therapy management. However, application of the surgical therapy requires the use of surgical instruments, the knowledge of anatomic specialties of skin, as well as of modification of surgical methods necessary for individual animal species.

In the therapy of extensive defects and skin defects at hard-to-access locations, an alternative method to surgical therapy, i.e. application of a simple stretched and rotated vascularised skin flap, seems to be very appropriate. The variety of the skin damage extent and of the localisation of skin defects in different animal species does not facilitate the creation of unified conditions to describe exact procedures of the surgical therapy. Nor does it aid the wound healing process as such, with regard to the occurrence of complications in the post-operation period. An example is the post-operation period in avian patients, during which the wound edge desiccation represents a great complication. This fact underlines the importance of creating ideal conditions for wound healing as well as possible application of supporting preparations increasing the efficiency and speed of the wound healing process.

### REFERENCES

**1. Bryden, J. S., 2007:** Reconstructive surgery. http://www.vin.com/proceedings/Proceedings.plx?CID=WSAVA2007&PID=18149&O=Generic [cit. 2007-08-19].

**2. Bullocks, J., Basu, C. B., Hsu, P., Singer, R., 2006:** Prevention of hematomas and seromas. *Semin Plast Surg.*, 20, 4, 233–240.

**3. Burke, H. F., Swaim S. F., Amalsadvala, T., 2002:** Review of wound management in raptors. *J. Avian Med. Surg.*, 16, 180—191.

**4. Chapnick, A., Wilkins, R. J., 2014:** Surgical wound management in dogs using an improved stable chlorine dioxide antiseptic solution. *J. Vet. Sci. Anim. Husb.* (http://www.annexpublishers.com/full-text/JVSAH/403/Surgical-wound-management-in-dogs-using-an-improved-stable-chlorine-dioxide-antiseptic-solution.php) [cit. 2015-09-15].

**5. Chrysopoulo, M. T., 2008:** Flaps, classification. http://emedicine.medscape.com/ article/1284474-overview [cit. 2008-01-11].

**6. Chrysopoulo, M. T., 2015:** Tissue flap classification. http:// emedicine.medscape. com/article/1284474-overview [cit. 2015-09-15].

**7. Ferell, S. T., 2002:** Avian integumentary surgery. *Semin. Avian Exot. Pet Med.*, 11, 125—135.

**8. Gelis, S., 2005:** Wild birds — assessment, first aid and handy tips. *National Wildlife Rehabilitation Conference*, 1—6. http:// www.awrc.org.au/uploads/5/8/6/6/5866843/awrc_stacey gelis.pdf.

**9. Gentz, E. J., Linn, K. A., 2000:** Use of a dorsal cervical single pedicle advancement flap in 3 birds with cranial skin defects. *J. Avian Med. Surg.*, 14, 31—36.

**10. Hannon, D. E., Weber, T. D., 1995:** Use of a single pedicle advancement flap for wound repair in a great horned owl (Bubo virginatus). In *Proceedings Assoc. Avian Vet.*, 285—289.

**11. Hernandez-Divers., J., Hernandez-Divers., S. M., 2003:** Xenogeneic grafts using porcine small intestinal submucosa in the repair of skin defects in 4 birds. *J. Avian Med. Surg.*, 17, 224—234.

**12. Joseph, V., 2010:** Wound management and bandaging techniques. http://www.ccwr.org/membership/members_only/ 2010_Symposium_Handouts/Josephs_Wound_Management.pdf [cit. 2010-11-10].

**13. Monnet, E., 2012:** Wound management. http://veterinarycalendar.dvm360.com/avhc/author/authorDetail.jsp?sort= null&pageNo=3&start=18&id=48790 [cit. 2012-12-10].

**14. Nesbit, R. D., Laub, D. R., 2009:** Flaps. Random skin flaps. http://emedicine.medscape.com/article/1284569-overview [cit. 2009-06-26].

**15. Pollock, CH., 2013:** Wound management in exotic animals. http://www.lafebervet.com/emergency-medicine/general-principles/wound-management-in-exotic-animals/ [cit. 2013-01-02].

**16. Slate, R. K., 1997:** Wound healing and management. In **Bongard, F. S., Stamos, M. J., Passaro, E.:** *Surgery — a Clinical Approach*. Churchill Lingstone, New York, 69—73.

**17. Stroud, P. K., Amalsadvala, T., Swaim, S. F., 2003:** The use of skin flaps and grafts for wound management in raptors. *J. Avian Med. Surg.*, 17, 78—85.

**18. Swaim, S. F., 2009:** Full-thickness mesh skin grafts. *Vet. Med-US*, 14, 554—558.

**19. The Merck Veterinary Manual, 2009:** Wound management. http://www.ultimatefowl.com/wiki/index.php?title=Wound_ Management [cit. 2009-05-09].

**20. Wilson, J. A., Clark, J. J., 2004:** Obesity: impediment to postsurgical wound healing. *Adv. Skin Wound Care*, 17, 426—435.

# TOLFENAMIC ACID IN THE THERAPY OF INFLAMMATION OF THE RESPIRATORY SYSTEM AND SKIN IN SWINE

**Novotný, J., Guľvasová, J., Kadaši, M., Reichel, P.**

Clinic for swine, University of Veterinary Medicine and Pharmacy, 041 81 Košice
The Slovak Republic

jaroslav.novotny@uvlf.sk

## ABSTRACT

The aim of this experimental work was to evaluate the influence of tolfenamic acid on: the health state, blood concentration of leukocytes, and acute phase proteins in pigs. Eight pigs (cross-breeds of the Large White and Landrace), body weight 30 kg with acute inflammation of the respiratory system and the skin were involved in the experiments. Four pigs (group A) were treated with tolfenamic acid (Tolfedine CS inj. 1 ml.20 kg$^{-1}$, i.e. 2 mg.kg$^{-1}$ i.m.). Another four pigs (the group B) were treated with a combination of tolfenamic acid (Tolfedine CS inj. 1 ml.20 kg$^{-1}$, i.e. 2 mg.kg$^{-1}$ i.m.) and macrolid antibiotic Tylovet B 200 inj. (1 ml.30 kg$^{-1}$ i.m.). A clinical investigation and blood collection were performed on each patient before the application of medicaments (0 sampling), and 48 hours after the application (1st sampling). The clinical investigation consisted of the examination of: the general health state, an examination of the particular body systems, and a body temperature measurement. The blood was collected with the purpose of determining: the concentrations of haptoglobin (a member of the proteins of an acute phase response), and the concentration of leukocytes. The statistic evaluation of the body temperature values showed a significant decrease (P < 0.01) in group A. The administration of tolfenamic acid improved the health status of the respiratory system and the skin. The treatment had no negative influence on: appetite, digestion, or the consistency of the faeces.

Key words: haptoglobin; pigs; tolfenamic acid

## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively used in both people and other animals for their anti-inflammatory, pain relieving, and anti-fever properties. Generally, NSAIDs work by blocking the biosynthesis of intracellular prostaglandins that are important in pain and inflammatory pathways [7].

Tolfenamic acid (acidum tolfenamicum) is a member of the anthranilic acid derivatives (or fenamate) class of NSAID drugs discovered by scientists in Finland [9].

Chemically, it is related to mefenamic and flufenamic acid and also to diclofenac. Like other members of this class, it is a COX inhibitor and prevents formation of prostaglandins. Furthermore, tolfenamic acid has inhibitory effects on prostaglandin receptors [12] and also bradykinin induced oedema in inflammatory exudates [13].

The aim of this investigation was to evaluate the influence of tolfenamic acid on: the health state, blood concentration of leucocytes, and acute phase proteins in pigs with acute inflammatory diseases.

## MATERIALS AND METHODS

### Animals

Eight pigs (crossbreeds of Large White x Landrace) with the acute inflammation of the respiratory system and the skin were involved in the experiments. The average body weight of animals was approximately 30 kg and the experiments were carried out at the Clinic for swine, UVMP in Košice.

### Experimental design

Animals were divided into two groups: group A and group B, with four animals in each group. The group A was subjected to therapy with tolfenamic acid (Tolfedine CS inj. 1 ml.20 kg⁻¹, i.e. 2 mg.kg⁻¹ i.m.). The group B was treated with a combination of tolfenamic acid (Tolfedine CS inj. 1 ml.20 kg⁻¹, i.e. 2 mg.kg⁻¹ i.m.) and macrolid antibiotic Tylovet B 200 inj. (1 ml.30 kg⁻¹ i.m.). The clinical investigations and blood collections were done before (0 sampling) and 48 hours (1st sampling) after the commencement of the treatments. The examination of the general health state, measurement of the body temperature and complete individual clinical examination were performed on each patient. The blood was sampled from sinus ophtalmicus.

### Laboratory analysis

The haptoglobin (Hp) was determined at the Clinic for ruminants (UVMP in Košice) by the colorimetric method using a commercial diagnostic tests (Tridelta Development, Ireland) in microplates, based on Hp-haemoglobin binding and preservation of the peroxidase activity of the bound haemoglobin at a low pH. The reading of absorbencies and the consecutive calculation of the final concentrations of Hp was performed on an automatic microplate reader Opsys MR (Dynex Technologies, USA).

The concentration of leucocytes was analysed by an automated veterinary analyser (ABC Hematology Analyzer Vet Animal Blood Counter, France) at the Clinic for swine UVMP in Košice.

### Statistical processing of results

The statistical processing of the results was performed by the assessment of the means (x) and standard deviations (SD) in both groups of pigs. The significance (P) of differences in the means of corresponding variables were evaluated by the Student's $t$-tests. The measurements before and after therapy, were evaluated by the paired $t$-test. The significance of differences between the groups were evaluated by the unpaired $t$-test at the same time. The results are presented as the mean (x) and the standard deviation (SD).

## RESULTS

The initial clinical investigation demonstrated pathological changes of the respiratory system and the skin. Skin lesions were found on the auricles and tails of the animals and were the result of cannibalism. The clinical findings of the respiratory system showed a picture of bronchopneumonia, with a demonstration of dyspnoea and spontaneous coughing. Despite this pathology, animals were alert and had normal appetites and consistency of the faeces.

The therapy lead to the stabilisation of the health state in both groups of pigs, and the bite wounds were dry without any exudation or a presence of inflammatory oedema. The upper respiratory system was without discharge, and inflammation. The lower respiratory system was only determined by the presence of sporadic coughing. The type of breathing was costo-abdominal and eupnoeic.

The statistical analysis of the body temperatures showed a significant reduction in group A (39.48 ± 0.42 to 39.18 ± 0.34 °C; P < 0.01; Table 1). On the contrary, the comparison of temperatures measured in group B showed no significant decrease (39.75 ± 0.31 to 39.35 ± 0.13 °C).

**Table 1. Mean body temperatures (°C) before and after the therapy (x ± SD)**

| Group of swine | 0 measurement | 1st measurement |
|---|---|---|
| A | 39.48 ± 0.42** | 39.18 ± 0.34** |
| B | 39.75 ± 0.31 | 39.35 ± 0.13 |

** — P < 0.01

An insignificant decrease in haptoglobin serum concentrations was observed in both groups of swine as a consequence of the therapy (2.33 ± 1.83 to 2.31 ± 1.49 mg.ml$^{-1}$ in group A, 4.86 ± 1.12 to 4.45 ± 1.56 mg.ml$^{-1}$ in group B, Table 2)

**Table 2. Mean values of haptoglobin (mg.ml$^{-1}$) in the blood of swine (x ± SD)**

| Group of swine | 0 sampling | 1st sampling |
|---|---|---|
| A | 2.33 ± 1.83 | 2.31 ± 1.49 |
| B | 4.86 ± 1.12 | 4.45 ± 1.56 |

The mean concentration of the leucocytes in the 0 sampling was 24.23 ± 4.51 G.l$^{-1}$ in group A and 36.75 ± 20.99 G.l$^{-1}$ in group B (Table 3). As a result of the therapy, the concentrations of leucocytes non-significantly decreased in both groups of animals (1st sampling; 22.43 ± 1.42 G.l$^{-1}$ in group A, and 29.4 ± 12.55 G.l$^{-1}$ in group B).

**Table 3. Mean values of leukocytes (G.l$^{-1}$) in the blood of pigs (x ± SD)**

| Group of swine | 0 sampling | 1st sampling |
|---|---|---|
| A | 24.23 ± 4.51 | 22.43 ± 1.42 |
| B | 36.75 ± 20.99 | 29.4 ± 12.55 |

## DISCUSSION

Although patients on the basis of the initial clinical examination, were divided into two equally affected groups, the laboratory analysis showed more obvious changes in group B. Because of that, group A was treated only with tolfenamic acid, and group B with a combination of tolfenamic acid and (on the presence of bacteria) tylosin.

The beneficial anti-inflammatory effects of tolfenamic acid became evident in the healing of the skin lesions and the respiratory system of the swine. The therapy lead to faster healing of the bite wounds in both groups of pigs, as the lesions were dry with scab formation. The state of the respiratory system was improved. The type of breathing was costo-abdominal and eupnoeic with the presence of only sporadic coughing. The treatment had no negative influence on: the general health state, appetite, digestion, or consistency of the faeces.

The intramuscular application of tolfenamic acid had an influence on the rectal temperature decrease in both groups of animals. The values measured after the therapies, decreased into the reference limits. The physiological range for this category of pigs is 38.7—39.3 °C [11]. The rectal temperature significantly decreased by about 0.30 °C (P < 0.01) in group A. On the other hand, an insignificant decrease was recorded in group B.

The C-reactive protein, haptoglobin (Hp), serum amyloid A (SAA) and the pig major acute phase protein (PigMap) belong to the most important proteins of the acute phase [1]; these parameters are also useful for diagnostics of the swine health state [10]. For our experiments, a determination of haptoglobin was selected. The mean concentration of haptoglobin in group A before application of the tolfenamic acid, was 2.33 mg.ml$^{-1}$. After 48 hours, this level decreased to 2.31 mg.ml$^{-1}$. On the other hand, group B had 4.86 mg.ml$^{-1}$ of haptoglobin at 0 sampling and this level decreased to 4.45 mg.ml$^{-1}$. The physiological level of haptoglobin in pigs varies between 1.2—1.47 mg.ml$^{-1}$ [2, 3], however, during infectious or inflammatory diseases these values could increase up to 5 mg.ml$^{-1}$ or even higher. Higher haptoglobin values have been associated with: diseases of the respiratory system, gastrointestinal system (diarrhoeas), locomotor system (laminitis), and skin disorders (caudophagia and auricles necrosis) [4, 5]. An increased haptoglobin level can be observed before the appearance of the first clinical symptoms of a disease [6].

The seriousness of the presented diseases was confirmed also by values of leucocytes that exceeded the physiological limit (11—18 G.l$^{-1}$) [14]. Forty eight hours after the administration of the medicaments, the mean value of leucocytes was insignificantly decreased by about 1.8 G.l$^{-1}$ in group A, and 7.35 G.l$^{-1}$ in group B. However, the measured values were still above the physiological range (22.43 ± 1.42 G.l$^{-1}$ in group A, and 29.4 ± 12.55 G.l$^{-1}$ in group B). These find-

ings prove the necessity of long term and repeated therapy of the lower respiratory system diseases. A single administration of tolfenamic acid has been found be sufficient in the prophylaxis and therapy of MMA syndrome in sows for example [8].

The intramuscular administration of tolfenamic acid improved the health state of pigs with acute inflammation of the respiratory system and the bite wounds of the skin. In the case of bacterial diseases, more effective therapy is possible by the simultaneous application of antibiotics. This fact is confirmed in our experiments by a more marked decline of haptoglobin and leucocytes concentrations in group B, which was treated by a combination of tolfenamic acid and tylosin.

## REFERENCES

**1. Carpintero, R., Alonso, C., Piñeiro, M., Iturralde, M., Andrés, M., Le Potier, M. F. et al., 2007:** Pig major acute phase protein and apolipoprotein A-I responses correlate with clinical course of experimentally induced African swine fever and Aujeszky disease. *Vet. Res.*, 38, 741—753.

**2. Clapperton, M., Bishop, S. C., Cameron, N. D., Glass, E. J., 2005:** Association of acute phase protein levels with growth performance and with selection for growth performance in Large White pigs. *Anim. Sci.*, 81, 213—220.

**3. Chen, H. H., Lin, J. H., Fung, H. P., Ho, L. L., Yang, P. C., Lee, W. C. et al., 2003:** Serum acute phase proteins and swine health status. *Can. J. Vet. Res.*, 67, 283—290.

**4. Eckersall, P. D., 2008:** Proteins, Proteomics and the Dysproteinemias. In **Kaneko, J. J., Harvey, J. W., Bruss, M. L.:** *Clinical Biochemistry of Domestic Animals*. 6th edn. United States, Academic Press, 896 pp.

**5. Grau-Roma, L., Heegaard, P. M. H., Hjulsager, C. K. et al., 2009:** Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viraemia and the clinical course of postweaning multisystemic wasting syndrome. *Vet. Microbiol.*, 138, 53—61.

**6. Harding, J. C., Baarsch, M. J., Murtaugh, M. P., 1997:** Association of Tumour Necrosis Factor and acute phase reactant changes with post arrival disease in swine. *J. Vet. Med. B*, 44, 405—413.

**7. Hera, A., Bureš, J., 2010:** The use of non-steroid anti-inflammatory substances in large animal practice (In Czech). *Veterinářství*, 60, 353—359.

**8. Kotowski, K., Žmudzki, J., 2007:** Tolfenamic acid in prophylaxis and therapy of agalactia syndrome in sows and their litters. *Medycyna Weterynaryjna*, 63, 1342—1345.

**9. Pentikäinen, P. J., Neuvonen, P. J., Backman, C., 1981:** Human pharmacokinetics of tolfenamic acid, a new anti-inflammatory agent. *Eur. J. Clin. Pharmacol.*, 19, 359—65.

**10. Sorensen, N. S., Tegtmeier, C., Andresen, L. O., Piñeiro, M., Toussaint, M. J. M., Campbell, F. M. et al., 2006:** The porcine acute phase protein response to acute clinical and subclinical experimental infection with Streptococcus suis. *Vet. Immunol. Immunopathol.*, 113, 157—168.

**11. Straw, B. E., Meuten, D. J., Thacker, B. J., 2003:** Physical examination. *Diseases of Swine,* Ames, Iowa State University press, 3—14.

**12. Tokola, R., Neuvonen, P., 1984:** Effects of migraine attack and metoclopramide on the absorption of tolfenamic acid. *The British Journal of Clinical Pharmacy*, 17, 67—75.

**13. Váczi, P., 2008:** Analgetics — antiphlogistics, antipyretics and antirheumatics (In Slovak). In **Čonková, E. et al:** *Veterinary Pharmacology – special part*. IInd vol., 1st edn., University of Veterinary Medicine, Košice, 280 pp.

**14. Vrzgula, L., Sokol, J. 1990:** Interpretation of haematological profiles. In **Vrzgula, L. (Ed.):** *Metabolic Disorders and their Prevention in Farm Animals*, University of Veterinary Medicine, Košice, 470—472.

# ADMINISTRATION OF BACTERIOCIN-PRODUCING AND PROBIOTIC BACTERIAL STRAINS ATTENUATE THE OUTCOME OF MOUSE *TRICHINELLA SPIRALIS* INFECTIONS

## Bucková, B.[1], Dvorožňáková, E.[1], Hurníková, Z.[1], Lauková, A.[2]

1Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice
2Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice
The Slovak Republic

buckova@saske.sk

## ABSTRACT

The aim of this investigation was to study the effects of probiotic and bacteriocin-producing bacterial strains on the intensity of *Trichinella spiralis* infections in mice during its intestinal and muscle phases and female larvae fertility. Bacteriocin-producing strains *Enterococcus faecium* AL41 and *E. faecium* 2019-CCM7421 significantly reduced the number of adults in the intestine. Application of probiotic strains *Lactobacillus fermentum* AD1-CCM7420, *L. plantarum* 17L/1, and bacteriocin-producing strains of *E. faecium* AL41 and *E. durans* ED26E/7 caused a significant decrease in the number of muscle larvae. The reproductive capacity index (RCI) of *T. spiralis* was reduced in mice after the administration of: *E. faecium* AL41, *E. durans* ED26E/7, *L. fermentum* AD1-CCM7420, and *L. plantarum* 17L/1. The significant inhibition of female fertility was observed only after the application of *E. faecium* AL41, whereas the administration of the other strains had only a limited effect on the reduction of the number of newborn larvae. We presume that the reduction of parasitic burden in the host muscles was probably caused by the augmentation of the immune defence mechanisms stimulated by the bacterial strains.

Key words: *Enterococcus*; *fertility*; *Lactobacillus*; mouse; parasitic burden; *Trichinella spiralis*

## INTRODUCTION

The gut of the host represents a complex ecosystem where the interactions between intestinal microbiota, the immune system, and pathogens occur. All of these components interact and play a relevant role in the maintenance of homeostasis. For health maintenance, it is crucial to form a balance between the gut microbiota and the host organism. Intestinal parasites (protozoans and helminths) also affect the gut microbial community and modify the balance between the host and commensal microbiota. In addition, gut microbiota represents a critical factor which significantly influences the pathophysiology of the parasitic infections. Moreover, the survival of parasites and the outcome of many parasitic infections depend on the gut commensal

microbiota, including the production of nutritious macromolecules. Thus, probiotic bacteria can play an important role in reducing the pathogenicity of many parasites.

There has been a growing interest among scientists to explain the interactions between the microbiota, immune response, inflammatory processes, and intestinal parasites [1]. Probiotic strains with their health benefits are able to inhibit, displace and compete with pathogens in the host gut, and enhance the mucosal barrier activity. These beneficiary properties are strain-specific [7]. In general, probiotics may be useful in the prevention and treatment of various health conditions and diseases [13]. A beneficial effect of probiotic bacteria in the treatment of gastrointestinal parasitic infections has also been studied during the last decade. These studies indicate that probiotic bacteria can provide a strain-specific protection against parasites, probably through multiple mechanisms [14]. The identification of factors mediating the beneficial effects of probiotics provide an opportunity to not only study and understand their fine mechanisms of action, but also to develop effective pharmacological strategies that could lead to the effective treatments for various pathogens [5].

The aim of this study was to evaluate the anti-parasitic effects of bacteriocin-producing and probiotic bacterial strains in mice infected with *Trichinella spiralis*. Since trichinellosis constitutes a serious zoonosis spread throughout the world, there is a need for the development of new therapies where the use of probiotic strains with anti-parasitic activity may be promising.

## MATERIALS AND METHODS

The experiments were performed on 126 pathogen-free BALB/c male mice, weighing 18—20 g. The animals were divided into seven groups consisting of 18 animals per group: Control — *T. spiralis* infection without the administration of bacterial strains; Group 1 — *Enterococcus faecium* EF55 + *T. spiralis*; Group 2 — *E. faecium* 2019-CCM7421 + *T. spiralis*; Group 3 — *E. faecium* AL41 + *T. spiralis*; Group 4 — *E. durans* ED26E/7 + *T. spiralis*; Group 5 — *Lactobacillus fermentum* AD1-CCM7420 + *T. spiralis*; Group 6 — *L. plantarum* 17L/1+ *T. spiralis*. Bacterial strains were administered daily *per os* at a dose of 100 μl ($10^9$ CFU.ml$^{-1}$ in Ringer's solution). Two groups of bacteria were examined: bacteriocin-producing strains with probiotic properties (*E. faecium* EF55 — chicken isolate producing an antimicrobial proteinaceous substance enterocin (Ent) 55; *E. faecium* 2019-CCM7421 — rabbit isolate producing Ent 2019; *E. faecium* AL41 — environmental isolate producing Ent M (all prepared at the Institute of Animal Physiology SAS, Košice); *E. durans* ED26E/7 — isolate from traditional ewes milk producing durancin-like bacteriocin (prepared at the Research Dairy Institute, Žilina), and probiotic strains (*L. fermentum* AD1-CCM7420 — canine isolate (prepared at the Institute of Animal Physiology SAS, Košice); and *L. plantarum* 17L/1 — isolate from stored sheep cheese (prepared at the Research Dairy Institute, Žilina). Mice were infected with 400 *T. spiralis* larvae/ mouse *per os* on the 7th day of the experiment.

*T. spiralis* adults were collected from the small intestine, which had been cut longitudinally and its content incubated in conical pilsner glasses in 37 °C NaCl (0.9 % saline) for 4—5 hours [4]. Muscle *T. spiralis* larvae were obtained by artificial digestion (1 % pepsin, 1 % HCl for 4 h at 37 °C) according to K a p e l and G a m b l e [9]. The fertility of *T. spiralis* females was determined by an *in vitro* test of adult females isolated from the gut of infected mice on day 5 post infection and incubated afterwards in RPMI medium enriched with antibiotics and foetal bovine serum (3 %) at 37 °C for 20 h [3].

## RESULTS AND DISCUSSION

The nematode *Trichinella spiralis* causes an intestinal and tissue disease — trichinellosis which is characterized by an enteritis (induced by adult worms) and inflammation with degenerative changes in the skeletal muscles (induced by larvae) [8]. The pathology of trichinellosis is primarily a reaction to the initial inflammatory response during the intestinal phase to begin with and later to the subsequent allergic and inflammatory responses during the larval migration and invasion into the host muscles. Due to the high production of newborn larvae and the strong immune response of the host [2, 10, 12], the pathogenicity of *T. spiralis* is greater than that seen with other intestinal parasites [11]. The severity of trichinellosis is ultimately affected by the number of migrating newborn larvae. Therefore, the effective immunity is characterized by reactions which: limit the fertility of *T. spiralis* females, damage or destroy the newborn larvae, and eliminate the adult worms from the gut.
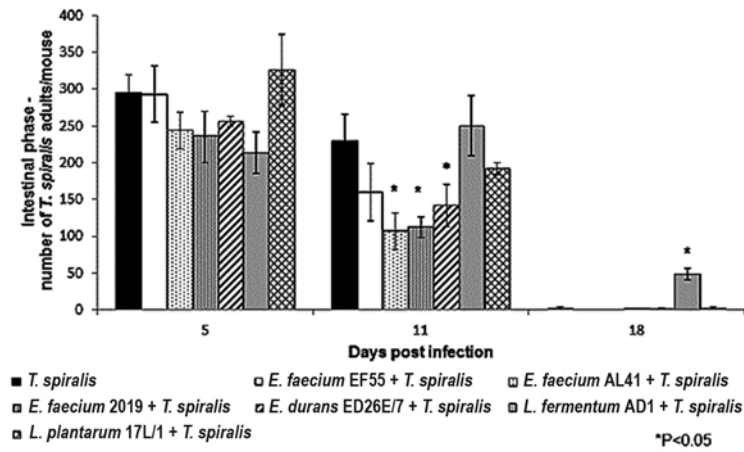
**Fig. 1. Numbers of adult worms isolated from the small intestine of mice treated with probiotic bacteria and infected with *T. spiralis***
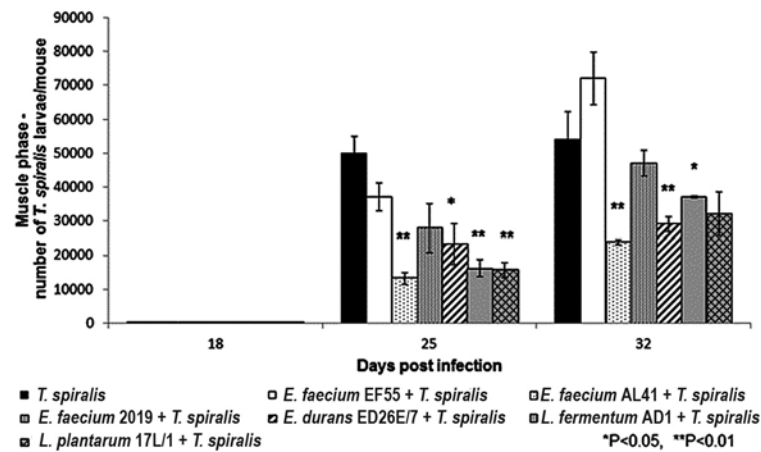


**Fig. 2. Numbers of muscle larvae isolated from mice treated with probiotic bacteria and infected with *T. spiralis***
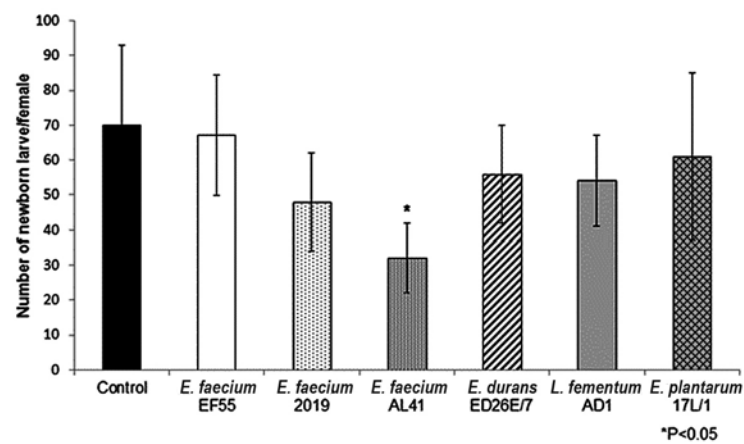


**Fig. 3. Fertility of *T. spiralis* females — Numbers of newborn larvae**

**Table 1. Reproductive capacity index in mice treated with probiotic bacteria and infected with *T. spiralis*.**

| Day p.i. | *T. spiralis* | *E. faecium EF55 + T. spiralis* | *E. faecium AL41 + T. spiralis* | *E. faecium 2019 + T. spiralis* | *E. durans ED26E/7 + T. spiralis* | *L. fermentum AD1 + T. spiralis* | *L. plantarum 17L/1 + T. spiralis* |
|---|---|---|---|---|---|---|---|
| 25 | 125.20 | 92.65 | 33.05 | 69.93 | 58.13 | 40.48 | 38.85 |
| 32 | 135.17 | 180.30 | 59.53 | 117.38 | 72.70 | 92.85 | 80.17 |

In our experiments, the adult worms were isolated from the small intestine on days 5, 11, and occasionally day 18 post infection (p.i.). A significant reduction of intestinal parasites occurred on day 11 p.i. in mice which have been administered with bacteriocin-producing strains *Enterococcus faecium* AL41 and *E. faecium* 2019-CCM7421 (Fig. 1).

In the evaluation of the muscle phase of the infection, a significant decrease in the number of muscle larvae on days 25 and 32 p.i. was detected. Particularly in the groups of mice which were administered with: *Lactobacillus fermentum* AD1-CCM7420, *L. plantarum* 17L/1 probiotic strains and *E. faecium* AL41 and *E. durans* ED26E/7 bacteriocin-producing strains (Fig. 2).

The application of *E. faecium* AL41 was linked, not only with a reduction of adult numbers, but also with a decrease in female reproductive potential. The production of larvae was evaluated by the reproductive capacity index (RCI), which is a biological indicator of *T. spiralis* infectivity. It is the ratio of, the total number of larvae recovered from an animal, compared to the number of larvae administered into it. Parasite infectivity is a result of the interplay of four components; 1 — number of females that develop into adults, 2 — their fecundity, 3 — the length of their survival in the gut, and 4 — the period during which the muscle larvae remain viable [6]. The RCI of *T. spiralis* females was reduced in mice which were administered with: *E. faecium* AL41, *E. durans* ED26E/7, *L. fermentum* AD1-CCM7420, and *L. plantarum* 17L/1 (Tab. 1).

It could not be determined whether the decreased reproductive potential of *T. spiralis* was associated with a reduced fertility induced by bacterial strains or these strains prevented the passage of newborn larvae into the blood and the lymph circulation. Perhaps it could be explained by female fertility test results isolated from the gut of infected mice and treated with bacterial strains. The number of ex vivo newborn larvae per one female was used as a measure of fecundity. In our experiments, the female fertility was significantly inhibited after the administration of *E. faecium* AL41. Based on this result, we suspect a direct impact of the bacterial strain on the female fertility. The administration of other strains has had only a modest effect on the reduction of the number of newborn larvae. Thus, we presume that immune mechanisms were further stimulated by the bacterial strains and could participate in the reduction of the parasitic burden in the host muscles.

Suggesting probiotics as an alternative to the classical treatments such as anthelminthics appears unreasonable. However, a complementary therapeutic approach in combination with probiotics to reduce the risks of infestation or to decrease the drug dosage to avoid adverse side effects seems to be more realistic. Studies on the effects of probiotics on parasites are still in their infancy and further investigations are necessary to move forward with this topic.

## ACKNOWLEDGEMENT

## REFERENCES

1. **Berrilli, F., Di Cave, D., Cavallero, S., D'Amelio, S., 2012:** Interactions between parasites and microbial communities in the human gut. *Front. Cell Infect. Microbiol.*, 2, doi: 10.3389/fcimb.2012.00141.

2. **Brushi, F., 1999:** How can *Trichinella* escape the host immune response. *Helminthologia*, 36, 139—145.

**3. Cabaj, W., 1990:** The effect of cyclosporine A on the course of infection by *Trichinella pseudospiralis* or *Nematospiroides dubius* on C3H strain mice. *Acta Parasitol. Pol.*, 35, 241—253.

**4. Campbell, W. C., 1983:** Epidemiology I. Modes of transmission. In **Campbell, W. C.:** *Trichinella* and *Trichinosis.* Plenum Press, New York, 425—444.

**5. Cicenia, A., Scirocco, A., Carabotti, M., Pallotta, L., Marignani, M., Severi, C., 2014:** Postbiotic activities of lactobacilli-derived factors. *J. Clin. Gastroenterol.*, 48, 18—22.

**6. Dvorožňáková, E., Hurníková, Z., Kolodziej-Sobociń-ska, M., 2011:** Development of cellular immune response of mice to infection with low doses of *Trichinella spiralis, Trichinella britovi* and *Trichinella pseudospiralis* larvae. *Parasitol. Res.*, 108, 169—76.

**7. Gupta, V., Garg, R., 2009:** Probiotics. *Indian J. Med. Microbiol.*, 27, 202—209.

**8. Jurášek, V., Dubinský, P., 1993:** *Veterinary Parasitology* (In Slovak). Príroda, Bratislava, 382 pp.

**9. Kapel, C. M. O., Gamble, H. R., 2000:** Infectivity, persistence, and antibody response to domestic and sylvatic *Trichinella* spp. in experimentally infected pigs. *Int. J. Parasitol.*, 30, 215—221.

**10. Morales, M. A., Mele, R., Sanches, M., Sacchini, D., De Giacomo, M., Pozio, E., 2002:** Increased CD8 (+) T-cell expression and a type 2 cytokine pattern during the muscular phase of *Trichinella* infection in humans. *Infect. Immun.*, 70, 233—239.

**11. Pozio, E., La Rosa, G., Rossi, P., Murrell, K. D., 1992:** Biological characterizations of *Trichinella* isolates from various hosts species and geographic regions. *J. Parasitol.*, 78, 647—653.

**12. Pozio, E., Varese, P., Gomez-Morales, M. A., Croppo, G. P., Pellicia, D., Bruschi, F., 1993:** Comparison of human trichinellosis caused by *Trichinella spiralis* and by *Trichinella britovi.* *Am. J. Trop. Med. Hyg.*, 48, 568—575.

**13. Singh, V. P., Sharma, J., Singla, A., Babu, S., Ullah, R., 2013:** Role of probiotics in health and disease: a review. *J. Pak. Med. Assoc.*, 63, 253—257.

**14. Travers, M. A., Florent, I., Kohl, L., Grellier, P., 2011:** Probiotics for the control of parasites: an overview. *J. Parasitol. Res.*, 2011, doi:10.1155/2011/610769.