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OCCURRENCE OF BUTYRYLCHOLINESTERASE (BuChE) — POSITIVE NERVE STRUCTURES IN THE SPLEEN OF RABBITS

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ABSTRACT

The occurrence of BuChE-positive components of the spleen in rabbits was investigated by the direct thiocholine method. BuChE-positive nerve components enter the spleen in a common bundle with the arteries. In the organ they form characteristic periarterial and periarteriolar plexiform arrangements, which are especially conspicuous around *aa. centrales* running through the white pulp. Nerve fibres extend away from these plexuses into adjacent layers of trabeculae, further into marginal layers of periarterial lymphatic sheath (PALS), as well as into the mantle zone of the follicles. Several scattered periarteriolar and individual nerve fibres can be seen in the marginal sinuses and cords of the red pulp. In the fibrous capsula BuChE-positive nerve fibres can also be seen, which have an evident connection with the trabecular and parenchymal nerves of the organ. Microscopic findings support the notion that BuChE-positive nerve components supply vascular as well as parenchymal components of the spleen, and they participate in the regulation of the immune processes in this organ.

Key words: BuChE-positive innervation; rabbit; spleen

INTRODUCTION

Based upon a more detailed analysis of the published data on the nerve supply of primary and secondary lymphatic organs in mammals and birds (1, 5, 6, 9, 10, 17, 18), it is evident that more precise information on the adrenergic innervation of the spleen is relatively plentiful. On the other hand, the published data about AChE-positive innervation of the spleen in mammals are somewhat controversial, because more investigators have been unable to find AChE-positive nerve fibres in the parenchyma of the organ (8, 13, 15, 16), while the others have described only a small amount of nerve fibres near the hilus (7). For this discrepancy in the published data about cholinergic innervation, we have also examined the BuChE-positive innervation of the spleen in rabbits, because the published data about BuChE-positive nerve supply of this organ in these are only incomplete.

MATERIAL AND METHODS

Clinically healthy adult animals of both sexes were used in the study. The spleen of 20 rabbits (Chinchilla 2.5—3.5 kg) were examined. The animals were kept in the Central Animal Husbandry quarters under veterinary care. The direction of the Council of European Communities (86/609/EEC) on animal care was observed. Handling of experimental animals was performed under the supervision of the Ethical Commitee of the Medical Faculty of P. J. Šafárik University. The animals were anesthetized with thiopental (50—60 mg.kg^{-1} i.p.). BuChE-positive nerve profiles were demonstrated by means of the direct thiocholine method of cytochemical evidence of AChE (3). Tissue blocks were fixed in 4% paraformaldehyde (pH 7.4) at 4 ° for 2—4 h. Sections (16—20 µm thick) were cut with a cryostat, incubated in an incubation medium (pH 5.5)

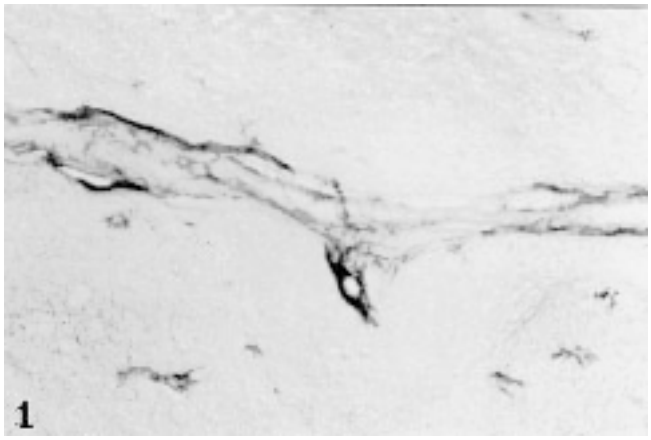


Fig. 1. The different density of the plexiform arrangement of BuChE-positive nerve fibres around the arteriolae running in the septum. Magn. × 250

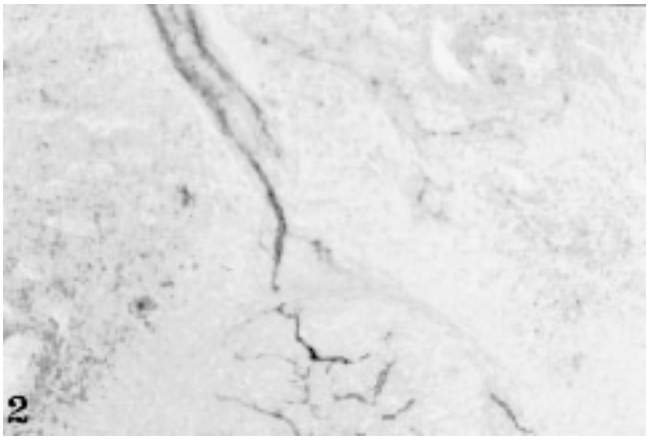
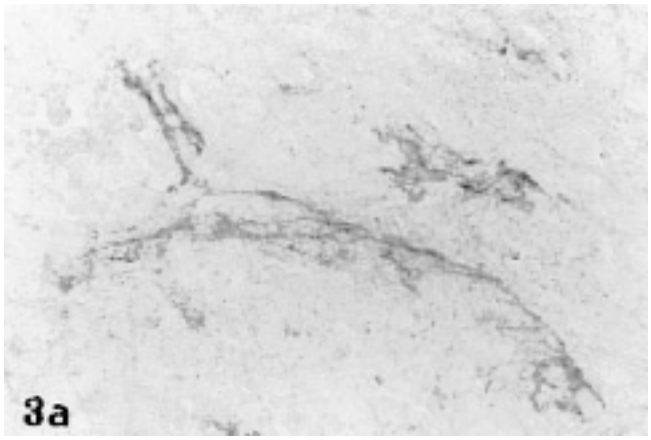


Fig. 2. BuChE-positive nerve fibres around the wall of the septum running arteriolae. Very fine linear BuChE-positive non-neural structures can be seen in the follicle. Magn. × 250



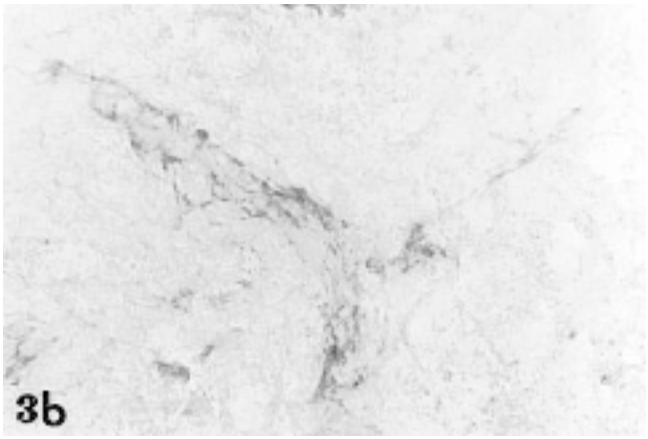


Fig. 3a, b. Very fine BuChE-positive nerve fibres around the wall of small arteriolar branches in the parenchyma of the organ. Magn. × 250

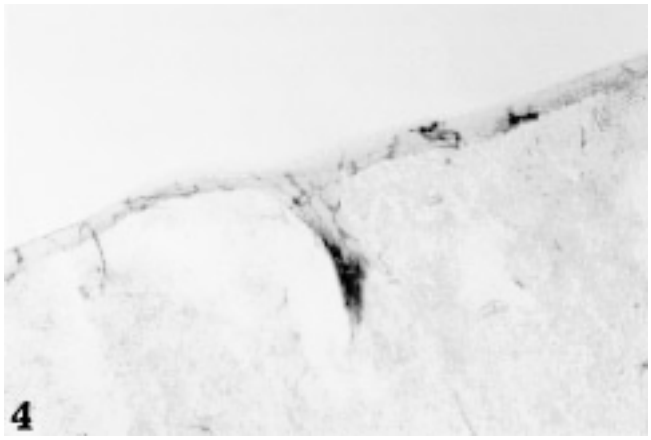


Fig. 4. BuChE-positive nerve fibres in the *capsula lienalis* and their connectiona with trabecular nerve profiles are evident. Magn. × 250

for two to four hours. Individual sections were mounted on glass slides. Control sections were incubated as follows: (*1*) incubation in a medium free of BW 284 c51 (Sigma, selective inhibitor of specific AChE; (*2*) incubation in a medium containing acetylthiocholine iodide and iso-OMPA (Sigma, selective inhibitor of nonspecific esterases). Both the microscopic examination and photographic documentation were performed using a Jenalumar 2 microscope (Zeiss, Jena).

RESULTS

BuChE-positive nerve components enter the spleen in a common bundle with arteries and its branches. In the organ itself, they border all its filial branches in the form of carrier nerve plexuses and also more delicate plexuses consisting mainly of the preterminal and terminal fibres (Fig. 1). These fine plexuses lie in close contact with the external side of the muscular media layer and are sometimes called “adventitial plexuses” BuChE-positive perivascular nerve profiles border arteries and arterioles that pass through the spleen partly

within the fibrous trabeculae and partly in the periarterial lymphatic sheath (PALS). Vascular and trabecular fibres enter the white pulp mainly in association with the central artery and its branches. Dense BuChE-positive profiles with an abundant amount of coloured reaction product accompany *aa. centrales* running through the white pulp. From these plexuses more fine nerve fibres radiate into the surrounding PALS, and in the vicinity of the white pulp, as well as in the inner region of the marginal zone within PALS, they can be seen most often in the marginal zone between the red and white pulp. BuChE-positive delicate nerve profiles, with a small amount of the coloured reaction product, were also recorded in the mantle zone of lymphatic follicles (Fig. 2), further around *aa. interfolliculares* in the close vicinity of lymphatic follicles.

However, as a rule, these nerve fibres do not penetrate into the germinal centres of the follicles. While BuChE-positive innervation of the white pulp within PALS is not conspicuous, the occurrence of periarterial or periarteriolar and individual nerve fibres on the borderline of the red and white pulp is evident (Figs. 3a, b). In addition, in the marginal sinuses and cords of the red pulp fine nerve fibres were also observed, but only rarely.

Some BuChE-positive nerve fibres also pass through the trabeculae and capsula of the organ without apparent connection with the vascular branches. BuChE-positive nerve fibres run more or less parallel to the adjacent artery or arterioles, mostly as isolated, and sometimes also branching nerve profiles.

The walls of large and larger splenic veins were innervated poorly and partially. BuChE-positive periarteriolar and individual nerve profiles were found to be relatively abundant in the *capsula fibrosa lienalis*. The direct connection between nerve components of capsula with intralienal neural structure was mostly quite evident (Fig. 4). Moreover BuChE-positive deposits of coloured reaction product were also present in non-neural structures. In lymphatic follicles, these deposits were linear or punctate of variable density with an appearance that resembled a reticular skeleton.

DISCUSSION

Our findings of BuChE-positive innervation of the spleen in rabbit is marked by the more common characteristics of the adrenergic nerve supply in other small mammals (5, 6, 19), and BuChE-positive innervation in rats (20), and with regard to that these nerve profiles enter the spleen together with arteries round which they form very conspicuous and typical thicker nerve arrangements that fulfil the role of a common carrying substrate for peripheral neuro-effector functions.

It is known that all the macro- and microscopic structural characteristics of various components of the organism are formed for certain functions. Reactions of smooth muscle cells to the released neurotransmitters both in the vasal media on the arterial and venous sides of the bloodstream as well as in fibrous *trabeculae* and *capsula* are among the factors that participate markedly in the regulation of blood flow. Furthermore, the spleen is among others as a filter for different non-immunogenic particles of organic or inorganic origin. It also has the function of a blood reservoir. Reactions of smooth muscular cells to the released neurotransmitter, as in the vascular media of intrasplenic vascular bed, as in the fibrous *trabeculae* and *capsula* of the organ, are the factors that could participate in the regulation of the blood flow as well as in accumalative functions of the organ (1, 2).

Studies on the histochemical detection of AChE have shown, as with our findings of BuChE, the presence of the enzyme not only in neural, but also in non-neural compartments of the spleen (13). There are authors (11, 13, 14, 21) who have suggested that a released neurotransmitter may influence the functions of the parenchyma-entering T- and B- lymphocytes as well as processes of antigen uptake by dendritic cells and macrophages. Lymphocytes isolated from spleen, thymus, lymph nodes, as well as from peripheral blood express muscarinic and nicotinic cholinergic receptors (4, 12). BuChE-positive nerve fibres in T-cell-dependent areas are in close vicinity or in contact with migrating lymphocytes, plasma cells, macrophages and mast cells, but also with arterioles where they participate in local modulation of humoral and cellular responses of the organism and indirectly in overall immunological responses (5, 12, 14).

Our findings of BuChE-positive nerve fibres in the red pulp are in contrast with the data of other authors (2, 17, 18) who suggest that AChE-positive nerve fibres in the spleen of the rats reached only the border of the white pulp, and that they do not enter the red pulp. Similarly these authors have not found any AChE-positive periarteriolar or individual nerve fibres in the *capsula lienalis*.

From comparative morphological study, it can be stated that the patterns of BuChE-positive innervation of the spleen in rabbits agree in principle with those in rats. Only the total number of nerve profiles supplying the larger vessels branches, and their occurrence in the perifollicular topography, are apparently higher in rabbits (20).

Our finding are in agreement with those found by the authors who state that nerve components supply not only the vasculature, but also the parenchyma of lymphoid organs with specific functional compartments, and do not enter their B-cell compartments.

On the basis of distribution patterns of BuChE-positive nerve profiles in the spleen of rabbits it can be concluded that, besides the regulation of vasomotor activity, direct or indirect effects of released neurotransmitters on immunocompetent cells cannot be excluded.

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THE EFFECTS OF CHROMATOGRAPHIC FRACTIONS OF *Phyllanthus amarus* Schum. et Thonn. ON THE HAEMATOLOGICAL PARAMETERS OF RATS

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ABSTRACT

The effects of the chromatographic fractions of *Phyllanthus amarus* Schum. et Thonn. on the haematological parameters in rats were investigated to determine the safety or otherwise of this plant as a medicinal herb. The ethanolic extract of this plant was subjected to chromatographic separation using vacuum liquid chromatography (VLC). With the aid of thin layer chromatography monitoring six fractions of this plant were obtained and were administered to rats in graded doses of 400 mg.kg^{-1}, 800 mg.kg^{-1} and 1600 mg.kg^{-1} orally for fourteen days. Fraction D of this plant caused a significant decrease in the levels of packed cell volume (PCV), red blood cell (RBC) count and haemoglobin (Hb) concentration. Fraction A, on the other hand, caused a significant decrease in the total white blood cell (TWBC) count. The results from this study showed that some chromatographic fractions of *P. amarus* have potentially deleterious effects on blood cells and therefore caution should be exercised in the use of *P. amarus* as medicinal plant.

Key words: chromatography; fractions; haematology; *P. amarus;* rats

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INTRODUCTION

Phyllanthus amarus Schum. et Thonn. is a common herb, occurring from Sierra Leone to Nigeria and Equatorial Guinea, and widespread elsewhere in tropical Africa. This plant is used for medicinal purposes. *Phyllanthus amarus* acts against against the Hepatitis B virus and related hepadnaviruses. This plant is known to have an inhibitory effect on endogenous hepadnavirus DNA polymerase – Morton (12), Oliver – Bever (15), Unander *et al.* (22), Unander *et al.* (23), Unander *et al.* (24), Unander *et al.* (25). Recent biochemical, pharmacological and clinical studies have confirmed and extended the medicinal uses of *Phyllanthus* species in traditional medicine – Venkateswaran *et al.* (26), Thyagaranjan *et al.* (21), Blumberg *et al.* (1), Ogata *et al.* (14), Shead *et al.* (19). Analgesia has been demonstrated using methanolic extracts of callus culture *in vitro* obtained from *P. tenellus, P. corcovadensis,* and *P. niruri* (*amarus*) – Gorski *et al.* (8), Santos *et al.* (18).

This plant belongs to the family Euphorbiaceae, which is a large family of trees, shrubs and herbs growing in habitats ranging from rainforest to desert. Although plants of this family have important economic uses as foodstuffs, medicinal, and in industry, particularly as sources of rubber and timber, nonetheless most members of this family are poisonous – Garner (7), Evans and Kinghorn (6), Burkill (2). The genus *Phyllanthus* grows wild among the pastures and is used as food supplement for rabbits in Ibadan. It is generally used for fencing and in traditional medicine in Nigeria – Hutchinson and Dalziel (11), Hartwell (10).

The study was carried out to investigate the activities of different plant fractions on certain haematological parameters of rats. Changes in certain haematological parameters were used as indices of toxicosis because it was thought that this plant is not toxic – Ogata *et al.* (14), Shead *et al.* (19), Raphael (17) and again, some of the dreaded side effects of poisons are haematological.

MATERIALS AND METHODS

Animals, Groupings and Experimental Design

Seventy six eight-week old albino rats (of both sexes), bred and maintained at the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan were used in this study. They were divided into seven groups. Six groups (n = 12) were dosed with six chromatographic fractions of *Phyllanthus amarus while* the seventh group (n = 4) served as controls. Each fraction was dosed orally at 400 $mg.kg^{-1}$, 800 $mg.kg^{-1}$ and 1600 $mg.kg^{-1}$ for 14 days to 4 rats per group. The animals in the control group received only distilled water for the same number of days.

Preparation of the Chromatographic fractions of *P. amarus*

Fresh leaves of the plant were collected within the campus of the University of Ibadan and were identified at the Department of Botany and Microbiology, University of Ibadan. Some of the air-dried leaves were ground into fine powder and the extraction procedure is as described by Harbone (9). The powdered leaves were continuously extracted using absolute ethanol in a soxhlet extractor until all the pigment was in the extract. The extract was concentrated *in vacuo* using a rotatory evaporator. The ethanol remaining in the extract was finally removed by placing the extract in porcelain dishes in a vacuum oven at 60 °C. The semi-solid extract was used for chromatography.

The crude extract was dissolved in just sufficient quantity of ethanol. Silical gel powder (TLC grade) was added to the solution with stirring until all the sample was adsorbed on silical gel and there was no free flowing liquid. The impregnated silical gel was spread on a watch glass and the solvent was allowed to evaporate in the fume cupboard on standing. The almost dried extract was afterwards dried in the oven at 60 °C.

Buckner filter assembly was used for the vacuum liquid chromatography. TLC grade of silical gel was used as stationary phase. The stationary phase (20 grams) was packed on top of a thick filter paper in the Buckner funnel. Another filter paper was placed on the column of silical gel, before the impregnated silical gel (4 g) was carefully spread on it with the vacuum line on. The mobile phase comprises of a gradient ranging from hexane, ethyl acetate and methanol, with a successive 10 % increment in the next polar solvent. A total of 50 ml was prepared for each gradient mixture. Each gradient mixture was added to the column and eluted with the vacuum line until the bed was sucked dry. The eluates were collected successfully as separate fractions.

The fractions were monitored by thin layer chromatography using silical gel GF_{254} as stationary phase and ethanol as mobile phase. The plates were visualized under UV lamp at 254 nm. With this procedure, fractions with similar resolutions were pooled together bringing the number of fractions to six. Samples were then obtained from these fractions after evaporation. These were designated fractions A–F based on their profile in terms of non-polarity to increasing polarity. In other words, while fraction A is non- polar, fraction F is polar.

Blood Samples Collection and Determination of Haematological Parameters

The animals were anaesthetized using diethyl ether and the blood samples were collected by cardiac puncture into heparinised clean bottles for haematological studies. Packed cell volume (PCV), red blood cells (RBC) and white blood cell (WBC) counts were determined using microhaematocrit and haemocytometer. Haemoglobin concentration was determined using the cyanomethaemoglobin method – Duncan *et al.* (5). Leukocyte differential count was determined following Giemsa staining under a light microscope.

Statistical Analysis

All data were expressed as means with standard error. The data were subjected to the pooled variance *t*-test for comparison and Duncan Multiple Range Test – Steel and Torrie (20).

RESULTS

The effects of chromatographic fractions of *P. amarus* on the haematological parameters of rats are presented in tables I to VI. Fraction D (400, 800, 1600 $mg.kg^{-1}$) caused a significant decrease ($P < 0.05$) in the levels of packed cell volume (PCV), haemoglobin concentration, and red blood cell count. Fraction A (1600 $mg.kg^{-1}$) caused a significant decrease ($P < 0.05$) in the total white blood cell count (TWBC). There was no significant difference in the number of monocytes, neutrophils, lymphocytes and eosinophils when compared to the controls.

DISCUSSION

Only fraction D caused a significant decrease in the level of the PCV, haemoglobin concentration, and red blood cell count. This may suggest that the compound(s) responsible for decreasing PCV, RBC count and haemoglobin concentrations are found in fraction D. Thus the use of *Phyllanthus amarus* as feed supplement may cause anaemia if fed to rabbits. Its use for medicinal purpose may also be fraught with danger. In a recent study on the phytochemical constituents of aqueous extract of *P. amarus*, the following were detected: alkaloids, saponins, tannins anthraquinones, flavones, carotenoids, anthacyanosides, reducing compounds, cardenolide (cardiac glycoside), sterols and triterpenes, coumarins, and volatile oils – Odetola and Akojenu (13). Tannins,

Table I. The effects of Fraction A on the haematological parameters of rats (n = 4)

Parameters	Control	TREATMENTS 400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	37.3 ± 5.3	38.0 ± 7.6	40.0 ± 1.3
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	6.1 ± 1.0	6.1 ± 1.0	6.7 ± 0.2
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	10.7 ± 2.5	10.4 ± 2.8	12.0 ± 1.7
Mean Corpuscular Volume (fl)	62.4 ± 7.6	61.8 ± 0.3	63.0 ± 0.2	61.0 ± 0.8
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	5.9 ± 1.4	5.3 ± 1.2	4.9 ± 1.2[a]
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.8 ± 0.6	2.6 ± 0.5	2.4 ± 0.6
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	2.9 ± 0.8	2.5 ± 0.6	2.3 ± 0.6
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.08 ± 0.1	0.14 ± 0.1	0.11 ± 0.05
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.11 ± 0.12	0.08 ± 0.07	0.08 ± 0.04

Superscripts indicate significant difference from control ($P < 0.05$)

Table II. The effects of Fraction B on the haematological parameters of rats (n = 4)

Parameters	Control	TREATMENTS 400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	38.8 ± 8.0	35.8 ± 2.5	34.0 ± 4.6
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	6.1 ± 1.1	6.0 ± 0.6	5.5 ± 0.5
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	11.4 ± 2.6	10.5 ± 2.1	10.1 ± 1.8
Mean Corpuscular Volume (fl)	62.4 ± 7.6	63.0 ± 0.4	60.0 ± 1.1	61.0 ± 0.6
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	4.5 ± 1.6	5.2 ± 2.6	5.2 ± 1.5
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.2 ± 0.9	2.5 ± 1.3	2.5 ± 0.8
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	2.2 ± 0.6	2.6 ± 1.4	2.5 ± 0.7
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.07 ± 0.06	0.06 ± 0.07	0.13 ± 0.11
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.1 ± 0.13	0.09 ± 0.13	0.07 ± 0.06

Table III. The effects of Fraction C on the haematological parameters of rats (n = 4)

Parameters	Control	TREATMENTS 400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	36.0 ± 4.6	37.8 ± 3.4	41.0 ± 7.9
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	5.9 ± 0.7	6.3 ± 0.8	6.2 ± 1.4
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	11.0 ± 1.1	11.2 ± 0.7	12.5 ± 2.6
Mean Corpuscular Volume (fl)	62.4 ± 7.6	61.0 ± 0.7	61.0 ± 0.8	67.0 ± 2.3
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	5.4 ± 0.5	5.7 ± 1.5	5.3 ± 2.9
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.6 ± 0.2	2.7 ± 0.7	2.6 ± 1.4
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	2.6 ± 0.3	2.9 ± 0.8	2.6 ± 1.4
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.1 ± 0.08	0.002 ± 0.003	0.004 ± 0.004
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.006 ± 0.005	0.009 ± 0.009	0.004 ± 0.004

for example are soluble in water, dilute alkalis, alcohol, etc., but generally only sparingly soluble in other organic solvents. C l a r k e and C l a r k e (3) reported that *Acacia decurrens* and *Acacia salicina* may be toxic on account of their high tannin content of 35 % and 16 % respectively. Clinical signs such as salivation, inappentence and ataxia of the hind limbs were noted. The pods of *Acacia nicotica* have been the cause of death in goats in South Africa giving rise to tachycardia, anorexia, ruminal stasis, anaemia, dyspnoe and recumbency.

Many members of the spurge family to which *P. amarus* belong are poisonous. For instance, *Mercuralis perennis* (Dog's Mercury) and *M. annua* (Annual Mercury) are poisonous. *Mercuralis perennis* gives rise to two distinct syndromes, the first, and the one usually encountered in field case, is a haemolytic anaemia, the second an acute oedematous gastroenteritis in cattle. In poisoning by *Mercuralis annua*, haematuria is also the most obvious clinical sign. D e p r e z *et al.* (4) have reported on two cattle farms that animals showed con-

Table IV. The effects of Fraction D on the haematological parameters of rats (n = 4)

Parameters	TREATMENTS			
	Control	400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	25.5 ± 5.2[a]	30.5 ± 7.9[a]	25.0 ± 5.6[a]
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	4.6 ± 1.5	4.9 ± 1.4	4.3 ± 0.8[a]
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	8.2 ± 1.7	9.3 ± 2.7	8.1 ± 1.6[a]
Mean Corpuscular Volume (fl)	62.4 ± 7.6	57.0 ± 2.6	63.0 ± 0.2	58.0 ± 2.3
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	5.0 ± 0.5	6.3 ± 2.0	5.4 ± 0.6
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.4 ± 0.2	3.1 ± 1.0	2.7 ± 0.2
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	2.4 ± 0.2	3.1 ± 0.9	2.6 ± 0.4
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.09 ± 0.008	0.09 ± 0.007	0.001 ± 0.002
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.009 ± 0.007	0.01 ± 0.008	0.003 ± 0.005

Superscripts indicate significant difference from control ($P < 0.05$)

Table V. The effects of Fraction E on the haematological parameters of rats (n = 4)

Parameters	TREATMENTS			
	Control	400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	37.3 ± 4.1	38.0 ± 3.6	36.5 ± 5.5
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	6.4 ± 0.8	6.0 ± 0.6	6.0 ± 1.1
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	11.5 ± 1.3	11.6 ± 1.1	10.3 ± 0.5
Mean Corpuscular Volume (fl)	62.4 ± 7.6	59.0 ± 1.8	64.0 ± 1.8	63.0 ± 1.0
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	5.2 ± 0.3	4.8 ± 1.4	5.5 ± 0.9
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.6 ± 0.2	2.3 ± 0.7	2.6 ± 0.4
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	2.6 ± 0.2	2.4 ± 0.7	2.8 ± 0.5
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.1 ± 0.008	0.06 ± 0.007	0.08 ± 0.009
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.03 ± 0.001	0.05 ± 0.007	0.07 ± 0.008

Table VI. The effects of Fraction F on the haematological parameters of rats (n = 4)

Parameters	TREATMENTS			
	Control	400 mg.kg^{-1}	800 mg.kg^{-1}	1600 mg.kg^{-1}
Packed Cell Volume (%)	34.6 ± 4.9	35.8 ± 4.2	32.5 ± 2.1	34.5 ± 3.7
Red Blood Cell count (10^6.µl^{-1})	5.6 ± 0.5	5.7 ± 0.8	5.4 ± 0.3	5.9 ± 0.8
Haemoglobin (g.dl^{-1})	10.8 ± 1.0	11.5 ± 0.9	11.3 ± 1.3	11.4 ± 1.0
Mean Corpuscular Volume (fl)	62.4 ± 7.6	63.0 ± 0.1	61.0 ± 0.9	59.0 ± 1.7
White Blood Cell count (10^3.µl^{-1})	6.4 ± 1.0	6.1 ± 0.9	4.5 ± 0.5	5.8 ± 0.4
Neutrophils (10^3.µl^{-1})	3.1 ± 0.4	2.9 ± 0.5	2.2 ± 0.2	2.8 ± 0.2
Lymphocytes (10^3.µl^{-1})	3.2 ± 0.5	3.0 ± 0.5	2.2 ± 0.02	2.9 ± 0.2
Monocytes (10^3.µl^{-1})	0.08 ± 0.01	0.09 ± 0.001	0.09 ± 0.001	0.06 ± 0.001
Eosinophils (10^3.µl^{-1})	0.04 ± 0.05	0.01 ± 0.001	0.008 ± 0.001	0.01 ± 0.001

stipation or diarrhoea, dullness, haemolytic anaemia and red urine after ingestion of *M. annua.* Welchman–Dde *et al.* (27) earlier reported that eleven lambs in a flock of 400 8-month old Romney lambs died from grazing *M. annua*. Pathological findings including haemolytic anaemia and haematuria were indicative of annual mercury poisoning. It has also been reported that the extracts of *Jatropha curcas* (family Euphorbiaceae) caused a progressive reduction in the measured haematological parameters (packed cell volume, red blood cell count and haemoglobin concentration) of rats – Oluwole and Bolarinwa (16).

The 1600 mg.kg^{-1} dose of fraction A caused significant decrease in the total white blood cell count. All the chromatographic fraction of *P. amaru*s tended to cause a decrease in the number of lymphocyte in rats. The continuous exposure, of all to these fractions may compromise the immune status of the animals. The significant decrease in white blood cell count may bring about immunosuppression, making the animals vulnerable to secondary opportunistic conditions.

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IMMUNE RESPONSE OF CHICKENS TO FEED SUPPLEMENTATION WITH SELENIUM

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ABSTRACT

The aim of this study was to assess the effects of feed supplementation with inorganic and organic Se sources on the cellular and humoral immune response in chickens. Selenium (Se) is well known to be an essential trace element with many vital functions in humans and animals. Studies on chickens have shown that selenium deficiency affects the maturation of lymphocytes as well as the functional abilities of fully differentiated cells. By the way of contrast, supplementation with selenium has been shown to have marked immunostimulant effects. In our work the Se dose correlation and T-cells expression has been determined. This phenomenon appeared predominantly in the second half of experiment (4th and 7th experimental weeks). Finally, the supplementation of chicken basal diet by selenium yeasts showed a trend to improve the protection of the chicken's body against pathogens.

Key words: cellular and humoral immune response; chickens; selenium

INTRODUCTION

Selenium (Se) is well known to be an essential trace element with many vital functions in humans and animals. Se exerts its function *via* specific selenoenzymes including glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases, and many others. To date, about 30 selenoproteins have been identified, but the precise function of most of them is not yet known. Apart from antioxidant defense, thyroid hormone function and others, Se is also essential in the immune system function of domestic animals, particularly cell immunity (9, 4).

Studies on chickens have shown that selenium deficiency affects the maturation of lymphocytes as well as the functional abilities of fully differentiated cells. The defects induced include impaired growth of primary lymphoid organs and damage to their epithelia, reduced lymphocyte production and a change in the balance of CD4/CD8 subsets (16, 13, 6). These findings have implied that the "education" of developing lymphocytes is impaired in Se deficient chicks and that the delivery of T-cell "help" is also shortened.

By way of contrast, supplementation with selenium has been shown to have marked immunostimulant effects, including an enhancement of proliferation of activated

T-cells in many animal species (9, 20, 7). Increased Se intake by people has resulted in the enhanced response of lymphocytes to antigen stimulation and their increased ability to develop into cytotoxic ones with the ability to destroy tumour cells (14, 11).

The consequences of insufficient selenium intake in chickens, including nutritional myodystrophy, exsudative diathesis, disorders pancreatic functions and many other syndromes, have been well described. Associated production problems are poor animal performance, reproductive disorders and considerable economic loss due to morbidity and mortality. Another situation induced by a marginal Se deficiency is the delayed immune system development of young chicks, poor

feathering with associated energy losses, increased mortality and culling, reduced egg production and increased embryonic mortality (18).

In most countries the natural selenium content of grain and forages is only 0.03—0.12 mg.kg^{-1} of dry matter (DM) with values more commonly at the lower end of this range. Intake of such feeds can result in serious selenium deficiency and health problems, especially in highly productive animals. For this reason, feedstuffs are routinely supplemented with various selenium sources, usually at the rate of 0.2—0.3 mg of Se per kg of DM.

The objectives of experiment described in this paper were to assess the effects of feed supplementation with inorganic and organic Se sources on the cellular and humoral immune response in chickens.

MATERIAL AND METHODS

Animals, diets and treatment

A total of twenty-four, day-old White Plymouth Rock chicks (obtained on the day of hatching from LP – Párovské Háje a.s., the Slovak Republic) were divided randomly into four groups of six birds and kept in large pens with wood shavings. From the first day of life till the age of seven weeks the chickens were fed treatment diets, which differed only in the total content and/or form of selenium. The first group (a control one) was given a basic diet (Table 1) containing 0.12 mg Se.kg^{-1} of dry matter (DM) arising only from the natural occurrence of selenium in the dietary components. The second received a basic diet to which sodium selenite was added to supply 0.2 mg.kg^{-1} Se resulting in a final selenium level of 0.32 mg.kg^{-1}. The third group received a basic diet supplemented with 0.2 mg.kg^{-1} Se in the form of selenium yest (Sel-Plex™) yielding the same final selenium level (0.32 mg.kg^{-1}) as in the diet fed to the second group of birds. The fourth group received the basic diet containing an added 0.7 mg.kg^{-1} Se from selenium yeast for a total dietary concentration of 0.82 mg.kg^{-1} Se.

Table 1. The composition of the basic diet for chickens

Component	%
Corn, ground, 9 % of crude protein (CP)	53.0
Soybean meal, 3.5 % CF, 48 % CP	23.00
Fish meal, 65 % CP	4.0
Wheat, ground, 11 % CP	11.45
Premix BR 1 Forte[1]	1.0
Dicalcium phosphate, 20 % P	0.3
Salt	0.25
Meat and bone meal, medium fat content, 48 % CP	5.0
Pulverised fat, BERGAFAT HPL 106	2.0

[1] — Supplied per kg of diet: vitamin A, 12500 IU; vitamin D_3, 3500 ICU; vitamin E, 150 mg; vitamin K_3, 4 mg; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 6 mg; cyanocobalamin, 40 µg; niacin, 60 mg; pantothenic acid, 15 mg; betain, 277 mg; biotin, 0,2 mg; folic acid, 2 mg; L-lysine, 1,2 g; DL-methionine, 1 g; Zn 80 mg; I, 1 mg; Co, 0,4 mg; Mn, 100 mg, Cu, 8 mg; Fe, 80 mg

The diets fed to groups 12 and 3 were supplemented with non-Se yeast extract (NuPro™, Alletch Inc.) to supply amounts of "yeast" equal to that added to the fourth diet (63.7 g in 100 kg of feed). Mineral and vitamin premixes were supplied by Biotika a.s. (The Slovak Republic).

The chickens were reared starting with a lighting regimen of 23 L:1 D, which was adjusted to 15 L:9 D after three weeks of age. The initial room temperature (32—33 °C) was reduced every week by 3 °C to a final temperature of 23 °C. All animals had free access to water and feed.

Samples collection and analysis

Each week six chickens from each treatment group were killed for the collection of blood and tissue samples. Following an IP injection of pentobarbital (Spofa, The Czech Republic, 60 mg.mg^{-1} body weight) blood was collected by intracardial puncture and placed in heparinised tubes or in tubes containing EDTA solution. Following euthanasia, samples of duodenum, caecum and *bursa Fabricii* were collected.

For the immunohistochemical analysis of lymphocyte differentiation the monoclonal antibodies (Moab, Scandic, The Czech Republic) against chicken CD (cluster of differentiation) 3 T-cells were used in duodenum and caecal tonsils, CD 4 T-cell subpopulation in the *bursa Fabricii* and CD8 T-lymphocytes in the duodenum (Table 2).

Table 2. Primary monoclonal antibodies use in the experiment

Specificity	MoAbs	Isotype	Dilution
CD3	RTMCA1378	mouse IgG1	1:25
CD4	SRTMCA1473	mouse IgG1	1:25
CD8	SRTMCA1377	mouse IgG1	1:25
BU1b	8370-01	mouse IgG1	1:25

Frozen sections from the caecal tonsils, duodenum and *bursa Fabricii* (7 mm thick) were immersed on cold acetone, rinsed with phosphated buffered saline PBS and incubated with 1 % skimmed milk for 30 minutes to reduce non-specific binding. Sections were then incubated for 1 hour with primary monoclonal antibodies. Biotin-Streptavidin amplified (B-SA) peroxidase detection was used to detect the lymphocytes (Biogenex, USA). Instead of primary antibody, the PBS was used as a negative control. The specific colour reaction was induced by 3.3 diaminobenzidine in Tris-buffered hydrochloric acid.

The lymphocytes in the duodenum and caecum were counted at the base of the villi as well as in the medullar part of *bursa Fabricii* follicles. In the duodenum lymphocytes were counted in the areas of villus epitelium and *lamina propria* from the *lamina muscularis mucosae* toward the point of the villi. Six appropriate areas were chosen at random from each of these sites in the gut and 100 areas in the *bursa Fabricii*. Measurements were taken by light microscope at 1000 × magnification in the duodenum and caecal tonsils and 400 × in the

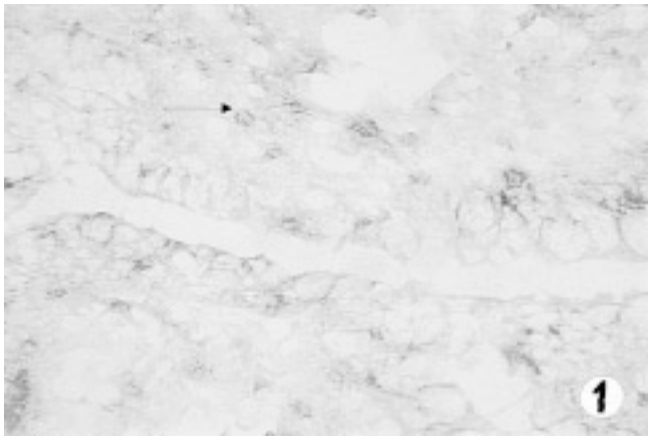


Fig. 1. The expression of CD3+ cells in the duodenum of chicken one week old which was fed a diet with the basic content of Se (0.12 ppm) only. Positive cells were round and showed a brown reaction in the cell membrane and cytoplasm (arrow). Labelling by mouse monoclonal antibody against chicken CD3+ lymphocytes; × 800

Fig. 2. Duodenum of 4th week old chicken fed a diet supplemented with 0.2 ppm of organic Se (Sel-Plex) shows an increased density of CD3+ cells. Labelling by mouse monoclonal antibody against chicken CD3+ lymphocytes; × 800

Fig. 3. Bursa Fabricii of seven weeks old chicken given a diet with the basic content of Se only (0.12 ppm) shows a low density of CD4 positive cells. Labelling by mouse monoclonal antibody against chicken CD4+ lymphocyte; × 400

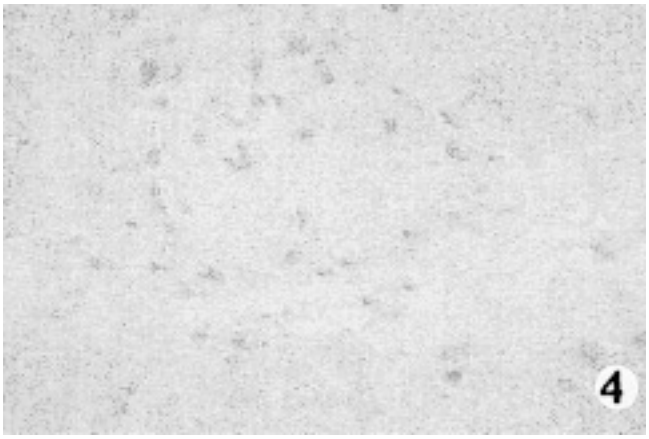


Fig. 4. The expression of CD4+ cells in the bursa Fabricii from a chicken seven weeks old. The bird was fed a diet supplemented with 0.7 ppm of organic Se (Sel-Plex). An increased density of positive cells can be seen. Labelling by mouse monoclonal antibody against chicken CD4+ lymphocyte; × 400.

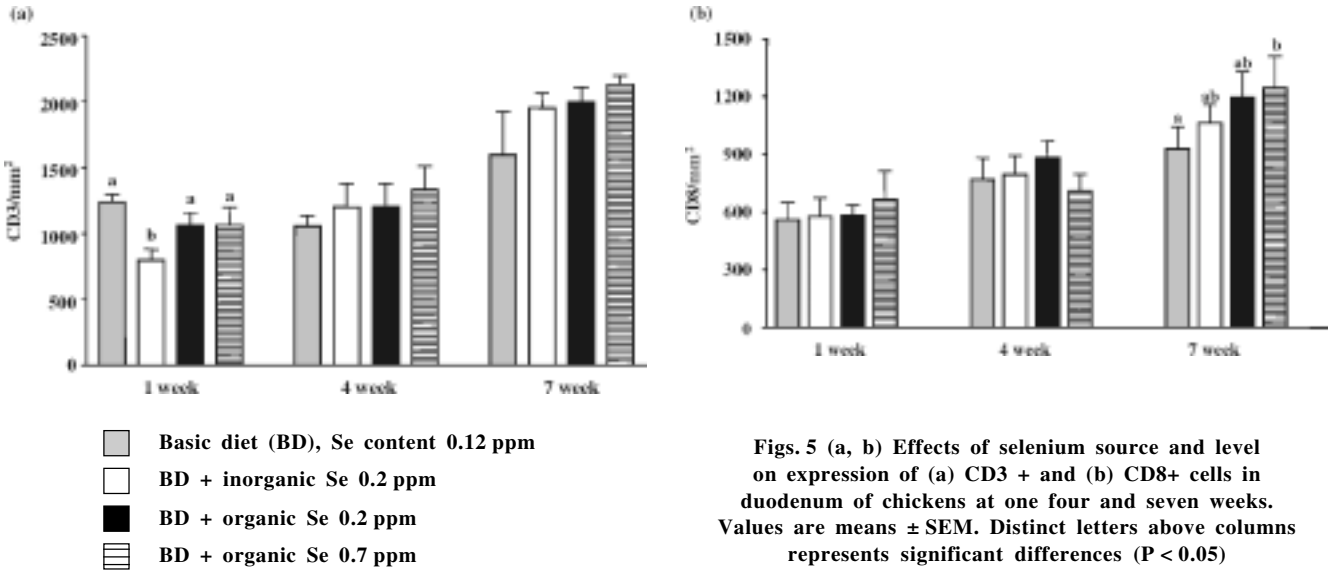
bursa Fabricii. The positive lymphocyte — stained cells within each randomly selected area were counted using of calibrated ocular graticule LTD 0.25 mm Id × Grd (Electronmicroscopy, UK). The appearance of positive lymphocytes is expressed in numbers per square millimeter.

For flow cytometry, the spleen was obtained from 1 and 4 week-old birds. Spleen lymphocytes were removed by teasing them through a 70 µm mesh screen (12) and isolated on a density gradient – Telebrix (Sevac, The Czech Republic). After separation, the lymphocytes were twice washed with PBS. Fifty µl of cellular suspension (1.10^6 lymphocytes in PBS) and 50 µl of specific MoAb (Table 2) were mixed and incubated at 4 °C for 30 minutes. After incubation the cells were washed twice in PBS and pellets were mixed and incubated with 25 µl of secondary antibody (FITC-conjugated goat antimouse immunoglobulin, Dakopatts, Germany) in a 1 : 50 dilution and incubated in the dark as describe above. The cells were washed twice in PBS and re-suspended in 0.5 ml of 1 % paraformaldehyde in PBS. Samples were analysed by FACScan flow cytometer (Becton Dickinson, Germany). Data on 1.10^4 viable cells were collected using the Cell Quest programme of Becton Dickinson.

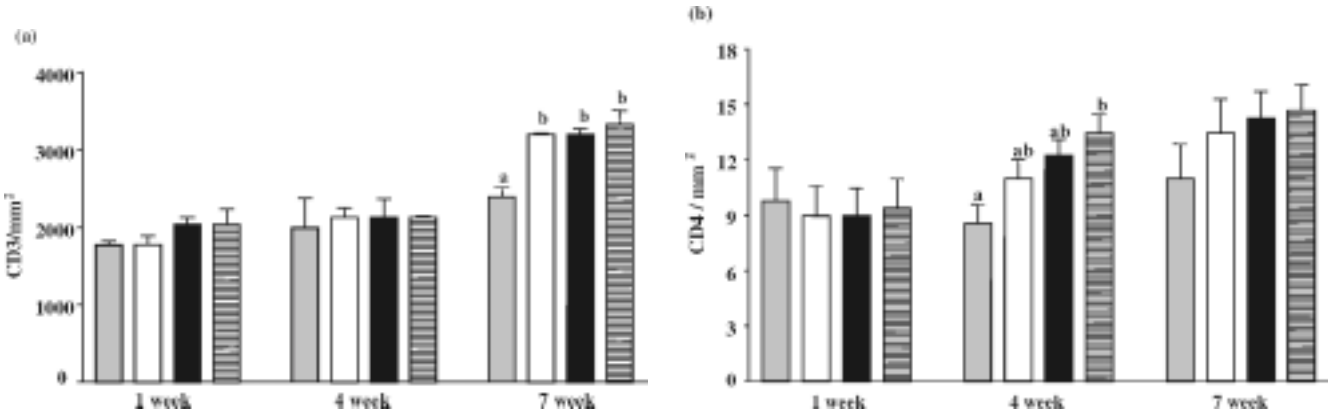
Functional immunity assays

Blood samples were collected by cardiac puncture into tubes containing sodium heparin (20 units·ml^{-1} of blood). The number of leukocytes and lymphocytes in the whole blood was evaluated by routine laboratory methods. Suspensions of heterophils and lymphocytes were isolated by a previously described method (1) with a slight modification (2, 5).

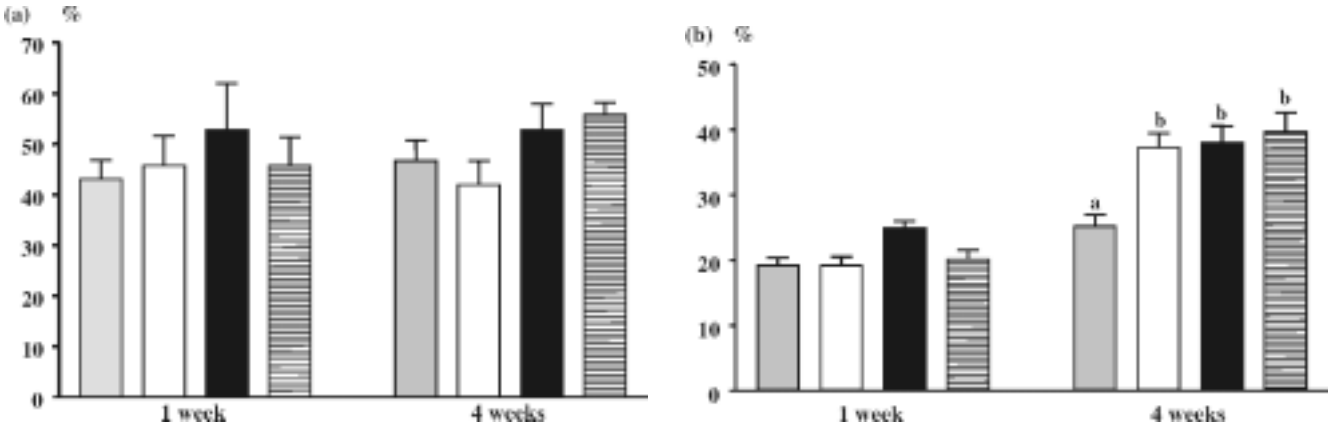
Iodo-nitro-tetrazolium reductase test (INTT) was carried out for the quantitative evaluation of the metabolic activity (MA) of phagocytes according to a familiar method (15). The index of metabolic activity was recorded as the ratio of mean



Figs. 5 (a, b) Effects of selenium source and level on expression of (a) CD3 + and (b) CD8+ cells in duodenum of chickens at one four and seven weeks. Values are means ± SEM. Distinct letters above columns represents significant differences (P < 0.05)



Figs. 6 (a, b). Effects of selenium source and level on expression of (a) CD3+ in caecal tonsils and (b) CD4+ in bursa of Fabricius in chickens at one, four and seven weeks. Values are means ± SEM. Distinct letters about columns represents significant differences (P < 0.05)



Figs. 7 (a, b). The flow cytometric analysis of effects of supplying the feed with inorganic (Na_2SeO_3) and organic form of selenium (Sel-Plex ™) on the expression of CD3+ and CD8+ cells in spleen of chickens at 1 and 4 weeks of age. Values are means ± SEM. Distinct letters above columns represents significant difference (P < 0.05)

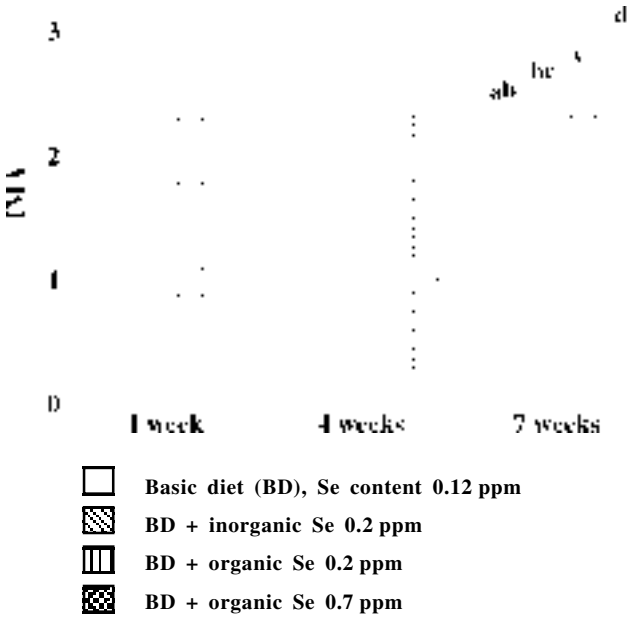


Fig. 8. Effect of various selenium source and level on index of metabolic activity (IMA) (ratio of heterophils with and without phagocyted starch) in chickens at one, four and seven weeks. Values are means ± SEM. Distinct letters above columns represent significant differences ($P < 0.01$ and $P < 0.001$)

optical densities (OD_{485}) of the suspensions of heterophiles with and without starch.

The lymphoproliferation test: A colorimetric immunoassay (5) was used for the quantification of lymphocyte proliferation, based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis (Cell Proliferation ELISA Kit, BrdU-colorimetric, Boehringer Mannheim, Germany). Briefly, suspensions of lymphocytes — 100 ml of 2.10^6 cells/well in RPMI 1640 with 10 % of foetal calf serum (5) — were cultured for 72 hours with 10 mmg·ml^{-1} of mitogen (phytohaemagglutinin, PHA, Sigma, Germany), and without mitogen in a 96-well microtiter test system at 37 °C in a humid atmosphere at 5 % CO_2. Each culture was tested in triplicate.

Cell activation rate was expressed as a stimulation index (SI) calculated on the basis of the ratio of absorbance of the stimulated cells to the non-stimulated cells according to formula:

$$SI = \frac{OD_{450} \text{ of the mitogen activated lymphocyte cultures}}{OD_{450} \text{ of the lymphocyte cultures without mitogen}}$$

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance with the *post hoc* Tukey and Dunett tests used to separate means where appropriate.

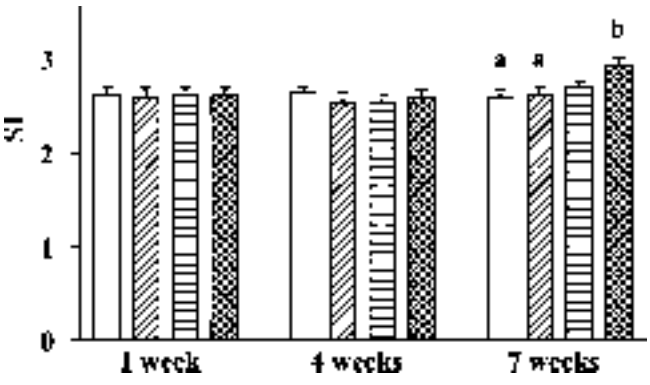


Fig. 9. Effect of various selenium source and level on stimulation index (SI) (ratio of stimulated to non-stimulated cells) in chickens at one, four and seven weeks. Values are means ± SEM. Distinct letters above columns represents significant differences ($P < 0.05$)

RESULTS

Immunohistochemistry and flow cytometry

The immunohistochemical observations (Figs. 1, 2, 3, 4) clearly demonstrated that the expression of avian T-and B-lymphocytes was correlated with the total intake of selenium.

The numbers of CD3+, CD4+, CD8+ and BU1b+, cells in the duodenum, caecal tonsils as well as of CD3+, CD4+ and CD8+ cells in *bursa Fabricii* showed a rising tendency from one to seven weeks of chicken life.

This tendency was observed mainly in CD3+ and CD8+ cells expression in the duodenum (Figs. 5a, b), in CD3 cells in the caecal tonsil (Fig. 6a) and duodenum and in CD4+ cells number in the bursa of Fabricius (Fig. 6b) and in the caecal tonsils.

In the 7th week the dose dependent pattern was found in: CD3+ cells in the caecal tonsils and duodenum, CD4+ cells in the caecal tonsil and *bursa Fabricii,* CD8+ expression in the duodenum and BU1+b in the caecal tonsils.

The results of *flow cytometry* analyses of CD3+ and CD8+ cells in the spleen are presented on Figs. 7a, b. The findings from 4 weeks old birds clearly showed a selenium dependent increase in numbers of CD8+ cells.

Functional immunity assays

The index of metabolic activity of peripheral blood phagocytes increased significantly ($P < 0.01$, 0.001) in chickens in the 4th group in week 7 only (Fig. 8).

The evaluation of the polyclonal activation of lymphocytes by PHA revealed no significant differences in the mean values of the stimulating index (SI) in lymphoproliferative test between the trial groups of chickens. In the two last weeks of the experiment a significant increase of SI was observed ($P < 0.05$) in the 4th group of chickens (Fig. 9).

DISCUSSION

In this work possible interference with the functional activities of chicken lymphocytes after *in vitro* supplementation with selenium was examined. Sodium selenite and selenium-yeast compounds were tested in parallel and their capability to inhibit or to increases the BU1b+ cells and T-lymphocytes subsets (CD3, CD4 and CD8) expression was investigated.

It is well known that selenium is necessary for an optimum performance of the immune system. To our best knowledge the immunohistochemical approach has not been applied to characterize the Se effects on the development of T-lymphocytes subpopulation in young growing chickens. The consequences of selenium deficiency on chicken splenocyte proliferation and cell surface marker expression by flow cytometry has been well described (13).

In general, the Se dose correlation and T-cells expression has been determined. This phenomenon appeared predominantly in the second half of the experiment (4th and 7th experimental weeks). An increased tendency in expression of CD8+ cells in the duodenum, CD4+ in the *bursa Fabricii* and CD3+ cells in the spleen after an organic selenium addition to the chicken diet could be seen in the same experimental period. While the Se effects on duodenal and spleen lymphocyte numbers appeared after the 7th week only the changes in bursal T-cells were observed in 4 weeks old birds. It is obvious because the development of *bursa Fabricii* is reached between 4 and 12 weeks of chicken life (10).

CD3 presents a complex of proteins acting as signal transducers found on all T-cells. That is why these cells have been used for the detection of the all expression of T-lymphocytes (19). The function of CD4+ T_H-cells as coordinators of the immune function include responsibility for the generation of antibody responses by providing help for the B-cells. They can also influence cytotoxic responses as well. Thus CD4+ cells have a central role in the avian immune system and their activation is a prerequisite for responses by other types of cells (3). CD8+ cells are known to be effector cells in cytotoxic response, by killing infected target cells (3). Selenium has been shown to stimulate the transformation of T-cells into cytotoxic ones (14).

According to R a y m a n, (17) supplementation with selenium has immunostimulant effects, including an enhancement of the proliferation of activated T-cells. Lymphocytes have shown an enhanced response to antigen stimulation and an increased ability to develop into cytotoxic lymphocytes and destroy tumour cells. Our experiment has revealed in the two last weeks of the experiment a significant increase of SI in the group with a higher dose of selenium yeast extract. A significant effect of the selenium yeasts supplementation in the higher dose group of chicken was also confirmed on the metabolic activity of phagocytic cells. A similar effect on phagocyte functions – killing of microorganisms and oxidative metabolism of neutrophiles has been described (8).

This experiment was carried out without the inclusion of any infection. The data from our experiment on the tendency of T-cell expression to increase with the feed selenium supplementation offers new knowledge about the density and functionality of cells responsible for cellular immunity. Actually it could indicate an improved status for the better preparedness of T-lymphocytes to respond to any antigen stimuli and the metabolic activity of phagocytes also increased.

Finally, the supplementation of a chicken basic diet by selenium yeasts showed a trend to improve the protection of the chicken's body against pathogens.

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THE PROCESS OF THE APPLICATION OF EUROPEAN UNION PHARMACO-VIGILANCE REGULATION IN THE CONDITIONS OF AQUACULTURES IN THE CZECH REPUBLIC

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ABSTRACT

The objective of this work was to arrange an assessment system for medicinal products intended for use on fish in conditions in the Czech Republic on the basis of European Union (EU) legislation requirements. The authors review the rules for preclinical trials and determination of pharmacological effects, clinical trials and residue studies for medicinal products for use on fish.

Key words: authorisation in accordance with EU regulation; drugs; fish

INTRODUCTION

The prosperity of aquaculture husbandries in the Czech Republic directly depends on the health conditions of fish. According the *Act on veterinary care* (No. 166/1999 Col., as amended) the breeder ought to monitor the state of health of animals and give skilled veterinary aid. Prevention and treatment are part of technological proceedings in all kind aquaculture husbandries. In practice, health state examination is more frequently required in fish, which is followed by a recommendation for treatment. However the recommended therapy in the Czech Republic can be supported by a small number of authorized products. With respect to pharmacovigilance requirements only one method for the legal enlargement of the range of medicinal products exists: that is the authorization of other veterinary medicinal products intended for use on aquatic species. Therefore rules for the testing of medicinal products for use in fish necessarily have to be designed corresponding fully with EU standards.

The headstone of the European Union in this sphere is Directive 2001/82/EC of the European Parliament and of the Council on the Community code relating to veterinary medicinal products, as amended. This directive is presently implemented in Czech legislation and is the background of Act No. 79/1997 Coll., on Pharmaceuticals and Amendments to Several Related Acts Amendments, and of the decrees that relate to this act.

Decree No. 288/2004 Coll., laying down details of registration, its variations, renewals, determination of the method of supply (legal status) of a medicinal product, transfer of marketing authorization, licensing of parallel import, details of reporting and evaluating of adverse reactions to a medicinal product and the way and extent of reporting of the use of a unregistered medicinal product, etc.

Decree No. 472/2000 Coll., on good clinical practice and more detailed conditions for clinical trials on pharmaceuticals, as amended.

Decree No. 504/2000 Coll., on good laboratory practice in the field of pharmaceuticals.

Decree No. 325/2003 Coll., concerning the rules for use of medicinal products for veterinary care, including the rules for prescription and supply of products, the rules for reporting procedures. This note sets rules for the utilisation of veterinary and human medicinal products in the therapy of animals, if the veterinary product is not available (veterinary medicinal product authorized according to § 23 of Act No. 79/1997 Coll. for acceptable indication and target species).

The directive is the legislative basis and corresponding guidelines are published for the clarification of veterinary medicinal products authorization procedure. They are stated as The Rules Governing Medicinal Products in the European Union in Volumes 5—9: Pharmaceutical legislation; Notice to Applicants; Guidelines; Maximum residue limits; Pharmacovigilance; which are the part of the EudraLex Collection. Guidelines from Volume 7 are subsequently harmonized by VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) in a range of rules in Europe, USA and Japan.

1. FISH – TEST ORGANISM

Fish used as test animals gain at present ground within the environmental monitoring as the indicator species and as the test organism in toxicological tests for chemicals and wastes. The precise methodology is designed in detail for acute and chronic toxicity tests according to OECD guidelines. These methodologies should be applied in the environmental risk assessment for veterinary medicinal products. The object of this article is the setting of specific rules for safety studies, efficacy testing, tolerance studies and studies of residues of medicinal products in fish which have to be carried out.

Certain particularities of fish as a test organism have to be taken into account. The carrying out of studies of medicinal products for fish is accompanied by a high importance factor, which is the environment quality of these organisms, e.g. water quality that can affect the results of a study in this case. The test organism fish should be taken as a shoal animal. The measurement and examination of a few individuals could be representative for the whole shoal, on the assumption that all fish are kept under the same conditions. But this means in practice that it is not suitable or possible in many cases to take samples frequently and to handle the animals often, because this could totally adversely affect the state of health of the fish organism. Despite this, samples for residue studies must be taken from several subjects of the same group (shoal) per required time point.

When injections or oral administration are used, the control group of fish has to be administered the same product deprived of the active substance and the same volume of this product in the same manner. With regard to negative effects on target species, the number and range of handling with fish have to be individually assessed within the study report and the investigator must justify and correctly interpret these circumstances.

Veterinary medicinal products intended for fish (including their reproductive stages and reproduction products, with the exception ornamental fish) have to satisfy all the legislative requirements for approval, that include the demonstration of safety for the consumer, user or operator and environment, and demonstration of efficacy, tolerance (tolerance for target species – fish) and quality.

The proposed approach for medicinal product testing should be considered in relation to every specific case. If some of the items are modified or omitted, justification has to be provided.

2. PRECLINICAL TRIALS

2.1. Tolerance study (tolerance for target species) has to be performed on the main target species. Tests performed in one species of fish are considered relevant for the evaluation of tolerance in another species of fish of the same genus (or taxonomic family). Extensive testing of tolerance is required for substances with a new molecular structure or substances not previously approved for fish or their reproductive and resting stages. Only limited investigation would be possible for substances, which are toxicologically well characterised in relevant fish species.

The test animals have to be healthy. A control group of fish is always required. The treated and control group are handled identically except for the exposure to the test medicinal product. If the tests of medicinal product are carried out with natural infection, it may be necessary to perform a tolerance evaluation in the diseased fish in reasonable cases.

The same approach concerns the testing of recipients used in medicines for fish as well. Based on serious literature data and preliminary data this testing could be omitted.

Tolerance assessment for target species is based on acute toxicity (single dose toxicity) and sub-chronic and chronic toxicity (repeated dose toxicity).

2.1.1. A quantitative evaluation of the approximate lethal dose and information on the dose-effect relationship has to be obtained **within acute studies of single dose toxicity** for test medicinal product or recipients. Acute toxicity tests generally include clinical observation and a necroscopy examination of all individuals, if possible. Acute test methodology follows the principles in OECD guideline 203: *"Fish, Acute Toxicity Test"*, the necessary variations have to be documented and justified in a study plan. The route of administration has to correspond with the intended one for final product. Administration of medicinal product for fish is possible in three ways:

1. Waterborne administration (bathing, dipping and top dressing) – control group has to have the same concentration administered to recipients as in the test medicinal product and in the same manner.

2. Oral administration (by gavage or mixed in feed) – the control group is administered the test product deprived of the active substance in this case. The maximum dose should not exceeded 2000 $mg.kg^{-1}$ fish. The volume given by gavage should not exceeded 0.5 ml test solution per 100 g fish for the required dose.

3. Injections (intraperitoneal or intramuscular) – control group has to have the same volume of test solution administered deprived of the active substance in the same manner.

The medicinal product or formulations used in the test have to be assayed for the concentration of the active substance before the start of trials! At least three dose levels have to be tested, the highest dose ensuring a high degree of serious toxic effects. The investigator must justify the choice of dose levels and duration of exposure. The test fish used in one study have to be fish of the relevant size, age and physiological status and breed for which the product is intended. Group size is minimally ten fish per group. Minimally two

tanks per dose and two control tanks must by stocked for each study, the allocation of fish in groups has to be done randomly. The fish to be tested must be acclimatized for two weeks in experimental tanks and be in good health.

If the lethal dose is above 2000 $mg.kg^{-1}$, further trials must not be performed. Finally post mortem examination has to be carried on all fish out.

All experimental techniques must be described in such detail as to allow them to be reproduced.

Further guidance compares with OECD guideline 204: "*Fish, Prolong Toxicity Test,* 14-Day Study".

2.1.2 Studies of repeated dose toxicity are relevant only for products intended for repeated administration.

The selection of test organism is the same as the selection in the acute test of single dose toxicity. The test animals used in one study have to be fish of the relevant size, age and physiological status and breed for which the product is intended. Group size is minimally ten fish per group. Minimally two tanks per dose and two control tanks must by stocked for each study, the allocation of fish in groups has to be done randomly. The fish to be tested must be acclimatized for two weeks in experimental tanks and be in good health.

Methods of administration agree with the principles of administration in acute tests of single dose toxicity. During oral administration detail records on feed uptake and concomitant daily dose of medicinal product have to be given. The controls are administrated the product deprived of the active substance in the same manner as the test fish.

Having regards to the recommended therapeutic dosage scheme, the duration of exposure has to be justified by the investigator.

Within whole test water quality parameters (temperature, salinity, O_2, NH_3/NH_4^+, hardness (?Ca a Mg), pH and flow) and clinical observations (behaviour of fish, appetite, sings of adverse effects and mortality) must be continuously monitored and recorded. A necropsy examination of all fish that die has to be performed, as well as histopathological examination of selected organs, and examination of injection site after injection, if necessary. Possibly also surviving fish should be examined at the end of the test.

2.1.3 Tolerance evaluation can be possible to derive from the documentation of toxicological and clinical studies. Determination of a **therapeutic index** is the purpose of this evaluation. The therapeutic index states margins between the maximum proposed therapeutic dose and the dose causing adverse effects. It may be sufficient to indicate a minimum or approximate value for this factor. For a product intended for repeated administration, the maximum proposed duration of use has to be considered. The nature and frequency of **adverse drug reactions** are to be stated on the basis of intensive monitoring during clinical trials. Observation of interactions applies to medicinal products likely to be used at the same time.

2.2. Evaluation of pharmacological effect

Pharmacological data concerning the product have to be compiled before evaluation medicinal product, any parameters must be stated in some cases. The product characteristics, such as chemical structure, molecular weight, pK_a and solubility must be exactly defined.

Studies in target species are required for the assessment of the pharmacological effect. Studies performed in one species of fish are considered relevant for the evaluation of pharmacological effects in another species of fish of the same taxonomic family (same genus). All studies must be performed under relevant conditions for the proposed use of the product.

In terms of **pharmacodynamics**, the pharmacodynamic effects of an active substance or substances, which form the basis for the recommended use of the product, have to be known. Desirable effects as well as adverse effects are to be reported. ED_{50} value should be provided if possible (effective dose required to produce a positive pharmacological effect in 50% of organisms).

Observation of **pharmacokinetic studies** in fish is very complicated. Assessment of the time course of the concentrations of the active substance and its metabolites in body fluids, tissues and excreta is possible only in different fish from one experimental group (shoal). Samples from randomly selected fish (ten is recommended, four minimum), which are required for analyses, include body fluids (blood plasma), intestinal content and muscle, liver eventually (hepato-pancreas in carp species of fish). If relevant, pharmacokinetic studies should be carried out at different water temperatures.

Performance of tests. For substances intended for single administration, single dose studies are sufficient. If the product is intended for repeated administration, data have to be given on the achievement and maintenance of a steady state or on a possible accumulation.

The objective of the evaluation pharmacological effects is the assessment of the time course of the concentration of the active substance in blood plasma and eventually in target tissues, where pharmacological or toxicological effects are obtained. Both **the rate and extent of absorption** should be described. The absolute bioavailability or relative bioavialability of the medicinal product must be stated for products (oral and injections) when their efficacy is dependent on tissues or plasma concentration. In terms of distribution, any **retention of the active substance or its metabolites** are to be found. **The metabolism or biotransformation** of the active substance has to be documented, if possible. The main **route of excretion** of the active substance and its principle metabolites must be stated.

2.3. Microbiology and parasitology. Proof of efficacy *in vitro* has to be given, if possible. For bacteriostatic and bactericid antibacterials MIC (minimal inhibitory concentration) or MBC (minimal bactericidal concentration) to be established and verified, it is recommended to establish the MIC/MBC ratio. Determination of values is conducted for at least ten pertinent strains of each pathogen. In the case of a pathogen of which several serotypes exist, the strains must represent the different serotypes. For antiparasitic agents, the approximate lethal concentration for the target parasites has to be indicated. Based on MIC values the estimated therapeutic concentration of the active substance in blood or serum and, if relevant, in target tissues are stated.

3. CLINICAL TRIALS

The main purpose of efficacy data is to prove the therapeutic value of a new medicinal product for fish and to define the optimal dosage and dosage scheme. Clinical trials are demanded for each proposed indication and for a group of the target species. The trials include control groups. Studies require performance under experimental conditions and full scale field trials. The studies have to be performed in accordance with the rules of GCP (good clinical practice) published in the note for guidance: Good Clinical Practice for the Conduct of Clinical Trials on Veterinary Medicinal Products in the European Union (in the Czech Republic Decree No. 472/2000 Coll., on good clinical practice and more detailed conditions for clinical trials on pharmaceuticals as amended.). Adverse effects, side effects and tolerance of target organisms have to be monitored during the course of the study.

3.1. Performance of trials. All studies must be performed under relevant conditions for the proposed use of the medicinal product. Positive and negative control groups are used in addition to the test groups. Control fish have to receive the same treatment with the placebo in the same manner as the product used on the test fish.

3.2. Dose determination and dose confirmation trials. The purpose of the trials is to determine the optimum dose, dosage interval and total period of treatment for the stated indications. The trials are performed as a combination of experimental studies and field trials. Data received from field trials are considered as giving the most valuable information. The need for differentiated dosage regimens studies exists if differences in pharmacokinetics or efficacy dependent on age, size or physiological status of fish or on water quality parameters occur.

3.2.1 Experimental trials have to be supported by controlled and standardised study conditions. Experimental trials are performed for the main target species – fish must be of similar age, size and origin, be susceptible to the tested disease and be in good health (examination of ten fish selected from shoal before trial). For the replication of all trials, the number and size of groups must be sufficient to give statistically significant data useful for statistical analysis. The test animals should not have previously been exposed to the challenge organism. The challenge organism must be of the strain relevant for the current disease situation and be isolated and characterised by the most appropriate methods (preferably a standard methods), which are described in detail. The results of the introduction of the challenge organism in different groups have to be reported and based on parasite counting, microbiological analyses or other acceptable investigation.

3.2.2 Field trials have to ensure that the medicinal product is efficacious in the various conditions for aquaculture in the Czech Republic. A minimum of three to five farms is selected for clinical trials, farms have to be geographically distant to optimise the possibility of heterogeneous environmental conditions. Each farm has to have several similar pens or tanks and sufficient number of fish of the relevant species, age, size, origin and state of health. The farmer has to be experienced in keeping detailed records (source of fish, disease history, previous medication, use of chemicals and vaccination, outbreaks of diseases, mortality and treatment, feeding, hygiene, parameters of water quality – temperature, oxygen, pH). All fish in one tank are considered as one group. A minimum of two groups must be used in each trial, one of these as a control group. The control group is in most cases a positive control group, in which there is a prevalence of disease which is not treated. Negative control groups are not demanded for contagious disease tests.

Field trials in commercial fish farms should preferably be performed under spontaneous outbreaks of diseases (a successful natural challenge) for which efficacy is applied. And identification of the causal agent must be performed. Field trials with anaesthetics or other non-therapeutics (for example for preventive treatment) are performed with healthy fish. Challenge studies in field conditions are accepted if the investigator justifies it.

The criteria for establishing the diagnosis must be given, these criteria are to be used in all trials and they include autopsy with histopathological examination of at least six individuals from each group. Bacterial diseases have to be diagnosed by isolating the agent from samples from at least six fish and characterising the pathogen by the most appropriate microbiological method.

The recommended therapeutic dose for treatment is presented based on dose-response relationship study, kinetic data and results from experimental clinical trials. The criteria used for the evaluation of efficacy of test product must be given. Concomitant therapy during trials has to be reported and discussed. The explanation of non-specific mortality and comments on any physiological abnormalities and on any behavioural changes and on the development of resistance as well. All field trials must be described in detail according to protocols, which are same for all fish farms included in trials.

4. RESIDUES STUDIES

The target tissues in fish for residues determination are muscle, including skin in natural proportion and liver, in particular cases (or hepato-pancreas in carp species).

Study contains a one to two week period of acclimatisation, a period of administration test medicinal product and period of sampling for the determination of depletion of residues. The samples are taken during the time at least twenty days, if the investigator does not justify another time period of sampling. The sample is always taken in ten fish from both test and control groups.

The requirements set out for residue study are same as the requirements on test animals, environment and handling with test fish for preclinical and clinical trials. The determination of the depletion of residues during clinical trial could be acceptable if sufficient number of individuals are involved in this trial and this number enables the required sampling.

The withdrawal period is established on the basis of depletion study for relevant residues. The units stated for fish

are the degree-days. One degree-day is presented by an average temperature 1 °C for one day (24 hours). If the withdrawal period cannot be established, the specific withdrawal period of 500 degree-days is used.

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RESULTS OF SPECIFIC PROPHYLAXIS OF SWINE PARVOVIRUS DISEASE BY PORCIVAC PARVO-ERY inj. a.u.v. VACCINATION

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ABSTRACT

Porcine parvovirus is ubiquitous, occurs currently round the world and also in the majority of herds in Slovak territory. In a new reproductive pig house at the School Agriculture Enterprise Zemplínska Teplica of UVM Košice some sows were immunized before assigning them to breeding. All categories of pigs housed in this building were examined with the exception of suckling and weaned piglets. Samples of blood were taken from young and older sows and boars and blood serum was tested by the haemagglutination-inhibition test (HIT) determining the titre of haemagglutination-inhibition antibodies (HI-Ab). Twelve sows, six to seven months old, and eight older sows, all before mating, were vaccinated against swine parvovirus disease using an inactivated bivalent adsorbent vaccine against parvovirosis and erysipelas of swine PORCIVAC – Parvo Ery inj. a.u.v. (MEVAK, Nitra, the Slovak Republic). Sows (young and adult) were vaccinated intramuscularly (i.m.) using a vaccine dose of 2 cm^3 or subcutaneously (s.c.) also with dose 2 cm^3. On days 14, 28 and 45 post-vaccination (or on day of re-vaccination and 14 and 28 days later) blood samples were examined by HIT for presence of HI-Ab. The PORCIVAC – Parvo Ery inj. a.u.v. vaccine is sufficiently effective as 1:256 titre of HI-Ab is considered protective and was detected in all vaccinated animals. The group of vaccinated young and adult sows with initial HI-antibody titre lower than 1:128 showed a marked increase in antibody production.

Key words: immunoprophylaxis; parvovirus disease; swine

INTRODUCTION

Reproductive disorders, embryonal mortality, decreased viability and death of piglets constitute at the present the most serious economical, health and breeding problems in swine rearing.

Many infectious and non-infectious diseases contribute to fertility disorders in sows; among them those of bacterial and viral ethiology are particularly important. Viral diseases associated with reproductive disorders of sows include porcine reproductive and respiratory syndrom (PRRS), classical swine fever, Aujeszky disease and last, but not least, parvovirus disease of swine. The parvovirus disease of swine is a contagious disease of domestic swine or other Suidae. Neither man nor any animal species are susceptible to this disease (15). Porcine parvovirus was isolated first by Cartwright and Huck in England (3, 4).

The causative agent of parvovirus disease of swine is *Porcine parvovirus (PPV)* belonging to the family Parvoviridae, subfamily Parvovirinae, genus *Parvovirus*. Porcine parvovirus is ubiquitous, occurs currently round the world and also in the majority of herds in the Slovak territory. The disease is associated with reproductive disorders of sows, most frequently with embryonal and foetal mortality. It develops particularly in serum-negative sows infected by the virus mostly in the oronasally up to day 70 of gravidity (9). The main source of

infection are infected animals. In the viraemic period the agent is eliminated practically in all excretions and secretions (11). The virus is eliminated for a relatively short time, approximately two weeks (8). An important role in the dissemination of this disease belongs to boars as they spread the virus mechanically to sows at oestrus. In addition to the above mentioned routes of transfer the spread through transfer in early embryos is rather important (16, 17). The infection acquires an enzootic character in the majority of herds and most of the sows in the infested herds are immune (14).

With regard to the high economic losses caused by this disease one of the effective preventive methods is immunoprophylaxis – vaccination of sows and boars before assigning them to breeding (5). Sows are vaccinated usually two to eight weeks before mating. Preventive vaccination induces sufficient immunity before first mating of young sows (2).

MATERIAL AND METHODS

In 2003 epizootiological screening was carried out in a new reproductive pig house at the School Agriculture Enterprise Zemplínska Teplica (SAE-ZT) focused on the occurrence of parvovirus disease of swine. Almost 70 % of all sows of entire SAE-ZT were housed in the investigated house. All categories of pigs housed in this building were examined with the exception of suckling and weaned piglets. Samples of blood were taken from young and older sows and boars and blood serum was tested by the haemagglutination-inhibition test (HIT) determining the titre of haemagglutination-inhibition antibodies (HI-Ab).

Twelve sows, six to seven months old, and eight older sows, all before mating, were vaccinated against swine parvovirus disease using a vaccine PORCIVAC – Parvo Ery inj. a.u.v. (producent: MEVAK a.s., Nitra, Slovak Republic). This is an inactivated bivalent adsorbent vaccine against parvovirosis and erysipelas of swine intended for vaccination of sows and boars to induce immunity and at the same time to protect the foetus throughout gravidity. Both categories of sows (young and adult) were divided into two groups. The first group was vaccinated intramuscularly (i.m.) using a vaccine dose of 2 cm^3, while the second was vaccinated subcutaneously (s.c.) and the dose administered was also 2 cm^3. Revaccination was performed on the fourteenth day after prime vaccination. Before vaccination, blood samples were taken from all animals and the titre or level of haemagglutination-inhibition antibodies was determined in the respective blood sera.

On days 14, 28 and 45 post vaccination (or on day of revaccination and 14 and 28 days later) blood samples were withdrawn from all vaccinated animals. Blood sera were obtained and examined by the HIT for presence of HI-Ab. During 21 days post-vaccination animals of both categories were observed for their overall health and occurrence of potential local reactions at the site of vaccination.

RESULTS

Of the 128 pigs examined in the new breeding house on SAE-ZT sera of 75 sows and 2 boars, i.e. 58.6 %, had HI-Ab titres higher than 1:256 (Tables 1, 2). Therefore, on the basis of results obtained by detection of HI-Ab, this breeding herd of pigs belongs among those with a medium risk of development of parvovirus disease (as medium risk herds are considered those in which 40—80 % of the basic stock is resistant to PPV infection, with HI-Ab titre exceeding 1:256) (2).

Table 1. Results of detection of HI-antibodies before prime vaccination to PPV in young sows on SAE-ZT, new breeding house, February 2003

Category	Number of animals	Titre of HI-Ab to PPV	
Young sows	12	2 sows	negative
		3 sows	1:8
		1 sows	1:16
		2 sows	1:32
		3 sows	1:64
		1 sow	1:128

Table 2. Results of detection of HI-Ab before prime vaccination to PPV in adult sows and boars on SAE-ZT, new breeding house, February 2003

Category	Number of animals	Titre of HI-Ab to PPV	
Sows	114	22 sows	1:64
		19 sows	1:128
		11 sows	1:256
		13 sows	1:512
		10 sows	1:1024
		23 sows	1:2048
		14 sows	1:4096
		2 sows	1:8192
Boars	2	1 boar	1:512
		1 boar	1:1024

Results obtained in twelve young and eight adult sows proved the effectiveness of the inactivated vaccine PORCIVAC – Parvo Ery inj. a.u.v. that in serum negative sows vaccinated either i.m. or s.c. induced production of antibodies to PPV on the level of titres 1:256–1:2048 (Tables 3, 4) after revaccination. The young sows that were serum-positive before assigning them to the experiment showed an increase in antibody titres following the vaccination using both methods. The antibody titre in serum-negative sows, vaccinated either i.m. or s.c., ranged between of 1:256 and 1:2048. Sows with a high titre of antibodies before vaccination (1:4096; 1:2048) exhibited a decrease in HI-Ab titre post vaccination or revaccination.

In the period of 21 days after vaccination neither local reactions at the site of vaccination nor changes in overall health of animals were observed. The method of administration of vaccines had no effect on production or HI-Ab titres in vaccinated or re-vaccinated sows and boars.

DISCUSSION AND CONCLUSION

Parvovirus infection is a serious disease of pigs not only from the health point of view, but also from the economic. Fertility disorders in sows and piglet mortality immediately after birth are still an important breeding problem (5). Inactivated vaccines are used for vaccination against parvovirus disease of swine at present in most countries. Most producers recommend the application of two vaccine doses at intervals of 14 to 21 days, so that the vaccination has to be completed fourteen days before mating (2).

In order to determine the risk to the herd it is necessary to detect haemagglutination-inhibition antibodies in 10 to 20 % of animals including all age and reproduction categories (2); the resulting detection is based on the haemagglutination-inhibition test (13). The titre 1:256 is considered protective with regard to HI-Ab as it imparts sufficient protection against infection to young and adult sows before mating and to foetuses (6, 10).

Vaccination of young sows with PORCIVAC vaccine induced production of HI-Ab to PPV; after prime vaccination the HI-Ab titre ranged between 1:32 and 1:256; after re-vaccination HI-antibodies varied between 1:256—1:1024 and 45 days later between 1:512 and 1:2048. Vaccination of serum-negative adult sows induced HI-Ab titres 1:256—1:2048 and vaccination of serum-positive sows resulted in an increase in antibody titres which, however, remained no lower than 1:512. The results on HI-Ab titres obtained in our study are comparable with the results of other authors (2, 5).

The epizootiological screening of swine parvovirus disease on SAE-ZT in the new breeding house which houses almost 70 % of the entire stock of sows indicates a high contamination of animals in the herd as higher than 1:256 HI-Ab titre was detected in 58.6 % of the examined sows and boars.

The PORCIVAC – Parvo Ery inj. a.u.v. is sufficiently effective as 1:256 titre of HI-Ab is considered protec-

Table 3. Haemagglutination-inhibition antibody titres in young vaccinated sows

Ear tag No.	Titre before vaccination	Way of administration	Titre day 14 post vaccination (re-vaccination)	Titre day 28 post vaccination	Titre day 45 post vaccination
788	1:64	2 cm^3/i.m.	1:256	1:1024	1:2048
789	1:8	2 cm^3/i.m.	1:128	1:512	1:1024
790	1:32	2 cm^3/i.m.	1:256	1:1024	1:2048
791	1:8	2 cm^3/i.m.	1:128	1:256	1:512
792	0	2 cm^3/i.m.	1:128	1:512	1:1024
793	1:128	2 cm^3/i.m.	1:256	1:1024	1:2048
794	1:8	2 cm^3/s.c.	1:64	1:512	1:1024
795	1:64	2 cm^3/s.c.	1:256	1:1024	1:2048
796	0	2 cm^3/s.c.	1:128	1:512	1:512
797	1:32	2 cm^3/s.c.	1:64	1:512	1:1024
798	1:16	2 cm^3/s.c.	1:128	1:1024	1:2048
799	1:64	2 cm^3/s.c.	1:32	1:256	1:1024

Table 4. Haemagglutination-inhibition antibody titres in adult vaccinated sows

No.	Ear tag vaccination	Titre before administration	Way of	Titre day 14 post vaccination (re-vaccination)	Titre day 28 post vaccination	Titre day 45 post vaccination
022	1:4096	2 cm^3/i.m.		1:2048	1:1024	1:1024
743	1:64	2 cm^3/i.m.		1:256	1:512	1:1024
118	1:64	2 cm^3/i.m.		1:512	1:1024	1:2048
827	1:2048	2 cm^3/i.m.		1:1024	1:512	1:512
105	1:128	2 cm^3/s.c.		1:512	1:1024	1:2048
118	1:64	2 cm^3/s.c.		1:256	1:1024	1:2048
023	1:512	2 cm^3/s.c.		1:1024	1:2048	1:2048
720	1:4096	2 cm^3/s.c.		1:2048	1:512	1:1024

tive (6, 10) and was detected in all vaccinated animals. The group of vaccinated young and adult sows with HI-antibody titre lower than 1:128 showed a marked increase in antibody production. The antibody titre detected in sera on day 28 after prime vaccination was higher than 1:256 (protective titre). The sows with high antibody titre before vaccination (1:4096, 1:2048) showed a decrease in HI-Ab titres after vaccination or re-vaccination. The method of administration had no effect on the production of HI-Ab. The most suitable way of verification of vaccine effectiveness is to vaccinate serologically and virologically negative young or adult sows (1, 7). With regard to the high level of infection in pig herds the selection of individuals for this testing is very demanding.

When considering the need for vaccination, it is necessary to determine the infection risk in the herd based on serological examination – detection of HI-Ab (2). In herds with a high risk of disease development vaccination against PPV should include all animals.

O l b e r t z (12) recommends observing all preventive measures against the introduction of infection in herds with the largest risk of an outbreak of the clinical form of swine parvovirus disease; to examine time all newly-bought sows, young sows and boars serologically in quarantine and to include in breeding only negative animals. In herds with a medium risk of disease development it is recommended to vaccinate individually while in those with minimum risk no vaccination is considered necessary. In herds with a medium risk of disease development 40 to 80 % of sows of the basic herd are protected against infection. In these herds it is difficult to decide about the performance of vaccination. It deals with swine with high antibody titres and swine which are insufficiently protected against infection.

Following the serological examination of sows and boards it is possible perform vaccination. The advantage of this procedure is that animals may be vaccinated individually and a high titre of post-vaccination antibodies may be achieved at the same time. Higher financial charges for serological examinations and for vaccination are the disadvantage of this method. It is essential to eliminate all possible ways of spreading the infection into a herd.

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IMMUNOREACTIVITY OF RABBITS TO BLV INFECTION

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ABSTRACT

Immune response of rabbits intravenously infected with bovine leukaemia virus (BLV) was observed during 82 weeks. BLV seroconversion of 8 experimental animals examined by AGID assay was observed from 2 to 42 weeks. The examination of lymphocytes showed positivity of BLV provirus in DNA by PCR. Despite BLV antibodies and proving of BLV provirus, complete virus particles were not found out in cultivated lymphocytes with mitogen by transmission electron microscopy. The examinations of leukocytes have shown no significant and lymphocytes significant decrease value of experimental animals from 54 weeks. The evaluation of lymphocyte subsets CD4, CD5, CD11c, CD25 and IgM in the peripheral blood demonstrated tendency of IgM positive cells decreasing by flow cytometry in the experimental rabbits. At the end of experiment, examination of the same lymphocyte subpopulations in the spleen of BLV infected rabbits shown increase, except of CD11c, with significance on IgM and CD25 positive cells (P > 0.05). Immunohistochemical staining of frozen spleen samples demonstrated more densely population of IgM, CD4, and CD5 with larger zone of white pulp around periarteriolar lymphoid sheats in BLV infected rabbits in comparison to control animals.

Key words: BLV; immunity; PCR; rabbit; subpopulations

INTRODUCTION

The bovine laeukaemia virus (BLV) is a causative agent of enzootic bovine leucosis. Several species of animals including cattle, sheep, capybara, water buffalo, goats, pigs and rabbits can be infected with BLV (2). The experimental infection in rabbits with BLV has been successful but no tumours developed during a long-term observation period of over one-year (11) in spite of provirus detection in the lymphocytes (6). The observation of lymphocytes from BLV infected cows demonstrated changes in lymphocyte subpopulation (4). In rabbits there is no similar report. The aim of this work was to study immunocompetent cells in the peripheral blood and in the spleen of BLV infected rabbits.

MATERIAL AND METHODS

Animals. Eight experimental and six control four-month-old New Zealand white rabbits of both sexes with an average weight 2.5 ± 0.3 kg were included in the experiment. Rabbits of a conventional outbreed strain came from a private BLV-free colony. They were housed separately in standard rabbit cages without bedding at room temperature, a relative humidity of 60 %. Rabbits were fed with commercial rabbit diet (Kendice, CZ) and offered fresh water *ad libitum*.

Experimental design. Commercial culture of foetal lamb kidney cells (FLKC) persistently infected with BLV (Mevak, Nitra, SR) was used for *intravenous* infection (*vena auricularis marginalis*) of experimental rabbits (3.10^3 in 0.5 ml PBS/

per animal). Peripheral blood was taken 2 weeks after infection and then monthly during 18 month (82 weeks). Isolated peripheral blood lymphocytes for TEM and PCR assays were used on 78 week of the experiment. At the end of the experiment rabbits were killed (ether inhalation and dislocation) and spleen was taken into PBS for immunohistochemical examination.

Blood collection. Blood for flow cytometry was withdrawn from the marginal ear vein into 1.5 % EDTA and for finding BLV seropositivity by AGIDT in tubes without anticoagulant solution.

White blood cell count. The total number of leukocytes in the peripheral blood was made by routine laboratory method using a Türk solution and Bürker chamber. Differential cell counts were made on blood smears after Haemacolor staining (Merck, Germany) by counting 100 cells per side.

AGIDT. BLV infection was diagnosed by a commercially available agar-gel immunodiffusion test for the detection of serum antibodies against BLV glycoprotein 51 (Agebion®, Mevak, Nitra, SK)

Flow cytometry. Indirect immunofluorescent method in the whole peripheral blood and isolated lymphocytes from spleen for measuring by FACScan (Becton Dickinson, Germany) was used. Mouse anti-rabbit primary monoclonal antibodies (Serotec, GB) in dilution 1:25 summarised Table 1. Goat anti-mouse FITC conjugated immunoglobulin (F 0479, Dakopatts, GB) in dilution 1:50 was used as secondary antibody.

Table 1. Mouse anti-rabbit primary monoclonal antibodies using for flow cytometry

MoAbs	Specificity	Clone	Isotype
CD4	MCA799	KEN-4	IgG2a
CD5	MCA800	KEN-5	IgG1
CD11c	MCA1144	3/22	IgG1
CD25	MCA1119	kei-a1	IgG2b
IgM	MCA812	NRBM	IgG1

Evaluation of flow cytometry. Cell Quest programme was used for acquisition and analysis of lymphocytes and monocytes by collection 10 000 cells in gate. Relative percentages were computed on absolute counts in the peripheral blood.

Immunohistochemistry. The spleens were collected into phosphate buffer saline (PBS; pH 7.6), frozen and cut in Cryocut E (Reichert, Germany) at –24 °C. Frozen 7 μm sections fixed in cold acetone and rinsed in PBS were incubated with primary monoclonal antibody 1 h. Biotin-streptavidin amplified peroxidase detection system (Biogenex, USA) was used to detect a positive reaction. All incubations were done at room temperature, and between the two consecutive 1 h incubations, the sections were rinsed three times with PBS; it was used instead of monoclonal antibodies as a negative control. The specific colour reaction was developed for 5 min with 3.5 mmol.l^{-1} 3,3´-diaminobenzidine (Sigma, Germany), and 30 ppm hydrogen peroxide in 200 mmol.l^{-1} Tris/HCl (pH 7.6). The sections were counterstain with haematoxylin and mounted into Entellan (Merck, Germany).

Transmission electron microscopy (TEM). Before the experiment finishing (at 78th week) peripheral blood lymphocytes were isolated by density sedimentation gradient on Telebrix N 300 (1.076 g.ml^{-1}; Léčiva, Praha, the Czech Republic) and cultivated days 3 in RPMI medium (Sigma, Germany) with PHA (5 ml.ml^{-1}). The cultivation was done in CO_2 thermostat (5 %). After centrifugation (250 *g*; centrifuge type MPW-340, Poland) the lymphocytes were immersed in a mixture of glutaraldehyde 2.5 % and paraformaldehyde 2 % in 0.1 mol cacodylate buffer (pH 7.2). Samples were postfixed in 1.0 % OsO_4, dehydrated in increasing concentration of ethanol, and embedded in Durcupan. Ultrathin sections were examined in an electron microscope (JEOL 1200 MX) with an accelerating voltage of 80 kV and 36 000 enlargement.

PCR assay. Two regions of the BLV proviral genome, located in *gag* and *pol* genes were chosen to be target of DNA amplification assay. Primers VZ1for (45 bp start from 462) and VP4rev (17 bp, start from 2589) were used. The amplified regions were 1919 bases long. Primers were synthesised by use of an automated DNA synthesiser. Thirty-five cycles were performed for each sample. The PCR products were subjected to electrophoresis in 2.5 % agarose gel, stained with ethidium bromide, photographed using a UV transilluminator. The isolated peripheral blood lymphocytes were used and PCR assay was done at the same time as TEM, at 78 week of the experiment.

Statistical evaluation. Unpaired Student's *t*-test, mean, standard error of mean (SEM) for value in the peripheral blood and standard deviation (SD) for spleen positive cells were used.

RESULTS

BLV infected rabbits converted to antibody-positive at 2nd—44th weeks by the AGID test. The antibody was non-continually detected during the whole experiment (82 weeks).

The number of leukocytes, lymphocytes, and their subpopulations were sequentially tested for experimental period. The total number of leukocytes was no significant lower from 54th week in BLV infected rabbits to the end of experiment (82 weeks) comparing to controls (Fig. 1). Absolute number of lymphocytes showed decrease in infected rabbits with significance in 58 and 69 weeks ($P > 0.05$) of the experiment (Fig. 2). Similarly, BLV infection have shown no significant lower value of IgM positive cells (B-lymphocytes) from 27 week post infection (Fig. 3). Absolute number of CD4 (Fig. 4) and CD5 T-cells (Fig. 5) was varied in two-tree sampling intervals during the experiment in infected rabbits. The value of CD11c and CD25 positive lymphocyte subsets showed decrease from 32 to 40 weeks of the experiment after BLV infection.

At the end of experiment (82 weeks), the flow cytometry evaluation of the spleen showed increase of CD4,

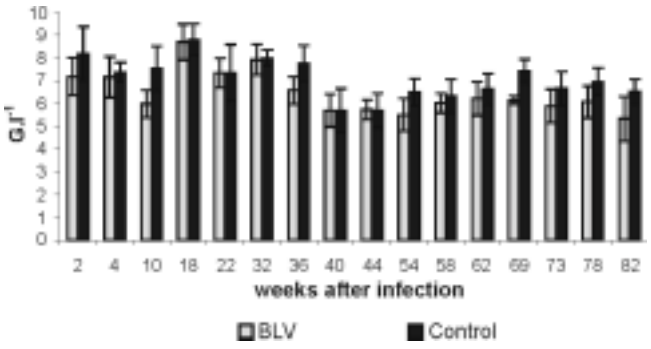

Fig. 1. Total count of leukocytes in the peripheral blood — G.l^{-1} (1.10^{9}.l^{-1}; mean ± SEM)

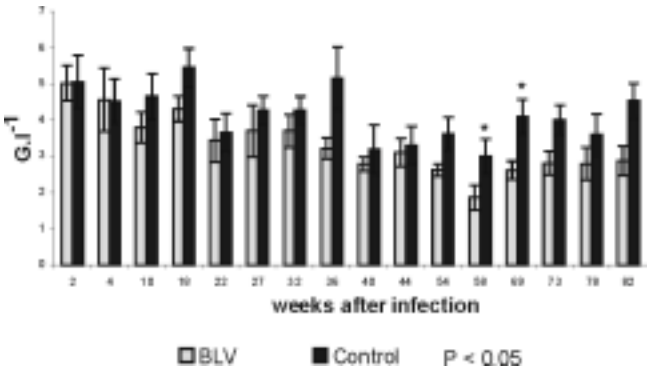

Fig. 2. Absolute number of lymphocytes in the peripheral blood — G.l^{-1} (1.10^{9}.l^{-1}; mean ± SEM); *P < 0.05 in control animals to BLV infected animals

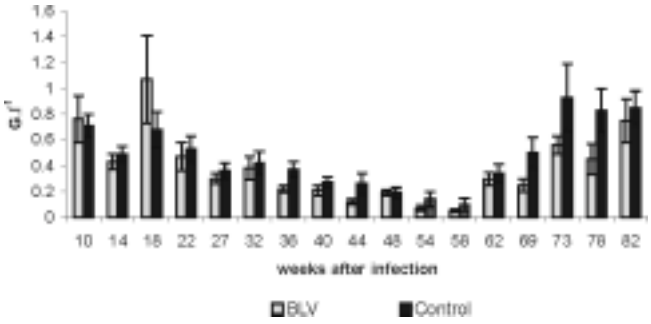

Fig. 3 Absolute number of IgM positive cells in the peripheral blood — G.l^{-1} (1.10^{9}.l^{-1}; mean ± SEM)

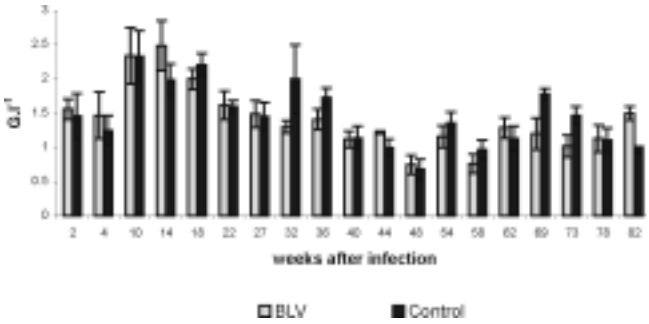

Fig. 4 Absolute number of CD4 positive cells in the peripheral blood — G.l^{-1} (1.10^{9}.l^{-1}; mean ± SEM)

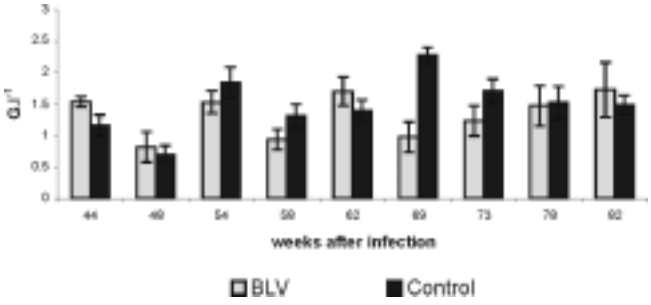

Fig. 5 Absolute number of CD5 positive cells in the peripheral blood — G.l^{-1} (1.10^{9}.l^{-1}; mean ± SEM)

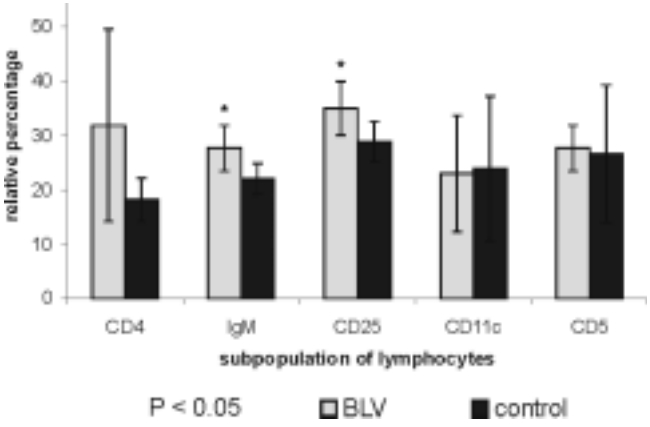

Fig. 6. Relative percentage of lymphocyte subpopulations in the spleen (mean; ± SD) at the end of experiment after necropsy (*P > 0.05 — higher value of BLV infected animal in comparison with controls)

CD5 relative percentage, with significance in IgM, and CD25 (P > 0.05) positive cells (Fig. 6).

Immunohistochemical staining of spleen by using CD4, CD5, and IgM monoclonal antibodies demonstrated larger zone with more densely population around periarteriolar lymphoid sheaths in BLV infected rabbits in comparison with control animals (Figs. 7, 8, and 9).

All BLV infected rabbits were BLV positive by PCR assay at 78 week of the experiment (Fig. 10).

Transmission electron microscopy examination did not prove BLV viral particles in lymphocytes after cultivation of these cells with mitogen at 78 week of the experiment.

DISCUSSION

Progression to neoplasia by bovine leukaemia virus (BLV) and related human T-cell leukemia viruses

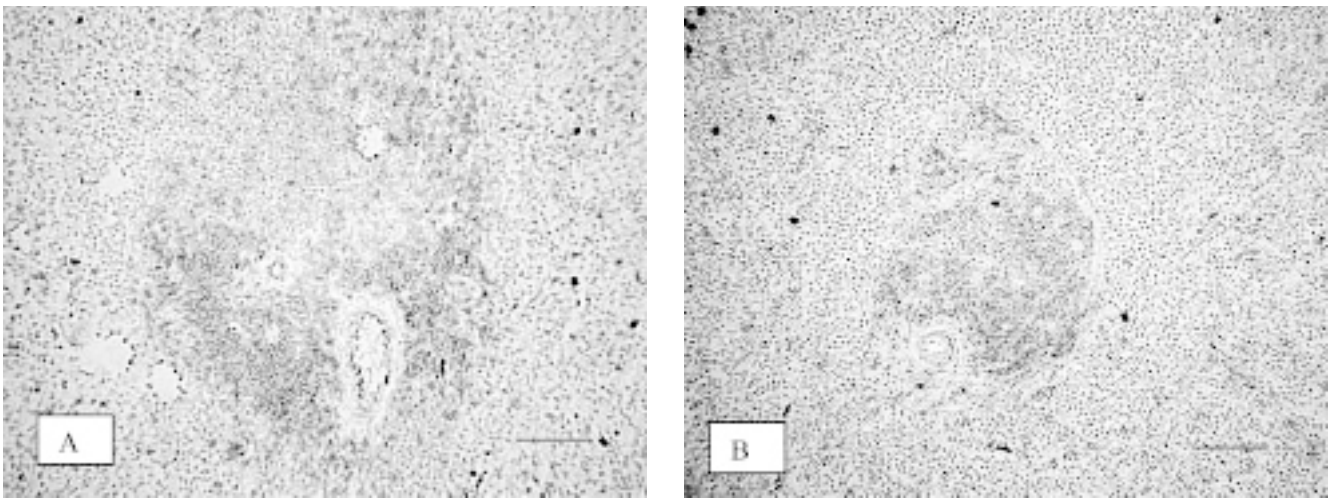

Fig. 7. Immunohistochemical staining of CD4 positive cells in the spleen (biotin-streptavidin amplified peroxidase detection system; bar 10 µm). A — control, B — BLV infection

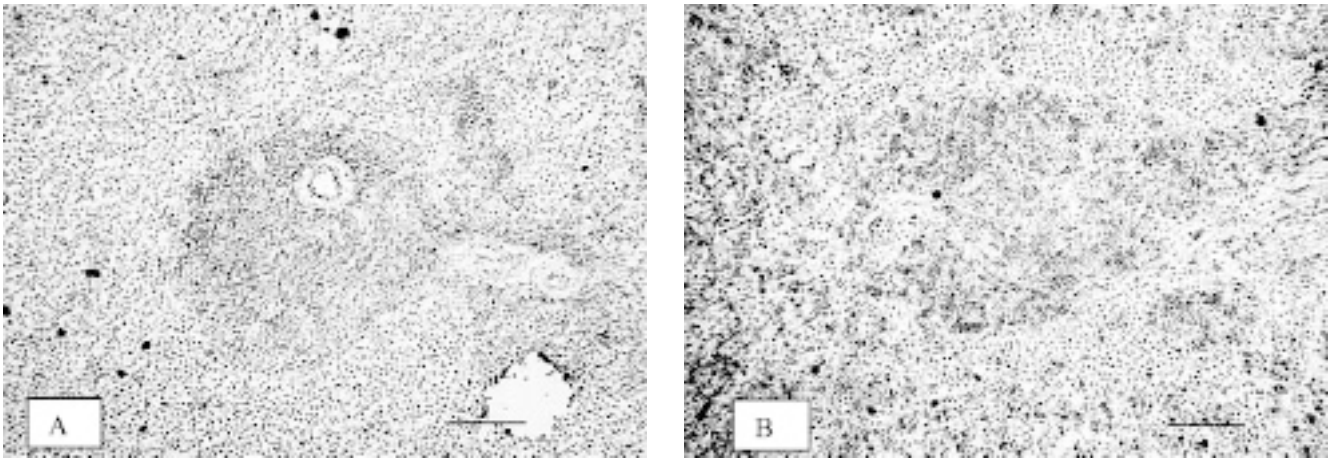

Fig. 8. Immunohistochemical staining of CD5 positive cells in the spleen (biotin-streptavidin amplified peroxidase detection system; bar 10 µm). A — control, B — BLV infection

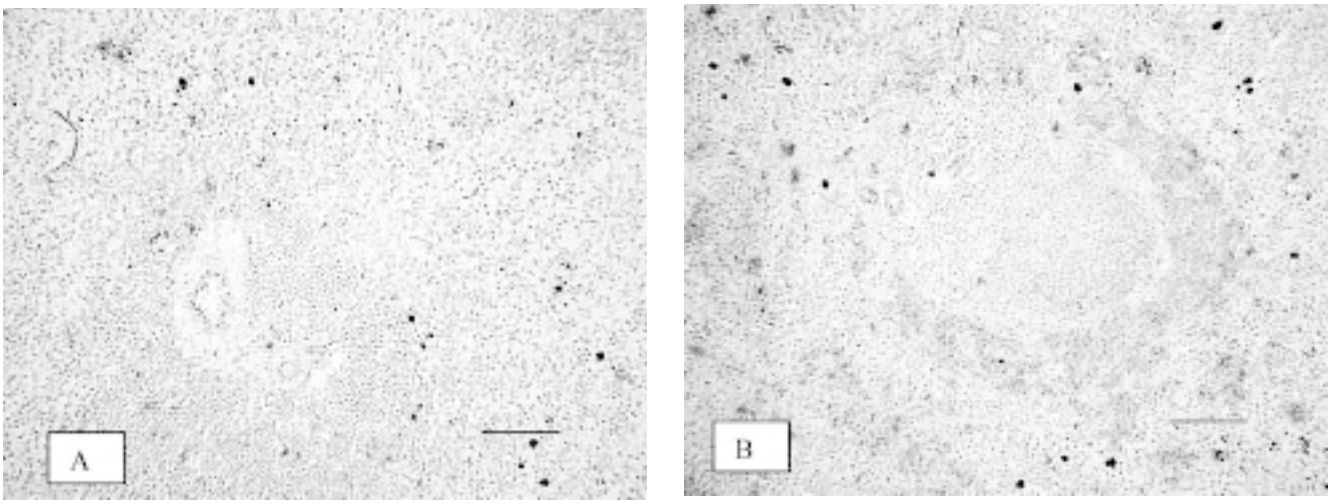

Fig. 9. Immunohistochemical staining of IgM positive cells in the spleen (biotin-streptavidin amplified peroxidase detection system; bar 10 µm). A — control, B — BLV infection

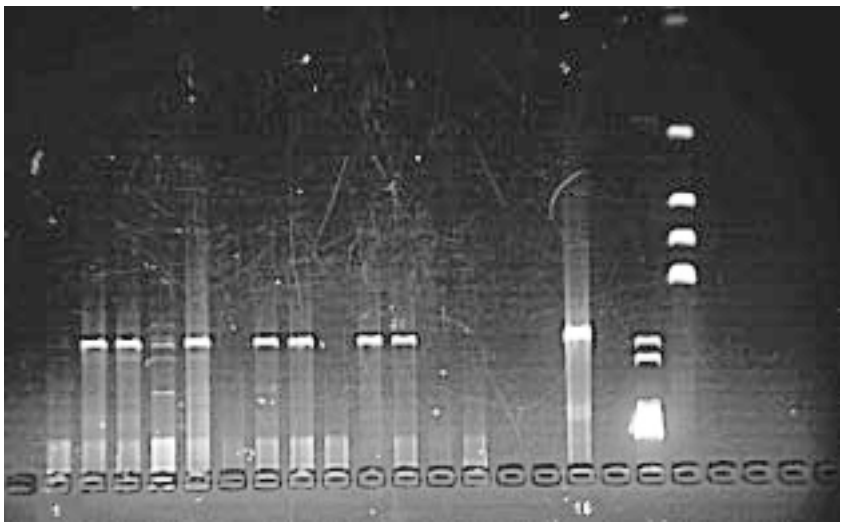

Fig. 10. Polymerase chain reaction (PCR) assay for BLV detection, using as target lymphocytes DNA from BLV infected rabbits. Lane 1 = negative control (without DNA), lanes 2, 3, 4, 5, 7, 8, 10, 11 = BLV positive rabbits, lanes 6, 9, 12, 13 = negative rabbits, lane 14 = negative cow, lane 16 = positive control (pBLV)

(HTLVs) is associated with long-term infection and indirect effects of virus-encoded oncoproteins on cell growth control (7, 9). BLV infection of rabbits by inoculation of cell-free BLV or cell-associated BLV typically causes an immunodeficiency-like syndrome and death by 1 year postinfection (8). Experimentally infected rabbits seroconvert to BLV shortly after inoculation, and antiviral antibody persist for life (1, 6).

In the present experiment, rabbits were *intravenously* inoculated with FLK (foetal lamb kidney) cells that were persistently infected with BLV. By 16 weeks after inoculation all eight experimental rabbits had converted to BLV-antibody positive. It was interesting that antibody response was not continually found for experimental period in all BLV infected rabbits. No animal died during 82 weeks of the experiment and no pathological changes were present by the autopsy, but some infected rabbits suffered from respiratory infection.

Examination of immunocompetent cells showed the decrease on peripheral white blood cells count and lymphocytes. Study of lymphocyte subsets demonstrated temporary tendency of IgM positive cells decreasing. There were found periodic alteration in two-tree sampling intervals of CD4 and CD5 T- lymphocyte subsets. It is known that rabbit CD5 antibodies also showed binding to the most B- lymphocytes, but anti-rabbit CD5 antibody (KEN-5) used in our experiment reacted with T-cells (5).

In BLV infected cattle, B/T ratio is modified concurrently to an increase of the total number of circulating lymphocytes (10). In our case BLV infected rabbits did not show dramatic changes in B- and T-cells.

The other monoclonal antibodies used in our experiment recognised the rabbit CD25 cell surface antigen, the interleukin-2 (IL-2) receptor á chain, and the rabbit CD11c cell surface antigen, also known as the $á^x$ integrin. Merely no significant decrease of these subpopulations was observed from 32—40 weeks of the experimental period after BLV infection.

IL-2 receptors are heterodimers of α (CD25), β (CD122), and γ (CD132) chains. A combination of β and γ chains binds IL-2 with low affinity, the presence of the α chain is required for high affinity binding. CD 25 is expressed on activated T-cells, B-cells, and monocytes. When it binds IL-2 it activates these cells (13). Pigheti and Sordillo (12) reported that IL-2 activity was altered in BLV positive cattle with persistent lymphocytosis. IL-2, a growth factor for T-lymphocytes, may be directly or indirectly responsible for excessive B-lymphocyte proliferation in this disease. Based on their previous research and that of human T-cell leukaemia virus, they believed that the progression of this disease was connected to altered IL-2/IL-2 receptor interactions.

CD11c functions in the adherence of neutrophiles and monocytes to stimulated endothelium and is important in the phagocytosis of complement C3b coated particles. CD11c has similar functions to CD11b/CD18 with which it cooperates. The major CD11/CD18 receptor is on tissue macrophages. CD11c/CD18 has been described to play important roles in cytotoxic T-cell killing, and on neutrophiles and monocyte adhesion to endothelium, although its ligands in these two cases have not been identified. Thus, leukocyte adhesion molecules including also CD11c are important in inflammation, systemic acute phase reaction, ischaemia perfusion injury and resistance against infections (3).

The findings in our experiment, significantly higher relative percentage of CD25 and IgM positive cells and intensive immunohistochemical reaction of these subpopulations in the spleen at the end of the experi-

ment indicated more proliferating signals then in the control animals.

In spite of antibodies production against BLV and proving of BLV genome in infected lymphocytes, the virus-like particles could not be found in cultivated lymphocytes. We supposed that there was expression of viral antigens on the surface of rabbit lymphocytes, mainly in the first weeks after infection, but there was no completisation of viral particles in cultivated lymphocytes at the end of experiment. O n u m a *et al.* (11) also have found differences between rabbits in their susceptibility to BLV infection.

In conclusion, long-term BLV infection of rabbits developed seroconversion. Genome of BLV in cultivated lymphocytes was proved in spite of the lack of detection of virus-like particles. The decrease of IgM positive cells and periodic alterations CD4 and CD5 T-cells in the peripheral blood during experimental period may tend to imbalance of the immune response because of interrupting some interactions and cause higher susceptibility to the other infections. More visible changes of immunocompentent cells observed in the spleen gives suggestion that long-term BLV infection causes higher proliferative activity in the secondary lymphoid organs. This maybe is one possibility to prevent from the development of tumour.

ACKNOWLEDGEMENT

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THE EFFECT OF FREEZING ON ULTRASTRUCTURE OF CHICKEN SKELETAL MUSCLES

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ABSTRACT

The effect of two methods of freezing on chicken skeletal muscle was investigated using transmission electron microscopy (TEM). Electron microscopy revealed various structural changes in frozen samples. We observed that cell morphology was preserved better by quick freezing than by slow one. IQF (individual quick-freezing) appears a very effective method of freezing. Comparisons between IQF frozen and control muscles showed no obvious differences. Cross section myofibrils and A and I bands in longitudinal sections were clearly visible. The structure of mitochondria and sarcoplasmic reticulum was changed. The samples frozen by the slow-rate method showed damage caused by ice crystals. Myofibrils lost their regular banding pattern. Cross striations disappeared, dark A bands and light I bands were not seen by TEM, but marked Z lines remained. Pronounced T bands were present. Myofilaments were interspersed with enlarged intracelular spaces. TEM demonstrated that IQF is a better method of cryoconservation of chicken meat than other conventional freezing methods.

Key words: effect of freezing; skeletal muscle; transmission electron microscopy

INTRODUCTION

Meat plays an important role in human nutrition. Meat is a highly perishable product and soon becomes unfit to eat because of microbial growth, chemical changes and breakdown by endogenous enzymes. These processes can be curtailed by simple forms of preservation, such as drying, smoking, salting, chilling and freezing. The way of meat preservation affects both the sensory properties and structure of meat tissue. Freezing is a method currently used to extend the shelf-life of meat. It is a process which lowers the inner temperature of products below the cryoscopic point with the aim to limit (but not completely to inhibit) microbial growth and reduce biochemical and physical processes during the storage (3, 9, 11, 12 15, 16). On the other hand, physical and chemical changes in proteins which occur during freezing may result in deterioration of meat texture (6).

The quality of meat is determined by a number of factors, for example perimortal treatment – before and after slaughter, thermal properties (end temperature to which the food is frozen, the length and temperature of the frozen storage) and the rate of transformation of water from the liquid state to crystalline one (4, 5, 8). When the temperature falls below the cryoscopic point "crystallization centres" surrounded by numerous ice crystals are formed. The more quickly the temperature falls the higher number of crystallisation centres appears and the smaller ice crystals are formed (9). When ice crystals develop in the freezing system, solute concentration in the

unfrozen medium increases. To minimise the concentration effect it is very important to ensure that the product passes through the zone of higher solute concentration as quickly as possible.

High ionic strength of solutions damages the food. Changing ratios of buffer components can lower the freezing point of a solution and cause changes in pH that can deteriorate protein stability. Freezing can promote off flavours due to rancidity of fats. All these factors affect the stability of molecules and can by reflected in morphological changes of tissue.

Modern technology recognises many methods of freezing. The aim of the present study was to investigate and compare the structural changes that occur in chicken skeletal muscles frozen by two different methods.

MATERIAL AND METHODS

The experiment was performed on chicken skeletal muscle (breast and thigh muscles). The samples were divided into three groups according to the methods of freezing. The control group (n = 3) — unfrozen samples came from Ústav hygieny a technológie mäsa, Košice. The 1st experimental group (n = 3) — air frozen samples obtained from HYDINA Z. K., a. s., Košice, and 2nd experimental group (n = 3) — immersion freezing known as IQF (individual quick-frozen meat) came from KOMES PLUS, s. r. o., Rozhanovce (10).

The samples intended for ultrastructural examinations were fixed in 3 % glutaraldehyde, post-fixed in 1 % OsO_4 (both in a phosphate buffer pH 7.2—7.4), dehydrated in acetone and embedded in Durcupan ACM. The ultrathin sections were cut on the ultramicrotome Tesla BS 490 (Brno, Czech Republic), stained with uranyl acetate and lead citrate and evaluated using a transmission electron microscope TESLA BS 500 (Brno, Czech Republic).

RESULTS

Characteristic structure of chicken skeletal muscle is seen in the longitudinal section of the control group (Figs. 1, 2). Myofibrils were a more clearly defined by transversal striations. This pattern was repeated throughout the myofibrils length. The striations consisted of alternate bright I bands and dark A bands. A distinct, narrow Z lines bisected each I band. Each A band had central zone, H band, with a dark M line. The regular segments between two successive Z lines are sarcomeres, the structural and functional units of myofibrils. Myofibrils are highly organised structures of actin (thin) and myosin (thick) filaments responsible for transversal striations. In the transversal section both thick and thin filaments are present. Each thick filament was surrounded by six thin filaments and gave rise to a precise hexagonal pattern (Fig. 3). The longitudinal sections contained also elliptical interfibrillar mitochondria, arranged at the level of I bands. Sarcoplasma contains an elaborate membrane system, sarcoplasmic reticulum and glycogen.

Myofibril of experimental group 1 (Figs. 4, 5) lacked the typical pattern. Neither thick nor thin filaments were observed in transversal sections as in the control group. In the longitudinal sections, changes between the A and I bands were manifested by the loss of their architecture. It was impossible to distinguish the A and I bands. On the contrary, T lines were clearly visible as those in control group. They were coherent, thicker and undulated. The margin was not observed among myofibrils, glycogen almost disappeared and mitochondria and sarcoplasmic reticulum were indistinct. In all samples frozen by slow method myofilaments were interspersed with clearly visible spaces associated with formation of ice crystals.

In comparison with the control group, the experimental group 2 (Fig. 6) frozen by the IQF method also showed structural changes. However, the changes were less pronounced than in experimental group 1. There were not gaps among the myofibrils and myofilaments. Myofibrils were unchanged and did not merge as in experimental group 1. Their structure resembled that in the control. Regularly alternating dark A and lighter I bands were observed. The Z lines merged and the structure did not cascade. The sarcoplasmic reticulum disintegrated into a number of small vacuoles. Mitochondria were present but their structures were not clearly visible.

DISCUSSION

Freezing is a traditional method of food conservation. Freezing of food products causes changes in their quality related directly to this preservation process. Water, the key component of meat amounts to approximately 71 %. The proportion of water depends on the species and age of animals and varies with the type of muscle, kind of meat, season of the year and pH of the meat. By freezing the meat the contained water turns to solid ice crystals. Water expands upon freezing and the sharp-edged crystals push into the surrounding tissue and rupture the cells. The rate of freezing and formation of ice crystals plays an important role. S j o s t r o m (13) observed the growth of ice crystals in fibres of skeletal muscles frozen by the rapid method. Just below the fiber surface the crystals were numerous but small, while deeper in the fibres they were fewer but larger.

The cells may also be injured by other then mechanical damage induced by ice crystal compression, for example by chemical damage (17). Crystallisation of water results in increased ionic strength of liquids leading to breakage of electrostatic bonds in proteins and thereby to their partial denaturation (6). Water is characterised by a specific polar structure. Hydrogen bonds are formed between its own molecules and various chemical groups of biological compounds, especially the proteins in meat. Water is capable of consolidating the newly created conformation structures in protein molecules (2). These factors can cause damage to the fragile cellular

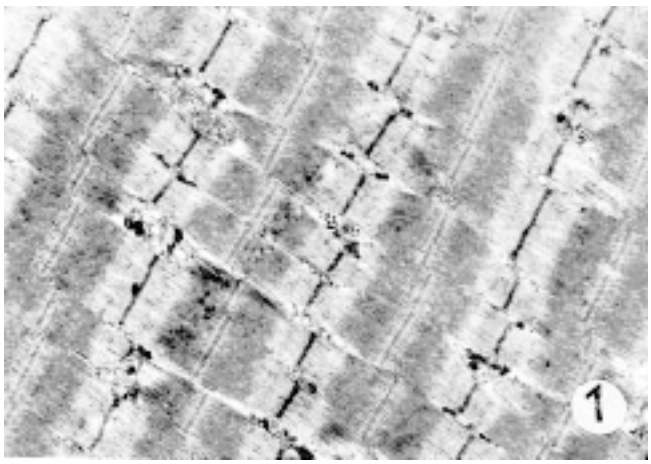

Fig. 1. Electronmicrograph of the poultry skeletal muscle (longitudinal section) of the control group. A bands are dark, I bands are light and are bisected by Z lines. Mitochondria are situated over I bands. Magnification: 6,400 ×

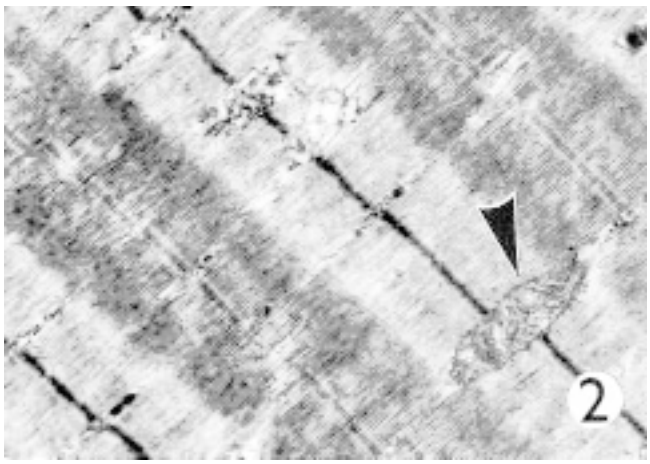

Fig. 2. Electronmicrograph of the poultry skeletal muscle (longitudinal section) of the control group at higher magnification. Mitochondrion (head). Magnification: 16,000 ×

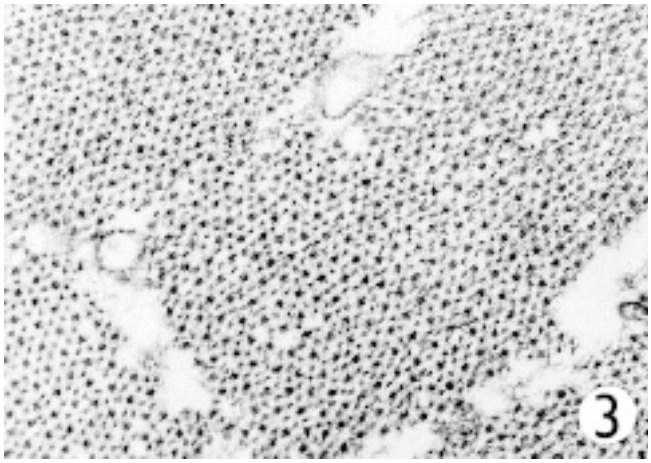

Fig. 3. Electronmicrograph of the poultry skeletal muscle (transverse section) of the control group. Both thick and thin filaments are present. They are regularly distributed and giving rise to hexagonal pattern. Magnification: 79, 800 ×

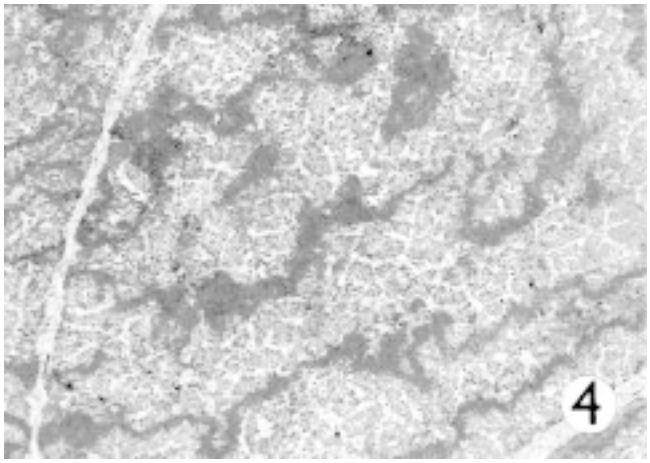

Fig. 4. Electronmicrograph of the poultry skeletal muscle (transversal section) of the experimental group 1. Boundaries of the myofibrils are not clearly defined and membranous structures are injured. Magnification: 3,638 ×

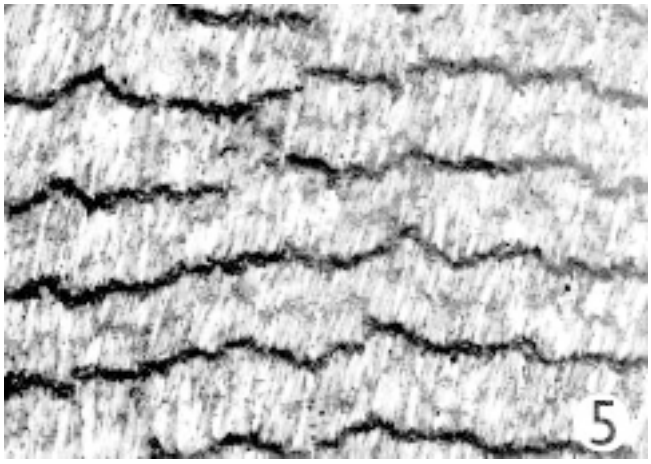

Fig. 5. Electronmicrograph of the poultry skeletal muscle (longitudinal section) of the experimental group 1. Myofibrils lost their architecture. There are irregular gaps among the myofilaments. Magnification: 6,000 ×

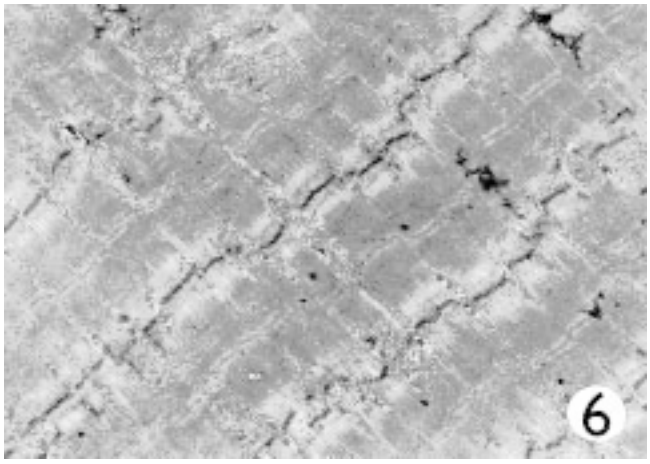

Fig. 6. Electronmicrograph of the poultry skeletal muscle (longitudinal section) of the experimental group 2. Myofibrils remain unchanged. Sarcoplasmic reticulum disintegrated into smaller vacuoles. Magnification: 9,684 ×

structures. They can also explain the morphological changes observed in samples.

Our study showed significantly different cryoinjury in samples experimental groups 1 and 2. The fact that fast frozen skeletal muscles (experimental group 2) exhibited less structural changes suggested that fast freezing might be a more effective method of meat preservation. This is directly related to less serious damage of the frozen tissue structure demonstrated in our study. Some intact myofibrils were present and no marked evidence of tissue damage by ice crystals was observed. Myofibrils were not separated by large intracellular space as in experimental group 1. The regular cross striation pattern of myofibrils was preserved. The sarcoplasmic reticulum lost its integrity and a number of small vacuoles were formed. On the other hand, D a l e n and S c h e i e (1) showed that the heart muscle sarcoplasmic reticulum exhibited high resistance to the formation of ice crystals. In general mitochondria in this tissue remained intact.

H o d s o n and W i l l i a m s (7) observed that ice crystals cavities formed in slow-rate frozen meat and all membranous structures were destroyed. Our slow-rate frozen samples (experimental group 1) exhibited most pronounced changes. All samples of tissue frozen by this method showed some empty spaces. The tissue lost its typical structure. Cross striated pattern was not seen in longitudinal sections, however, no obvious differences in electron optic density of A and I bands, were found. The Z lines observed were dark, thicker and wavy in comparison with control samples. Similar effect of freezing was observed by other authors (6, 9, 13, 14). Our results confirmed the general opinion that the slow-freezing process causes the more serious damage to muscle tissue.

The present study supplies ultrastructural evidence of different structural changes in chicken skeletal muscle in relation to the method of freezing. Our results should help to select the best method of cryopreservation of chicken meat. Electron microscopy revealed that IQF is a better method of cryopreservation of chicken meat than freezing in air. This finding corroborates the biochemical investigations.

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THE RECOVERY OF ENTEROHAEMORRHAGIC *Escherichia coli* O157 FROM COTTAGE CHEESE

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ABSTRACT

The presence of enterohaemorrhagic *E. coli* O157 was determined in 65 samples of raw sheep cheese and 18 samples of cottage cheese produced from pasteurized cow's milk. In accordance with the requirements of government regulations, selective enrichment, immunomagnetic concentration and separation were performed on each sample. Typical strains of *E. coli* O157 have not been found in any sample of raw sheep cheese. However, one sorbitol-positive mutant has been recovered from the sample of cottage cheese made from pasteurized cow's milk. This *E. coli* isolate did not utilize rhamnose and showed a positive serological reaction with *E. coli* O157 antiserum. As seen from results of this study, the method of immunomagnetic concentration is not exclusively specific for enterohaemorrhagic *E. coli* O157. Due to non-specific serological cross-reactions *Escherichia* sp., *Klebsiella* sp., *Enterobacter* sp., *Proteus* sp. and *Pseudomonas* sp. have also been captured on the surface of anti-*E. coli* O157 immunomagnetic particles. Moreover, high microbial contamination of cheese samples resulted in a significantly lower specificity and sensitivity of the immunomagnetic method.

Key words: cottage cheese; *Escherichia coli* O157; immunomagnetic separation

INTRODUCTION

Bovine products (beef and cow's milk) have been associated with *E. coli* O157:H7 food-borne infections in several countries (6, 8). Epidemiological investigations have revealed that dairy cattle, especially young animals, are the main reservoir of this pathogen (9, 16, 26).

Several pathogenity factors have already been described for enterohaemorrhagic *E. coli* O157:H7. First of all, these pathogens are able to synthetize verocytotoxins (15) and are generally known as verocytotoxic *E. coli* (VTEC). For the time being, three verotoxins have been isolated and purified (24). Their properties, as well as the course of disease ressemble the infection caused by *Shigella dysenteriae*. Thus, the toxins are also known as *shiga-like toxins* (*Stx*). The verotoxin group includes the following toxins: VT1 (*Stx1*), VT2 (*Stx2*) and VTe (*Stx2v*). Moreover, verocytotoxic strains also produce other factors of pathogenity including adhesins, haemolysins (10), as well as intimin (1, 18). The majority of *E. coli* O157:H7 strains posses 60 MDa plasmid, which is responsible for the adhesion of pathogen to gastro-intestinal cells (23).

Most sheep cheese in The Slovak Republic is made from raw (non-pasteurized) ewe's milk. Thus, the presence of *E. coli* O157:H7 is also expected in the final products (sheep cheese and bryndza-cheese) because of its circulation in the farm animal population (sheep and goats). The consumption of such products can endanger a consumer's health.

MATERIALS AND METHODS

Sixty-five samples of raw sheep cheese produced at sheep farms, as well as eighteen samples of cottage cheese produced from pasteurized cow's milk in the dairy plant, were examined for the presence of *E. coli* O157 in accordance with the requirements of government standard (21) as follows:

— the initial suspension was prepared from a test portion of 25 g and 225 ml of modified tryptone soya broth with novobiocin and incubated at 41.5 °C for twenty hours;

— bacteria were further concentrated and separated by means of immunomagnetic particles coated with antibodies to *E. coli* O157 using the magnetic separator (DYNAL, Norway);

— washed and re-suspended magnetic particles were inoculated in an amount of 50 µl on to pre-dried plates of cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and TXB agar (OXOID, Great Britain);

– after an incubation at 37 °C for twenty-four hours were the suspected (sorbitol-negative and β-glucuronidase-negative) colonies confirmed biochemically (Enterotube II, Becton & Dickinson, Germany) and also by agglutinatiom with *E. coli* O157 antiserum (OXOID, Great Britain).

RESULTS

The presence of typical enterohaemorrhagic *E. coli* O157 was not determined in any of 65 sheep cheese samples inspected. One sorbitol-positive serotype O157 has been recovered from the sample of cottage cheese made from pasteurized cow's milk. This *E. coli* isolate did not utilize rhamnose and gave a highly positive serological reaction with *E. coli* O157 antiserum.

As follows from the results of biochemical confirmation, the method of immunomagnetic concentration is not exclusively specific for enterohaemorrhagic *E. coli* O157. Colonies with a typical appearance on the surface of CT-SMAC and TBX medium included numerous genera of the family Enterobacteriaceae (*Escherichia* sp., *Klebsiella* sp., *Enterobacter* sp. and *Proteus* sp.). Pseudomonads have also been captured on the surface of immunomagnetic particles as a result of non-specific serological cross-reaction. Thus, a high microbial contamination of cheese samples resulted in a significantly lower sensitivity in the immuno-magnetic method.

DISCUSSION

Recent food legislation requires the production of wholesome and safe foods (19, 22). To fulfil this, the elimination of pathogens, including the enterohaemorrhagic serotype of *Escherichia coli* O157, is of great importance.

As described in the literature, various foods of both animal and vegetable origins can be involved into outbreaks of food-borne *E. coli* O157 infections. Insufficiently heat-processed meat and raw milk have been recognized as the most frequent causative agents (16). Although dairy cattle were considered to be the only natural reservoir of *E. coli* O157:H7 (11), the results of current studies have confirmed the presence of this microorganism in other slaughter animals, e. g. pigs, chickens, sheep, and goats (2, 8).

Outbreaks connected with the consumption of dairy products contaminated with *E. coli* O157:H7 have been reported in many countries (14, 17, 25, 27). Fermented or low-pH foods can also be involved in these outbreaks, since this pathogen can survive in a low acid environment (6, 11, 13). Some results also confirm the survival and multiplication of *E. coli* O157:H7 in raw milk kept at 8 °C (12), as well as its survival in the presence of 6.5% salt concentration (7). Enterohaemorrhagic *E. coli* O157:H7 is able to survive both phases of cheese ripening (Feta and Telemes cheese) at temperatures of 16 °C and 4 °C and the pH-values of 4.6 and 4.1 for 1 to 1.5 months (4).

The conditions of fermentation, especially the temperature and the composition of starter cultures can considerably influence the survival of this pathogen. The reduction of viable cells occurs more quickly in products being contaminated before fermentation, which confirms the fact, that the fermentation significantly contributes to the reduction of undesirable micro-organisms (3).

As seen from the results of this study, both milk pasteurization and fermentation have resulted in the absence of typical *E. coli* O157 in any out of 83 cheese samples inspected. The detection of an sorbitol-positive *E. coli* O157 mutant in the sample of cottage cheese made from pasteurized cow's milk could be explained by milk pasteurization. Heat processing has probably resulted in a change of typical sorbitol-negative *E. coli* O157 strain occurring in raw milk before pasteurization to that sorbitol-positive.

Sorbitol-positive *E. coli* O157 mutants are also reported to produce *Stx* toxins (5). In contrast to typical sorbitol-negative *E. coli* O157 strains they do not ferment rhamnose (20), which has also been confirmed in this study.

As reported, the infection dose for food-borne *E. coli* O157 infection is extremely low. Even the presence of less than 5 viable cells per 1 gram of food is reported to be sufficient to cause food-borne illness (14). Negligible counts of pathogen can often become unnoticed due to the presence of the higher contamination of accompanying bacteria. Therefore, special methods based on selective enrichment and concentration of food samples are used worldwide.

The results of this study confirm, that a high microbial load of the sample, as well as numerous serological cross-reactions can considerably decrease both the specificity and sensitivity of immunomagnetic concentration.

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THE DETERMINATION OF OXYTETRACYCLINE RESIDUES IN MILK AFTER THE MEDICATION OF COWS

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ABSTRACT

The purpose of this study was to determine the presence of oxytetracycline (OTC) residues in milk from cows with clinical signs of mastitis being administered with OTC and also to compare the sensitivity between the agar diffusion test with *B. stearothermophilus,* the Four-plate Test (FPT) and the HPLC method to the oxytetracycline residues in milk. Milk samples were collected from six cows, which were intramuscularly treated with Engemycin over five days. Milk samples were collected and tested for the presence of OTC residues intervals of 0, 24, 48, 72, 96, 120, 144, 168, and 192 hours after the first drug administration. The highest concentration OTC of 2.74 mg.kg^{-1} was detected by the HPLC method five days after the first drug administration was given (the last day of therapy). Two positive samples (zone diameters 1 mm) were detected using a microbiological assay with *B. stearothermophilus* var. *calidolactis* C 953 in milk samples before the first drug administration. As seen from the results of this study, positive milk samples confirmed by the HPLC method, in which the concentrations of OTC residues exceeded the MRLs, were not always detected by the microbiological assays. Thus, the microbiological inhibition assays, which we used in our experiment, are not sufficiently sensitive to the presence of OTC residues in milk.

Key words: HPLC; microbiological inhibition assays; milk; oxytetracycline

INTRODUCTION

Tetracycline antibiotics are intensively used in the therapy and prophylactic control of bacterial infection in human and veterinary medicine and as food additives for growth promotion in the farming industry. The first naturally occurring antibiotics chlortetracycline and oxytetracycline were discovered in the late 1940s. Today, nearly 1000 tetracycline derivatives exist, but only seven have been in extensive clinical and/or veterinary use. Tetracyclines are bacteriostatic agents, which inhibit rather than kill bacteria, and they exhibit activity against a wide range of Gram-negative and Gram-positive bacteria. The mode of action lies in its binding to 30S ribosomal subunits of bacteria, thus inhibiting protein synthesis (4).

However, the use of tetracyclines and other antibacterials in the farming industry has certain drawbacks: antibiotic residues in food products, such as in milk, may provoke allergic symptoms in humans, or may lead to monetary losses in the dairy industry, e. g., by inhibiting starter cultures in food technological processes. Tetracycline resistance is widespread in Gram-negative and Gram-positive bacterial species. Therefore, the detection of tetracycline residues in different biological samples is indispensable.

The control of tetracycline residues is commonly accomplished using three different methods: microbial inhibition tests, group- or substance-specific tests, and quantitative confirmatory methods. Microbial inhibition tests are based on the inhibition of growth and the metabolism of test bacteria by antibiotic residues, and they usually detect a broad spectrum of antimicrobials in a non-specific manner. Several

microbial inhibition tests have been developed for the detection of antibiotic residues in milk. Most of them detect very sensitively the widely used β-lactam antibiotics but are usually not able to detect tetracyclines with the required sensitivity, i.e., below the European Union maximum residue limits (MRL; 100 ng.ml^{-1} for tetracyclines) (6, 10). Group- or substance-specific tests include an enzymatic tests as well as receptor assays and immunoassays. The final identification and quantification of antibiotic residues is performed with sophisticated confirmatory methods, such as liquid chromatography.

The purpose of this study was to determine the presence of oxytetracycline residues in milk from OTC-administered cows with clinical signs of mastitis. Milk samples were tested for the OTC residues during the whole period of antibiotic intramuscular treatment (5 days), as well as within four days after the last drug administration. In order to compare their sensitivity, three methods were used, simultaneously for the determination of OTC residues in milk: the HPLC method, the agar diffusion test with *B. stearothermophilus* and Four-Plate Test (FPT).

MATERIAL AND METHODS

Black-red pied cows with clinical signs of mastitis, confirmed at the University of Veterinary Medicine in Košice, were involved in this study. Engemycin (oxytetracyclinum Intervet, Holand: 100 mg.ml^{-1}; 0.1 ml.kg^{-1} body weight) intramuscularly was administered to six cows at 24-hour-intervals. The equal volumes of foremilk from each quarter of the same cow were taken during milking in the morning and in the afternoon, and then mixed together. Milk samples were collected immediately before the first drug administration (0 h), and then after 24, 48, 72, 96, 120, 144, 168, and 192 hours (the withdrawal period of OTC is five days).

The oxytetracycline residues in milk were detected using the HPLC-procedure (11) with a sensitivity of 50 mg.kg^{-1}; the agar diffusion assay with *Bacillus stearothermophilus* var. *calidolactis* C 953 as the test micro-organism; as well as the FPT using the spores of *Bacillus subtilis* BGA at three different pH-values (6.0, 7.2 and 8.0) and *Micrococcus luteus* (3). Commercially distributed test media and the spore-suspensions of sensitive micro-organisms (MERCK, Germany) were used for both microbiological assays.

The discs of filter paper (Whatman 1, ϕ 12 mm) were moistened with 0.1 ml of milk samples, and then placed on the surface of the test-medium containing the spores of sensitive test micro-organisms. The plates with *B. stearothermophilus* were then incubated at 63—65 °C for three to five hours, the plates with *B. subtilis* at 30 °C for 18 to 24 hours, and the plates with *M. luteus* at 37 °C for 18 to 24 hours. Positive results (the presence of OTC residues) were manifested by the formation of transparent zones around the discs.

RESULTS

The results of HPLC analysis are shown in Table 1. All the milk samples showed positive results of the presence of antibiotic residues 24 hours after the first drug administration. However, the average OTC concentrations were lower than those determined 48 hours after the first administration (0.35 ± 0.09 mg.kg^{-1} *versus* 1.73 ± 0.97 mg.kg^{-1}). The highest concentration of OTC was observed 96 hours after the beginning of the therapy (2.74 ± 4.32 mg.kg^{-1}). One hundred and forty-four hours after the first drug administration, the residues of oxytetracyclines in milk were only found in four of the six cows (67 %) involved in the experiment. The milk from this cow still showed the presence of OTC residues three days after the last drug administration.

The results of both the microbiological assays performed are recorded in Table 2. At the beginning of the trial with *B. stearothermophilus*, two positive samples (zone diameters 1 mm) were detected. Twenty-four hours after the first Engemycin administration, positive results were not observed. Forty-eight hours after first drug administration, three positive samples were observed and simultaneously with the highest inhibition (zone mean

Table 1. Oxytetracycline (OTC) concentrations in milk samples determined by the HPLC method

Periods after the first OTC administration (hours)	Concentrations of OTC in milk (mg.kg^{-1})			Positive samples/	
	Range	Mean	SD	Total number	Percentages
0	ND	ND	ND	0/6	0
24	0.25—0.46	0.35	0.09	6/6	100
48	1.03—3.56	1.73	0.97	6/6	100
72	0.90—1.24	1.07	0.20	6/6	100
96	0.88—11.52	2.74	4.32	6/6	100
120	0.13—0.64	0.49	0.19	6/6	100
144	ND—0.23	0.09	0.09	4/6	67
168	ND—0.06	0.02	0.03	2/6	33
192	ND	ND	ND	0/6	0

Legends: ND — not detected; SD — standard deviation

Table 2. The diameters of inhibitory zones determined by microbiological assays

Periods after the first administration (h)	*Bac. stearothermophilus*			*M. luteus*			*Bac. subtilis; pH 6.0*			*Bac. subtilis; pH 7.2*			*Bac. subtilis; pH 8.0*		
	Range (mm)	Mean (mm)	Posit./ Total	Range (mm)	Mean (mm)	Posit./ Total	Range (mm)	Mean (mm)	Posit./ Total	Range (mm)	Mean (mm)	Posit./ Total	Range (mm)	Mean (mm)	Posit./ Total
0	0–1	0.3	2/6	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6
24	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6
48	0–4	2.0	3/6	0–2	1.0	3/6	0–12	8.0	4/6	0–5	2.7	4/6	0–5	1.5	3/6
72	0–6	1.0	1/6	0–2	0.3	1/6	0–12	4.0	2/6	0–6	1.0	1/6	0–5	0.8	1/6
96	0–2	0.7	3/6	0	0	0/6	0–7	3.8	5/6	0–4	1.5	4/6	0–3	0.8	2/6
120	0–2	0.3	1/6	0–3	0.5	1/6	0–3	1.0	2/6	0–4	0.6	1/6	0–3	0.5	1/6
144	0–3	0.5	1/6	0	0	0/6	0–2	1.3	4/6	0	0	0/6	0	0	0/6
168	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6
192	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6	0	0	0/6

diameters 2 mm). Seventy-two hours after the first drug administration, one milk sample was positive (zone diameter 6 mm). No inhibition was observed in milk samples 168 hours after the first drug administration.

As to the results obtained with FPT, no inhibition was detected at the beginning of the trial. Using the spores of *Bacillus subtilis* BGA at pH 6.0, four milk samples gave positive results 48 hours after the first OTC administration. Residues were not detected in all samples 168 hours after the first OTC administration. The highest inhibition was observed 48 hours after the first administration (zone mean diameters 8 mm). Ninety-six hours after first OTC administration, the highest numbers of positive samples were observed.

When the spores of *Bacillus subtilis* BGA at pH 7.2 were used, four positive milk samples were found 48 hours after the first OTC administration. Forty-eighty hours after the first drug administration, the highest inhibition was observed too. No inhibition was observed in the milk samples 144 hours after the first drug administration.

When *Bacillus subtilis* BGA at pH 8.0 was used, three positive milk samples were detected 48 hours after the first OTC administration. The most intense inhibition was observed 48 hours after the first drug administration (zone mean diameters 1.5 mm). After 144 hours, no inhibition was observed in the milk samples.

Using the test with *Micrococcus luteus* ATCC 9341, the highest numbers of positive milk samples were detected 48 hours after beginning of the therapy. No positive milk samples were detected 144 hours after the first OTC administration.

As can be seen from the results, *B. subtilis* BGA at pH 6.0 showed the highest sensitivity (zone diameters between 0 and 12 mm) and *M. luteus* the lowest sensitivity (zone diameters between 0 and 3 mm).

DISCUSSION

For the detection residues of antibiotics and sulfonamides, microbial inhibition methods were widely used. Their sensitivity to different drugs depends mainly on the indicator micro-organism used and the concept of the test.

The milk sample for our experiment confirmed by the HPLC method, in which the concentrations of OTC residues exceeded the MRLs, were not always positively detected by the microbial inhibition assays. The Council Regulation (EEC) No. 2377/90 sets the MRLs for tetracyclines in milk at 0.1 $mg.kg^{-1}$ (5).

As can be seen from the results, from all micro-organism tests used *B. subtilis* at pH 6.0 had the highest sensitivity, which appeared to be what we expected.

On the basis of the results obtained this study, *B. stearothermophilus* is not sufficiently sensitive to the residues of oxytetracycline in milk. As recorded in Table 2, two positive samples (zone diameters 1 mm) were detected by the test with *B. stearothermophilus* var. *calidolactis* C 953 in milk samples at the beginning of the trial before the first OTC administration to cows with mastitis. As is known, the amount of both the natural inhibitors lysozyme and lactoferrin is often abnormally high in colostrum, as well as in milk from cows with mastitis. The latest may even contain three $\mu g.ml^{-1}$ of lysozyme and eight $\mu g.ml^{-1}$ of lactoferrin, these compounds being responsible for false-positive results of microbiological residue assays both in isolation and in a combination (1, 2, 14).

Worldwide the most often applied microbial inhibitor tests utilise *B. stearothermophilus* as a test micro-organism, which is especially sensitive to the detection of b-lactam antibiotics and whose evaluation is based mainly on penicillin controls. However, its sensitivity to other antibiotics and sulphonamides (7, 9, 12, 13,) is described as insufficient. As reported, the sensitivity of *B. stearothermophilus* to tetracycline ranges between 400 and 500 $mg.kg^{-1}$ (6).

The lower detection limits ranging from 0.1 to 0.2 $mg.ml^{-1}$ are described (12) for *Bacillus cereus* microtitre test with an indicator. Its sensitivity can be increased by the pre-treatment of samples with ammonium oxalate. The detection limits ($\mu g.kg^{-1}$) for tetracyclines (oxalate pre-treatment in parentheses) are reported as follows: tetracycline 100 (60); chlortetracycline 60 (20); oxytetracycline 100 (70); doxycycline 60 (40), and rolitetracycline 0 (80).

In their proficiency study, Suhren and Heeschen (13) sensitivities to the detection of antimicrobials at fixed levels (FDA "safe/tolerance" level, EU-maximum residue limits), in three different batches of seven microbial inhibitor tests (BR-Blue Star, BR-AS, BR-EC,BR-6, Charm AIM-96, Delvotest SP special and *Bacillus cereus* microtitre test). Defining the sensitivities of this study as those concentrations where at least 80 % of the results were indicated as positive, the following sensitivities ($mg.kg^{-1}$) can be derived: Oxytetracycline: BR-6 > 100 < 150, *B. cereus* > 30 < 100; sulphadimidine: Delvo SP special > 150 < 200; sulphadimethoxine: BR-Blue Star, BR-AS, BR-EC and Delvo SP special > 30 < 100; gentamicin: Charm AIM-96 > 30 < 100.

Kurittu (8) describes a new group-specific microbiological test (the Tet-Lux test) for the detection of tetracycline residues in raw milk. The detection limits for tetracycline, oxytetracycline, chlortetracycline, doxycycline, methacycline, demeclocycline and minocycline were between 2 and 35 $ng.ml^{-1}$. The sensitivity of the Tet-Lux test was compared to Snap, an enzyme-linked immunosorbent assay for tetracyclines, and to Delvotest SP, a widely used screening assay for antibiotic residues in milk (9). The Tet-Lux test was clearly more sensitive to all seven tetracyclines tested, than Delvotest SP, and for five tetracyclines out of seven more sensitive than Snap.

Following our results and the results from writers abroad for the detection tetracycline residues below the MRL (100 $ng.ml^{-1}$) in milk a new sensitive screening test will be needed.

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IRON DEFICIENCY IN SUCKLING PIGLETS: ETHIOLOGY, CLINICAL ASPECTS AND DIAGNOSIS (A Review)

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ABSTRACT

The presented paper is the first part of a complex review. The authors survey the knowledge of the ethiology, pathogesy and diagnosis of iron deficiency in suckling piglets.

Key words: anaemia; haemoglobin; iron; piglets

ETHIOLOGY OF IRON DEFICIENCY

Nutritional or iron-deficiency anaemia has long been recognized as a serious problem in pig production. Iron is an important part of the body especially in growing animals. Iron is mostly (60 %) located as functional iron in haemoglobin, the rest forms myoglobin (20 %) and different enzymes (73).

A piglet is born with limited iron reserves (50 mg Fe) (70, 73). The daily requirement of iron during the first few weeks of life is ca 7—10 mg (70). The sow's milk however only provides the piglet with approximately 1 mg Fe per day (6, 36). An early supply of iron to suckling piglets is therefore essential. Without iron supplementation, suckling piglets develop anaemia 10 to 14 days after birth (22, 73).

The following factors contribute to the development of iron deficiency: the high intensity of piglet growth, an inadequate iron level in the sow's milk, low foetal iron reserves and blood loss at farrowing.

All suckling animals are iron-deficient for a time, but this is regularly critical only in pigs (66). The most important factors in the high incidence of anaemia in piglets is the rapidity with which they grow during early postnatal life and the low iron content in sow's milk. The high growth intensity makes them more prone to iron deficiency anaemia compared to other farm animals (64). During the first week, a piglet doubles its weight from 1.5 to 3 kilograms. At the same time plasma volume expands with 30 % (32). Piglets normally reach four to five times their birth weight at the end of three weeks and eight times their birth weight at the end of eight weeks (54). The more intensive the piglet's growth rate is, the more the piglets are in danger of becoming iron deficient and anaemic (64).

The iron content in milk can be increased only to a limited extent by increasing the iron content in a sow's feed. The amount of iron received by piglets from milk cannot in any case cover their iron requirements and prevent them from becoming anaemic (64). The amount of iron taken up by piglets from colostrum and milk shows high variability between sows. Milk consumption of a piglet varies between 0.5—1 l per day. Iron content in sows' milk varies from 0.2—4 $mg.l^{-1}$ and the piglet can absorb 60—90 % of milk iron (53).

It is interesting to note that the milk iron content of sows with higher milk production (i.e. high growth intensity of piglets) is smaller than that of poorly lactating sows (21).

Low foetal iron reserves and blood loss at farrowing can also contribute to an overall iron deficit. One of the peculiarities in pigs in comparison with uniparous animals is, that within a single litter, foetuses with low iron reserves and with normal iron reserves can be present. The nutrition of sows under modern housing conditions as well as increased litter size are apparently the main factors influencing the occurrence of low foetal iron reserves (64).

Liver is the most important organ for iron storage. The liver storage of iron is not large enough to prevent the rapid onset of anaemia in piglets which do not receive iron (19). Steinhardt *et al.* (64) have found significant variability in the iron liver content between piglets from different litters and even in piglets from the same litter, immediately after birth. The different iron supply of foetuses results also in a significant variability in values of Hb in blood plasma of piglets immediately after birth (35, 47). Also the iron levels in blood plasma of the piglets immediately after parturion show variability (5 and 35 $mmol.l^{-1}$) (1).

The supplementation of swine foetuses with iron proceeds in two ways:

a) During pregnancy a specific transport glycoprotein for iron (Uterofferin) is produced. It passes through allantochorion and accumulates in the allantois fluid. The maximal production of Uteroferrin takes place between the 40th and 75th day of pregnancy (37).

b) Another possibility is the direct transfer of Fe from blood through the epitheliochorial placenta, although the capacity of such a transport is limited (11).

It can be concluded that the iron supply of a sow in the first half of pregnancy is the most important factor affecting foetal iron reserves. Significantly, a positive effect on foetal iron reserves and Hb concentration in piglets after birth was achieved when iron-dextran was injected to sows between the 40th and 60th days of pregnancy (200 mg Fe as iron-dextran per kg body mass split in three injections – administered on day 40, 50 and 60) (13). The intramuscular injection of iron dextran preparations to sows during late pregnancy does elevate the haemoglobin levels of the piglets during the first few weeks of life but not sufficiently to prevent their anaemia.

According to Dilov and Chakurov (10), neither hypochromia nor erythropenia of the newborns could be overcome by intramuscular injection of iron dextran preparation (doses of 4.0 g Fe^{3+}) to the sows 21 days prior to farrowing. Also attemps to prevent piglet iron deficiency anaemia by administering iron chelates to sows in late gestation have not been succesful. A slight increase in Hb and RBC in the piglets from the amino acid-chelated iron treated sows compared with the controls was found, but this was considered to be of no practical importance (16). The iron content in foetuses could not be increased by adding iron salts to a sow's feed (57).

Positive correlation has been found to exist between litter weight and litter size, on the one hand, and qualitative blood properties of sows. In an adequate adjustment of quantitative blood parameters, mainly erythrocytes volume and total haemoglobin, was found to be of considerable importance in the reproductive performance of sows (63).

According to Guise and Penny (26) intramuscular injection of iron (gleptoferron) preparation to sows in late pregnancy do not prevent suckling piglets from becoming anaemic, it has an overall benefit of approximately 0.45 pigs/sows/year in favour of the iron-injected sows.

Bleeding from the navel cord is associated with greater or smaller blood loss. Sandholm and Honkanen-Buzalski (55) have reported bleeding with significant blood loss due to a deficient contractile ability of the navel cord. Supplementation of a sow's feed with vitamin C prevented further occurrence of this disorder. Direct blood loss during teeth clipping, tail docking and castration can also occur (30).

Over the years, different forms of iron supplementation have been used in order to prevent the development of iron deficiency anaemia in piglets. A total dose of 200 mg is usually recommended as being required to avoid clinically manifested iron-deficiency anaemia, but in order to avoid any chance of a subclinical deficiency the feed should contain additional iron at a minimum level of 240 $mg.kg^{-1}$ (54). It is generally agreed, that after single iron administration (either oral or parenteral) in order to get optimal red blood cell parameters, a good quality prestarter with iron content should be offered to piglets *ad libitum* (14, 25, 45).

Often it has been thought that the older suckling piglets that already take prestarter or recently weaned piglets overcome eventual iron deficiency quickly and without problems. It has been suggested that the risk of anaemia exists only in first 14 to 21 days of age. However, according to Steinhardt *et al.* (64) the frequence of anaemia in weaned piglets is considerable. These authors conclude that more attention should be paid to the control of the iron supply during weaning. Attention should be paid mainly to the accessibility of creep feed and the iron concentration in creep feed. Radostis *et al.* (54) recommend that creep feed should contain additional iron at a minimum level of 240 $mg.kg^{-1}$.

It is accepted that the amount of prestarter consumption depends mainly on its tastiness for piglets (39). For piglets there are so far no objective data evaluating the dependence of iron absorption on the type of prestarter, the form of iron presented, the concentration of iron and tastiness for piglets. Piglets can also to some extend receive iron from other sources (sow's faeces, iron containing impurities, creep feed) (64).

The addition of calcium carbonate to the diet of weaned fattening pigs is known to cause a conditioned iron deficiency and moderate anaemia but this effect is not apparent in mature pigs. Manganese may exert a similar antagonistic effect (54). The iron requirements for mature animals can be adequately met from the usual dietary sources (59).

Piglets kept in extensive husbandry systems can supply their iron requirement by ingesting iron presented in the soil. Access to earthen yards in most cases provides sufficient iron to overcome the deficiency in the sow's milk (54). The history of the increasing importance of anaemia parallels efforts to produce pork more efficiently. To control disease and parasites in swine, there has been a maximal use of concrete which can be thoroughly cleaned and disinfected. In doing this, iron deficiency anaemia develops because the piglets are separated from their natural source of iron in the soil (60).

Stern *et al.* (65) have studied the red blood cell parameters in piglets reared in- and outdoors in order to determine whether iron supplementation is necessary in outdoor pig production. Live weight at 35 days of age was slightly higher outdoors. The outdoor values were similar or exceeded indoor values although no prophylactic treatment of iron was given to the outdoor piglets (Table 1). The outdoor piglets had, compared to indoor piglets, a significantly higher Hb at 35 days of age, this is in agreement with values from Klein-

b e c k and Mc G l o n e (36) measured at 28 days of age. The results indicate that prophylactic treatment with iron was not necessary in outdoor production.

Iron requirements for domestic animals are influenced by age, growth rate and the availability of a dietary iron source (67). According to S t e i n h a r d t *et al.* (64) iron demands can be calculated, in dependence on live weight development, against the background of a low (110 $g.l^{-1}$) and an optimun (130 $g.l^{-1}$) haemoglobin level of the blood as well as in the context of blood volume development in the first two month of age.

The amount of iron supplies should depend on the conditions given at any specific pig production unit (nutrition and keeping of sows and piglets, severity and incidence of inadequate foetal iron reserves, actual increase of live weight, consumption of supplementary feed). The effects of iron supplies should be haematologically checked at regular intervals (64).

PATHOGENESIS

In the case of iron deficiency iron stores i.e. ferritin and haemosiderin are used first (prelatent phase), transport form i.e. transferrin follows (latent phase) and in the last stage the iron is released from the haemoglobin (30). Since iron is an essential component of haeme, its absence results in a reduction of circulating haemoglobin. A decrease in total Hb reduces the oxygen-carrying capacity of the blood (9). Insufficient supply of tissues with oxygen (hypoxia) causes anaerobic glycolysis, production of lactate and metabolic acidosis (7, 56). There is decrease of basic haematological parameters (haemoglobin, haematocrit and red blood cell count) and anaemia develops. This condition is characterized as microcytic hypochromic anaemia (17, 30, 54).

The direct effects of iron deficiency complicates the picture. Iron is essential for normal cell function and is a component of many enzymes (59). Cells of nearly all forms of life require well-defined amounts of iron for survival, replication and expression of differentiated processes (48). Iron has the capability to accept and donate electrons readily, switching between ferric (Fe^{2+}) and ferrous ($Fe3^{+}$) forms. This capability makes it a useful component of cytochromes, oxygen-binding molecules (i.e. haemoglobin, myoglobin) and many other enzymes (44). By activating or assisting enzymes, such as succinate dehydrogenase, iron is involved at every stage of the tricarboxylic acid (Krebs) cycle (68).

Iron-containing catalase and peroxidases remove potentially dangerous products of metabolism, while iron-activated hydroxylases influence connective-tissue development (51). Severely iron-deficient rats show evidence of thyroid hormone deficiency (2). It is involved in the transport of oxygen, in electron transfer, in the synthesis of DNA, in oxidations by oxygen (O_2) and hydrogen peroxide (H_2O_2) and in many other processes maintaining normal structure and function of virtually all mammalian cells (48).

The signs of iron deficiency are only partly due to a compromised delivery of oxygen to the tissues from decreased haemoglobin concentration. Iron deficiency depletes important iron-containing compounds in solid tissues (59).

Significantly lower iron concentrations in the brains of iron deficient rats have been reported by M a c k l e r *et al.* (46) and E r i k s o n *et al.* (20). Altered behaviour, that is, apathy and irritability, has been associated with iron deficiency. Brain iron decreases rapidly during iron deficiency and the low level persists after iron repletion of other tissues. Aldehyde oxidase, a key enzyme in serotonin degradation, is decreased, and serotonin level is elevated in iron deficiency. The accumulation of catecholamines may explain the mental changes associated with the condition (46). In piglets low

Table 1. Means and range of haematology for outdoor and indoor raised piglets (by S t e r n *et al.*, 2000)

	4 days of age			14 days of age			35 days of age		
	Indoor	Outdoor	Sign. level	Indoor	Outdoor	Sign. level	Indoor	Outdoor	Sign. level
Hb ($g.l^{-1}$)	75 60—97	82 60—107	*	109 90—128	112 95—134	*	119 97—141	127 109—146	***
PCV ($l.l^{-1}$)	0.25 0.19—0.30	0.26 0.20—0.32	n.s.	0.35 0.30—0.41	0.36 0.27—0.43	n.s.	0.38 0.31—0.46	0.41 0.34—0.48	***
RBC ($T.l^{-1}$)	4.23 3.1—5.2	3.98 3.2—5.1	*	5.61 4.2—7.0	5.42 4.7—0.8	*	6.79 5.7—8.8	6.96 5.3—8.4	n.s.
MCV (fl)	58.6 51—66	64.3 51—72	***	64.8 57—73	64.5 53—73	n.s.	56.4 51—63	59.2 52—66	***
MCHC ($g.l^{-1}$)	318.5 297—326	323.4 304—507	n.s.	311.4 299—326	312.5 291—396	n.s.	312 300—307	309 302—321	n.s.

* — $p < 0.05$; *** — $p < 0.001$ between groups on the same day; n.s. — not significant

haemoglobin and serum iron concentration were associated with orientation disorders in newborn piglets (5).

Apart from the effect on haemoglobin levels, iron-deficient piglets make considerably slower weight gains than supplemented piglets (54).

This could be explained by the fact that iron ions influence number of basic reactions (e.g. syntesis of DNA, transformation of ADP to ATP and so on) (12, 28). Also the function of cytochromic enzymes, especially peroxidase and katalases is dependent on the amount of iron in cells (4).

Gainer and Guarnieri (24) have found depletion of neutrophil count in iron deficiency anaemic piglets. A low leukocyte count, caused by low neutrophil counts, has been found also in a study conducted by Egeli *et al*. (17) on day 35 of age in the anaemic group compared to the healthy piglets. The eosinophils were also lower in the anaemic group than in the non-anaemic group at this time. A lowering of the neutrophil count in the anaemic piglets may increase their susceptibility to infection. The clinical significance of lowered eosinophil counts is more uncertain (17).

Iron deficiency causes anaemia and suppression of immunocompetence. Impaired resistance to infectious and parasitic diseases, growth retardation and increased mortality rate result in considerable economic losses (12).

Inadequate iron in rapidly growing pigs often seriously impairs their ability to synthesize the antibodies necessary to combat several diseases because iron is involved in number of enzymes essential to antibody production (9).

Iron deficiency is also associated with impaired cell-mediated immunity and the ability of polymorphonuclear granulocytes to kill ingested bacteria. Neutrophils have many iron-containing compounds. Myeloperoxidase is an iron-containing enzyme that is found in primary granules and contributes to antimicrobial activity. Cytochrome is another iron-containing compound that is found in specific granules and is required for the oxidative burst that follows phagocytosis (49).

Dubanský *et al*. (12) have found a marked deficiency of myeloperoxidase activity in iron deficient piglets. The impaired immunity was accompanied by the growth of facultatively pathogenic microorganisms. The increased virulence resulting from the passaging of these microorganisms eventually leads to infections of whole litters including piglets with a sufficient supply of iron (12).

Pedersen *et al*. (52) have found that iron deficiency increases the severity of *Trichuris suis* infection in pigs.

On the other hand an iron overdose actually promotes bacterial growth (38). When iron is present in the body in excess of the ability to properly bind it, an increase in the susceptibility to a variety of infections has been shown to occur (71).

Piglets with iron deficiency anaemia are, due to their impaired immunity, more prone to diarrhoea often associated with *E. coli* infections (8, 9). There are also other factors contributing to the higher susceptibility of piglets to diarrhoea. A marked impairment of the gastric secretion of chloride acid and atrophic gastritis occurs in iron-deprived piglets (40). Villous atrophy of the small intestine (41) and changes in gastrointestinal flora also occur in iron-deficient piglets which may contribute to the increased susceptibility to diarrhoea (42). The gut contents at several sites in iron-deficient animals has a higher pH and contain greater numbers of coliforms, lactobacilli, total aerobes and total anaerobes (42). Iron deficiency decreases energy dependent intestinal reabsorption, which can cause malabsorption of disacharides and leads to diarrhoea (7).

CLINICAL SIGNS

Anaemia is clinically apparent usually at the age of 10 to 14 days (22, 73). In a study conducted by Ast *et al*. (1), keeping piglets on a slatted metal floor, without a complementary iron supply, caused anaemia within seven days from parturition. Considerable variation occurs in the incidence of cases between litters kept under identical conditions. Black pigs are more prone to the disease than white animals (54). Early anaemia can be seen as pale mucousal membranes, especially *conjunctiva.* In the next phase the external ears become pale. In the advanced phase of anaemia the skin becomes pale over the whole body surface, severe dyspnoe, lethargy and a marked increase in the amplitude of the apex beat occur during exercise. There may be oedema of the head and forequarters, giving the animal a fat, puffed-up appearance. A lean, white hairy look is more common. Death can occur suddenly as a result of hypoxia and circulation failure. A high incidence of infectious diseases, especially enteric infection with *E. coli*, is associated with the anaemia. Piglets slightly may be well grown and in good condition but the growth rate of anaemic piglets is significantly lower than that of normal pigs. The losses that occur include those due to mortality, which may be high in untreated piglets, and a failure to thrive (30, 54, 67).

NECROPSY

The carcasses of affected pigs are pale, with thin watery blood and a moderate anasarca. Fluid exsudates in the body cavities are common. The heart is always dilated, sometimes extremely so. Cardiac dilatation and hypertrophy occur consistently (43). The liver in all cases is enlarged, and has a mottled appearance and the grayish yellow color of fatty infiltration. The mucosa of gastric fundus in cases of experimentlly induced disease is characteristically shallower and less cellular and shows a pronounced decrease in the parietal cells (40) and there is a greatly reduced capacity of the stomach to secrete acid (54, 67).

DIAGNOSIS

Diagnosis in piglets is based on clinical signs, laboratory examination and upon a history of no access to iron (67). Anaemia is usually associated with decreased concentration of haemoglobin and a decreased values of highly correlated haematocrit (packed cell volume – PCV). Therefore, the pri-

Table 2. Body weight and haematological parameters in piglets the day after birth (day 1), and in one group of anaemic and one group of healthy piglets on days 21 and 35 (Mean ± SD)

Parameters	Day 1 n = 133	Day 21 Healthy piglets n = 60	Day 21 Anaemic piglets n = 42	Day 35 Healthy piglets n = 60	Day 35 Anaemic piglets n = 42
Weight (kg)	1.79 (0.38)	7.27 (1.91)	6.78 (1.54)	12.20 (3.18)	10.73 (2,51)
Hb ($g.l^{-1}$)	81 (10)	102 (10)	47 (10)***	101 (10)	66 (22)***
PCV ($l.l^{-1}$)	0.26 (0.04)	0.34 (0.03)	0.14 (0.05)***	0.32 (0.03)	0.21 (0.08)***
RBC ($T.l^{-1}$)	3.85 (0.65)	5.35 (0.50)	2.93 (0.80)***	5.79 (0.68)	4.41 (1.58)***
MCV (fl)	69.4 (6.6)	63.6 (6.4)	46.8 (7.8)***	56.5 (6.6)	44.8 (6.5)***
MCH (pg)	21.2 (2.0)	19.2 (1.9)	16.5 (2.0)***	17.6 (2.1)	15.5 (2.3)***
WBC ($G.l^{-1}$)	9.07 (4.23)	8.40 (3.38)	8.47 (3.11)	13.58 (3.94)	11.25 (3.24)**
NEUT ($G.l^{-1}$)	6.56 (3.90)	3.08 (1.72)	2.68 (1.61)	5.56 (2.94)	3.73 (1.56)***
LYMP (G.l)	1.77 (0.61)	4.57 (2.53)	5.02 (2.33)	6.78 (2.19)	6.51 (2.47)
MONO ($G.l^{-1}$)	0.36 (1.13)	0.19 (0.10)	0.21 (0.12)	0.21 (0.10)	0.20 (0.10)
EOS ($G.l^{-1}$)	0.08 (0.05)	0.14 (0.13)	0.09 (0.10)	0.20 (0.14)	0.12 (0.08)**
BASO ($G.l^{-1}$)	0.03 (0.03)	0.08 (0.11)	0.05 (0.04)	0.12 (0.12)	0.10 (0.17)

Legends: SD in parentheses; * — $p < 0.05$; ** – $p < 0.01$; *** — $p < 0.001$ between groups on the same day

mary diagnosis of anaemia involves assessing the haematological status of an individual. This includes tests for haemoglobin, haematocrit, erythrocyte count and reticulocytes (44).

The anaemic limit, i.e. the point when the anaemia begins to exert a detrimental effect on weight gain or gives rise to clinical symptoms of anaemia, is by most authors set at a haemoglobin concentration of 80 $g.l^{-1}$ (23, 69).

Piglets at birth have haemoglobin concentrations of about 90—110 $g.l^{-1}$, they may fall as low as 30—50 $g.l^{-1}$ by the age of 3 to 4 weeks (67). The packed cell volume is lower than 0,3 l^{-1} and red blood cell count declines to 3—4 $T.l^{-1}$ (54).

Iron is preferentially shunted from other iron pools to haemoglobin (50). Thus, haemoglobin may be the last pool to show the effects of iron inadequacy. Inhibition or impairment of some processes in the animal through lack of iron occur long before haemoglobin formation becomes adversely affected (68).

In all species, sustained iron deficiency eventually leads to the release of small, new erythrocytes into the circulation with less than the normal concentration of haemoglobin (68). If the erythrocyte number (RBC), packed cell volume (PCV) and haemoglobin are determined, three erythrocytes indices can be calculated: MCH – mean corpuscular haemoglobin (pg), MCV – mean corpuscular volume (fl), MCHC – mean corpuscular haemoglobin concentration ($g.l^{-1}$). Classically, all three indices decrease in iron deficiency anaemia (59). The classification according to RBC indices helps to determine the type of anaemia, which assists with further investigation for differential diagnosis (44). If the MCV is within the normal range, the RBCs are considered to be normocytic. MCV has been found to be a sensitive indicator of active erythropoiesis in piglets (31). A rise in MCV indicates active production of new erythrocytes, with greater number of large immature cells being released into the circulation (15).

The reference values of haematological parameters for piglets are given by Egeli *et al.* (17) (Table 2).

Steinhardt *et al.* (61, 62) are more critical as the values of red blood cell parameters needed for optimal erythropoiesis are concerned. In their studies normal levels at which optimum erythropoiesis was possible were derived under functional aspects for piglets aged between one and eight weeks. Such normal levels were between 110 $g.l^{-1}$ and 130 $g.l^{-1}$ for haemoglobin concentration, haematocrit 0.36 to 0.42 $l.l^{-1}$, mean corpuscular haemoglobin concentration 320 to 350 $g.l^{-1}$, erythrocytes count 5.5 to 6.8 $T.l^{-1}$, mean corpuscular volume 58 to 62 fl, and mean corpuscular haemoglobin level 19 to 21 pg.

Three categories of iron deficiency exist in animals: iron deficiency (prelatent-iron stores deficit), iron deficient erythropoiesis (latent-decreased serum iron, decreased transferrin saturation) and iron deficiency anaemia (clinically apparent-low haemoglobin concentration) (29).

For practical conditions it has been stated that the growth retardation associated with iron deficiency anaemia is not distinct when haemoglobin concentration is over 80 $g.l^{-1}$. According to Gürtel *et al.* (27), the goal of anaemia prophylaxis is that no individual piglet has Hb concentration lower than 80 $g.l^{-1}$. Based on the knowledge of diagnosis and pathogenesis of iron deficiency, such a goal is no longer sufficient in order to assure the optimal iron supplementation of piglets in the first two month of life. Although no signs of anaemia and distinct growth retardation are present at Hb concentration of 80 $g.l^{-1}$, the genetic potential of piglets can not be fully utilized (18, 64).

Steinhardt *et al.* (61 and 62) concludes that using only Hb concentration the latent iron deficiency can be surely excluded, when haemoglobin concentration reaches as high as 110 $g.l^{-1}$. Prelatent anaemia can be excluded when haemoglobin concentration reaches 130 $g.l^{-1}$.

Table 3. Iron analytes in various stages of iron deficiency: Modified according to H a s t k a *et al.* (29)

Analyte	Iron deficiency erythropoiesis	Iron-deficient anaemia	Iron deficiency
Serum iron	N	↓	↓
Trasferrin saturation	N	↓	↓
Haemoglobin	N	N	↓
Erythrocyte size	N	N	↓

When using only haemoglobin concentration prelatent and latent iron deficiency can not be diagnosed. Serum iron concentration determination should be included in the diagnosis.

***Serum iron*:** Serum iron is measured in order to assess the iron transport compartment. Although plasma may be used, the anticoagulant should be tested for iron content. Samples should be handled carefully to avoid haemolysis and postsampling contamination (59). A large variation in serum iron concentration has been recorded by several authors (3, 72). This could be contributed to different factors: serum iron declines in severe iron deficiency, acute-phase inflammatory reactions, hypoproteinaemia, hypothyroidism, renal disease, and chronic inflammation. It may be elevated in haemolytic anaemia, iron overload, and liver disease (33). The effect of bacterial infection and subsequent acute-phase reaction on serum iron is especially dramatic, with levels decreasing to 10 to 20 % of normal within 15 hours. Serum iron then may return to normal within two to three days, if the stimulant is removed (58).

***Serum total iron binding capacity*:** Total transferrin can be measured by immunological methods, but the technique is not used commonly. Transferrin usually is measured in terms of iron content after it has been saturated with iron. When transferrin is saturated with iron, the iron content is called the total iron-binding capacity (TIBC). Because transferrin can bind more than is normally present, the TIBC is greater than the serum iron and the difference between them is the unsaturated iron-binding capacity (UIBC). Thus, serum iron can be expressed as a percentage of the TIBC and reported as the percent saturation (59).

The reference values have been given by S t e i n h a r d t *et al*. (62): 22—36 mmol.l^{-1} for plasma iron concentration, 80—100 mmol.l^{-1} for TIBC, and 25 to 40 % for saturation of total iron binding capacity.

More recently were reference values given by K a n e k o (34): 21.7 ± 5.9 mmol.l^{-1} for serum iron concetration, 56.8 ± 6.8 for TIBC, and 62 % for UIBC.

Care should be taken when interpreting serum iron concentrations because misdiagnosis of iron deficiency is possible (59).

Conventional laboratory findings of iron deficiency anaemia are a microcytic, hypochromic anaemia with normal or increased reticulocyte counts. Serum iron is decreased, serum TIBC is increased. Because the serum iron is decreased while the TIBC is increased, the percent saturation is decreased (59).

Requirements for iron supplies can be influenced by conditions given at any specific pig production unit. Therefore it is necessary to control regularly an adequate supply of iron for piglets through laboratory examination. The laboratory examination should be done ideally each quarter of the year and always in case of changes in feeding and housing conditions (64).

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CHRONICLE

48th YEAR OF STUDENT SCIENTIFIC CONFERENCE (SSC)

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INTRODUCTION

The first Student Scientific Conference (SSC) in UVL in Košice took place in the year of 1955, to share the results of experimental works carried out by the students of the past University of Veterinary Medicine in Košice, in which student scientific creativity was published by several departments in which student members of scientific groups were associated. There were not many contributions, 11 in total. Some of them were later presented at the National Student Conference in Prague. There were more reports presented at the Second Student Conference in the 1957 in Košice and at the third conference in 1958, which took place in the Veterinary and Pharmacy University in Brno. The conference included international participation and the crowning success was when a student of the University of Veterinary Medicine in Košice won the highest honors. It was the beginning of the alternating organization of those scientific work presentations in the field of veterinary medicine, which took place both at the University of Veterinary Medicine in Košice as well as in Veterinary and Pharmacy University in Brno. Students from Czechoslovakia, Hungary, DDR, Poland, Bulgaria, Yugoslavia, Italy and other countries took part in these conferences.

48th YEAR OF SSC

The 48th annual Student Scientific Conference (SSC) took place on the 27th of April 2005 at the University of Veterinary Medicine in Košice. International representation among the 42 participants was as follows: 4 members of the Veterinary Medicine Faculty in Zagreb, a student from the Veterinary and Pharmacy University in Brno, 4 foreign students of the University of Veterinary Medicine in Košice from Israel and Canada. In total 37 works were presented.

Student participation in the scientific conference was divided into three categories: pre-clinical, clinical and category composed of topics relating to the subjects of food hygiene and ecology. The event sponsored by the following companies and organizations: Waltham, Pfizer, Merial, Intervet, Pharmacopola, Vetis, Bioveta SK.

The quality of the works was reviewed by special commissions, which consisted of a chairman and two eminent teachers.

Summary of commended works in the first category, pre–clinical disciplines

1st place: **Viki-Nitzhona Aronov, 4th year, UVM Košice:** "*PCR based detection of IS900 of Mycobacterium avium* subsp. *paratuberculosis fro*m *buffy coat of cattle: A promising method of Johne's disease diagnosis*"

2nd place: **Václav Benedikt, 4th year, VFU Brno:** *"Blood parasites in free living birds in Bukov mountains, Slovakia"*

3rd place: **Viktor Paluš, 6th year, UVM Košice:** *"Pheasant's ectoparasites in breeding farms"*.

Summary of commended works in the second category, clinical discipline

1st place: **Oľga Halasová, 6th year, UVM Košice:** *"The effect of tooth canal obturation on fractured teeth in dogs"*

2nd place: **Tatiana Hazuchová, 6th year, UVM Košice:** *"Embryonic losses in pigs"*

3rd place: **Elizabeth Ruelle, 6th year BSc, UVM Košice:** *"Comparison of chemical immobilization effectiveness in the Przewalskii (Mongolian wild) horse using either medetomidine/etorphine or medetomidine/butorphanol/ ketamine"*.

Summary of commended works in the third category, food hygiene and environment

1st place: **Peter Supuka, 3rd year, UVM Košice:** *"Evaluation of meat parameter: breed quality of swine and type, tested at fattening and slaughterhouse value station in Bučany"*

2nd place: **Štefan Kulina, 6th year, UVM Košice:** *"Contribution to verification of germicide efficacy of choice, germicide preparation used in poultry farms"*

3rd place: **Zuzana Kostyová, 5th year, UVM Košice:** *"Comparison of microbiological quality of mechanically separated poultry meat and BAADER meat"*.

Recommendations for the premium Literal Fond SR

Each of the special commissions recommended from the works presented in each category for the premium of best student scientific work announced by Literal Fond SR:

Dana Malčeková, 3rd year, Peter Skladaný, 5th year, UVM Košice: *"Influence of Cadmium on male gonads structure of Japanese quail"*, **Veronika Lučaničová, 2nd year, Dávid Daniel, 6th year, UVM Košice:** *"Monitoring of foetus vitality in gravid female Boa constrictor by ultrasonografic apparatus"*, **Darina Labanská, 5th year, UVM Košice:** *"Sensitivity of microbial inhibition test for screening residues of sulfonamides in vitro"*.

Works commended by sponsoring companies representatives

Róbert Maňko, DVM, veterinary manager scientific center WALTHAM, brand name Pedigree and Whiskas, appreciate work of **Oľga Halasová, 6th year, UVM Košice** (1st price), **Peter Supuka, 3rd year, UVM Košice** (2nd price) and **Viktor Paluš, 6th year, UVM Košice** (3rd price) – see above:

Peter Lehocký, DVM, chief of veterinary division Co. PFIZER for Slovakia, commended the following work: **Peter Gál, 3rd year, PJŠ University Košice, Lukáš Weiss, 4th year, UVM Košice, Tomáš Toporcer and Boris Vidinský, 4th year, PJŠ University Košice:** *"Biomechanical and historical study of healing skin wounds"* and **František Zezula, 6th year, UVM Košice:** *"Monitoring the efficacy of mechanical correction in root canals of broken teeth and three root irigants in dogs"*.

Veterinary representative Co. **MERIAL** Vladimír Bony, DVM, presented "*MERCK Veterinary Manual"* to the following students: **Viktor Paluš, 6th year, UVM Košice** – see above, **Zuzana Štubňová, 6th year, UVM Košice:** *"Monitoring the effect of heptakain during the infiltrate and epidural anesthesia"* and **Štefan Kulina, 6th year, UVM Košice** – see above.

The representative of VETIS, Erika Konrádyová, DVM, presented valuable prices – foreign literature to: **Ivona Jagar, 3rd year FVM Zagreb:** *"Determination of sexual dimorphism on rudiments of pelvic bones in bottlenose dolphin (Tursiops truncates) from the Adriatic sea"*, **Peter Gál, 3rd year, PJŠ University Košice,** *et al.* – see above, **Jana Ottingerová, 6th year, UVM Košice:** *"Monitoring the efficacy of xylasine and his combination with trimekain during the epidural anesthesia in bovines"*.

Mária Bírešová, DVM, representative of PHARMACOPOLA gave *"Royal Canine The Dog Encyclopedia"* and *"Royal Canine The Cat Encyclopedia"* to the following students: **Viktor Paluš, 6th year, UVM Košice** – see above, **Eva Cvikelova, 6th year, UVM Košice:** *"Ultrasonografic treatment of some eye disease in small animals"*.

Director and administrator of BIOVETA SK, Juraj Salaj, DVM, PhD, gave valuable prices – foreign literature to: **Dana Malčeková, 3rd year, Peter Skladaný, 5th year, UVM Košice** – see above, **Tatiana Hazuchová, 6th year, UVM Košice** – see above, **Darina Labanská, 5th year, UVM Košice** – see above.

Gabriel Varga, DVM, president WSAVA and Ivan Rosival, DVM, representative of INTERVET for Slovakia participated in the preparation and evaluation of the 48th annual ŠVOČ.

A similar undertaking – *the 7th Conference of Young Scientific Workers*, with international participation will be held on the 2nd of June 2005 at the Veterinary and Pharmacy University Brno, where some of the students from *Student Scientific Conference (SSC)* will present their works.

SUMMARY OF THE APPRECIATED WORKS ABSTRACTS

Viki-Nitzhona Aronov, 4th year, UVM Košice

PCR based detection of IS900 of *Mycobacterium avium* subsp. *paratuberculosis* from buffy coat of cattle: A promising method of Johne's disease diagnosis

University of Veterinary Medicine, Košice

Johne's disease is one of the major diseases causing economic losses and health hazards in developing as well as

developed countries. To date, many microbiological, serological and molecular methods have been tried for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In the study we attempted PCR-based detection of IS900, distinct insertion sequences of MAP. The sensitivity as well as the specificity of the IS900-PCR proved to be high. The specificity of the test was determined by challenging the template from non-MAP strains. No amplification of non-MAP DNA confirmed the specificity of the primers. The presence of MAP in tested Slovakian cattle was 0 %. Positive control (MAP-detected diseased animals) in each test was amplified indicating the success of the test. Although, in recent years IS900-PCR detection of MAP has been used in humans, its use in animals is still limited. Our work not only supports its use in animals but also suggests further genotyping coupled with DNA sequencing, which can be a promising tool for rapid and effective surveillance of Johne's disease.

Václav Benedikt, 4th year, VFU Brno

Blood parasites in free living birds in the Bukov mountains, Slovakia

Institute of Biology and Diseases of Free-living Animals, Veterinary Hygiene and Ecology Department, Veterinary and Pharmacy University Brno, Czech Republic

Blood parasites in free-living birds were checked after the nesting period in two localities in Bukov Mountains, in the Slovak part of eastern Carpathian Mountains. In 2001 in the locality of Ruské at a height of 500 metres above sea level 595 birds were checked, in the year 2003 in the locality Kurników Beskid at a height of 1,000 metres above sea level 289 birds. Blood smears from them were examined. The presence of the following parasitic species was established: *Haemoproteus attenuatus, H. balmorali, H. belopolskyi, H. fringillae, H. killangoi, H. picae, H. zosteropis, Leucocytozoon dubreuili, L. fringillinarum, L. majoris* and the rarely found *Plasmodium* sp. and *Trypanosoma* sp. The prevalent parasitic infection in the locality of Ruské was *Haemoproteus, Leucocytozoon* and *Plasmodium* 21 %, 6 % and 0.5 %, in the locality of Kurników Beskid there was a prevalence of the parasites: *Haemoproteus, Leucocytozoon, Trypanosoma* at 17, 0.7 and 0.3 %. The difference between the prevalence of infection caused by genus *Leucocytozoon* in the localities was significant and probably it was caused by differences in the presence of proper vectors present at different altitudes in the monitored locations. Birds infected by *Haemoproteus* species had a significantly higher individual portion with low intensity of infection in the locality of Ruské and a high intensity of infection in the locality of Kurników Beskid. This difference was probably caused by a higher proportion of a recent acute infection of young birds in Kurników Beskid, where there is generally higher probability of the presence infections with high intensity. Birds infected by the parasites *Leucocytozoon* sp. have significantly the highest proportion of birds infected with a low infection intensity in the locality of Ruské; in the locality of Kurników Beskid it was not possible to statistically measure because of the low number of birds examined.

Viktor Paluš, 6th year, UVM Košice

The pheasant's ectoparasites in breeding farms

Institute of Parasitology, Diseases of Fish, Bees and Game UVM Košice

Our intention was to monitor the ectoparasite species representation in the intensive breeding of the field pheasant (*Phasianus colchicus*). Breeders usually do not take care of ectoparasitic problems. The high density of birds in relatively small places in farms breeding play an important part with regard to the speed of ectoparasite multiplication and its quick spread. The greater presence of ectoparasites can create an overall weakening, loss of appetite, anaemia, growth retardation and fertility disorders, skin damage with the chance of secondary infection and of course the transport of infectious diseases (Marek disease, Newcastle disease, bird pox, chlamidiosis and other). Research has been carried out in UVM Košice-Účelové stredisko in the locality of Rozhanovce. Our primary interest was to check the presence of ectoparasites in pheasants. The species representation we made from randomly chosen individual pheasants, while we considered that in the whole flock the same parasites would be present. We found the following species of mites: *Dermanyssus gallinae, Ornithonyssus sylvarium, Cnemidocoptes mutans*, and the following species of Mallophaga: *Goniodes colchici, Goniocotes chrysocephalus, Amyrsidea perdicis*, and *Lipeurus maculosus*. Our future aim is the establishment of prevalence values which was not done due to time constraints.

Oľga Halasová, 6th year, UVM Košice

The effect of tooth canal obturation on fractured teeth in dogs

Clinic of Surgery, Orthopaedics and Radiology UVM Košice

Endodontonic treatment of the tooth is a way of keeping the tooth functioning, and it is better alternative than extraction. Most of the works, dealing with the obturation of root canals of teeth, are directed at the effectiveness of filling narrow root canals of developed teeth. But most of the corona fractures of the front teeth are limited to an age up to two years. Teeth at that age are still developing, with expressive (marked) wide root canals. The aim of this work was to compare the individual obdurate techniques for filling the wide root canal of the teeth in young dogs. We used 49 teeth in this experiment, from young dog cadavers, put to sleep from various reasons. We used these techniques:

1. lateral condensation of cold gutta-percha
2. lateral condensation of cold gutta-percha and root cement
3. lateral condensation of heated gutta-percha
4. a) vertical condensation of heated gutta-percha
   b) hot vertical condensation after root's dentin primer treatment
5. vertical condensation of soften gutta-percha by halothane in apical part and vertical condensation by heat in the rest of the root's canal

After filling the canal with several techniques the quality was checked by X-rays examination and observing the permeability of liquates in histological slits. Quality was evaluated

in 4 scales: (1 – excellent, 2 – good, 3 – not so good, 4 – not enough). The quality of root filling checked by X-rays in the correspondent groups was: 1) 1.83; 2) 2.0; 3) 1.66; 4) 1.58 5) 1.5.

Tatiana Hazuchová, 6th year, UVM Košice

Embryonic losses in pigs

Clinic of Obstetrics, Gynaecology and Andrology UVM Košice

The success of the large animal reproduction depends on a series of coordinated relations between conception and the mother. After the fertilized zygote in its early stages is transported to the uterus, it must prevent the regression of *corpus luteum* by the production of specific substances. This mechanism is knows as "mother cognition of gravidity". The fertilization capability of oocyte is almost 100 %, but there are losses during the whole period of gravidity, highest at the earliest period, in the embryonic period. Most studies in this field have been carried out on bovines. In economic terms pigs are not a less important species. The aim of this work was to check embryonic loss in two breeds of pigs. The embryonic loss was checked in profit breed "1" and in profit breed "2", on the base of the interinsemination interval in 5,456 inseminations in one year. Pigs were from White Nice mixed with the Landrace breed. The groups of the swine were based on their interinsemination interval period. We created 5 groups in total. For embryonic loss in pigs *intra vitam* valuation is apt to use the interinsemination interval between 18 to 24 days (regular estral cycle period), respectively an interval of 25 to 35 days. 22.2 %, from whole 5456 inseminations repeated insemination from the 1st up to the 49th day and more after insemination (total amount within 5 groups). From all the highest percentage was in the zygote period, respectively the embryonic period. 45.9 % of the loss was in the period from the 24th day after insemination. Repetition of the heat from the 25th up to 35th day was present in 18.3 % of swine. Total gravidity loss up to the 35th day after insemination was almost 53 %. Loss of the fertilization of the swine in the embryonic period is very significant for its prevention and reduction within optimalization of the reproduction of swine in productive breeds.

Elizabeth Ruelle, 6th year, BSc, UVM Košice

Comparison of chemical immobilization effectiveness in the Przewalskii (Mongolian wild) horse using either medetomidine/etorphine or medetomidine/butorphanol/ketamine

Calgary Zoo Animal Health Centre, 1625 Centre Ave. East, Calgary, Alberta, Canada

The *Equus Przewalskii* (P. horse), which is noted for its unpredictable, and potentially violent nature, is one captive animal where routine procedures, such as hoof trims, require full immobilization. Dependable, safe, chemical immobilization is the goal of zoo and wildlife veterinarians, who must balance animal welfare and personal safety during such routine procedures on non-domesticated animals. Advances in anaesthesiology have led to the development of newer, safer pharmaceuticals, providing veterinarians with more options than ever before. The safety of chemical immobilization has been increased by the development of specific antagonists to anaesthetic agents. The purpose of this study is to compare the effectiveness of two chemical immobilization protocols that were administered to healthy P. horses for the purposes of hoof trims. Intramuscular (I.M.) administration of Medetomidine and Etorphine, or I.M. administration of Medetomidine and Butorphanol, followed by intravenous (I.V.) administration of Ketamine, was given to four healthy animals. Vital signs (temperature, pulse, respiration and O_2 saturation), time of induction, and time of recovery after administration of antagonists were recorded, and the results were compared. Both anaesthetic protocols were effective in immobilizing P. horses, and rendering them in a state of light anaesthesia, but neither drug combination demonstrated itself to be clearly superior to the other.

Peter Supuka, 3rd year, UVM Košice

Evaluation of meat parameter: breed quality of swine and type, tested at fattening and slaughterhouse value station in Bučany

Department of Food Hygiene and Technology UVM Košice

In recent years most of the breeds of pigs were selected for their higher or a high proportion of meat in the carcase. In some cases, one-sided selection for outstanding muscle can create an unexpected attendant phenomenon. A decrease in the reproduction profit, loss during or after transport and handling of the pigs, a quality anomaly in meat, these are the attendant phenomenon, when unexpected genotypes with a high sensitivity to stress are used. We can value the total economic loss of animals and loss caused by reproduction problems, but it is very hard to check and evaluate the loss caused by an anomaly in the quality of meat. It is necessary to select the animals for their sensitivity to stress and meat quality aspect, and by this support gain the desired genotypes. The aim of this work was to characterize breeds and profitable kinds of pigs with regard to meat quality breeds in Slovakia.

Štefan Kulina, 6th year, UVM Košice

Contribution to the verification of the efficacy of choice of germicide, the preparation of germicide used in poultry farms

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A preventive disinfection problem in free-range poultry breeds are considered solved by the professional community. Present conditions in the poultry industry allow the implementation of germicide programs to be included robustly in the technological process. The effect of disinfections on the environment is one of the most important questions. Chemical substances, such as chloric preparations and formaldehyde, belong among those with a very negative effect on the environment. The aim of this work was to evaluate the effi-

cacy of three tested germicide preparations on the base of *acidum peraceticum* and active chlorine, after spray and aerosol application in places with growing broilers. Used preparations were tested in laboratory and farm conditions.

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A comparison of the microbiological quality of mechanically separated poultry meat and BAADER meat

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Putting the mechanical separation process into practice in the poultry industry creates the more effective evaluation of residue meat from the trunk of a slaughtered fowl, after division into wing, breast and drumstick muscle. Mechanically separated meat (MSHM) is used as a base for heat-treated meat products, semi-finished product and products. Most of the separating machines work on the principles of pressure of the poultry bodies with high pressure in special machines with average holes on the performance net of less than 1.3 mm. During the separation due to pressure, the temperature increases, muscle cells are destroyed, meat juice is released and the whole mass is aired, thus making excellent conditions for the growth and multiplication of present microorganisms and pathogens. Besides pressure, the technique of the BAADER company is used in the poultry industry. “Mechanically deboned” or “thrifty separated poultry meat” is produced. The aim of this work was to compare microbiological parameters of mechanically separated poultry meat produced by the Hydina ZK Košice company on a discontinued machine type Protecon MRS 30 E and BAADER meat made in the Komes Plus Rozhanovce company. In both kinds of separated meat we monitored the total amount of microorganisms by quantitative examination, the amount of coliform bacteria’s, mezofil and anaerobe sporulates, koagulasis-positive staphylococcus and amount of *Escherichia coli*, the presence of *Salmonella* sp. and *Listeria monocytogenes* was monitored by qualitative examination. The results of microbial analyses confirm a lower level of microbial contamination and higher quality of poultry meat separated by BAADER company techniques.