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LOCALIZATION OF ACETYLATED TUBULIN-POSITIVE NERVE FIBRES IN THE SPLEEN OF SHEEP

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SUMMARY

The distribution of acetylated tubulin in the sheep spleen was studied using an indirect immunohistochemical method. A strong positive reaction for acetylated tubulin was observed on the nerve trunks and fine nerve fibres localized in the spleen hilus, and fine nerve fibres were seen in the fibrous capsule and trabeculae. Accompanying large and medium-sized branches of the splenic artery, nerve fibres enter the splenic parenchyma and as such are distributed in the white pulp. Most of the spleen nerve fibres positive for acetylated tubulin were found in the perivascular area, particularly arteries, arterioles, and the central artery of the splenic follicle. A fine network of nerve fibres was observed in the red pulp. After using the immunohistochemical method and acetylated tubulin antibody (Sigma), the present study displays the localization of nerve fibres common in mammals.

Key words: acetylated tubulin; nerve fibres; spleen; sheep; immunohistochemistry

INTRODUCTION

Nerve components in the lymphatic organs are supposed to link the nervous system and the immune system. Though morphological studies of the innervation of lymphoid organs are available, they have not provided a clear morphological link between the nervous and immune systems. Histochemical studies perfomed by Giron *et al.* (1980); Williams and Felten (1981); Williams *et al.* (1981); Walcott and MacLean (1984); Livnat *et al.* (1985); Ackerman *et al.* (1987) and others have demonstrated the presence of autonomic nerves in the specific regional sites of primary and secondary lymphoid organs. Attention has been paid mainly to the inervation of the spleen. Williams and Felten (1981) studied noradrenergic spleen innervation in the mouse, Felten *et al.* (1987) in the rat; Gillespie and Kirpekar (1966) and Fillenz (1970) in the cat; Zetterstrom *et al.* (1973) in the dog. In the spleen of man nerve fibres were describeed by Kudoh *et al.* (1979) and Heusermann and Stutte (1977). The presence of nerves in the spleen was also proved immunohistochemically by using S-100 protein, which is considered to be a specific antibody for glial elements of nerve tissue (Ueda *et al.*, 1991; Sugimura *et al.*, 1990; Marettová *et al.,* 1998). The aim of this study was to localize nerve components in the sheep spleen by the indirect immunohistochemical method using an acetylated tubulin antibody.

MATERIAL AND METHODS

The experiment was performed on 5 adult sheep and the spleen samples were taken in the slaughterhouse. After sampling the tissue was placed in 0.1 mol phosphate buffered 10 % formaldehyde for 24 hours at room temperature, dehydrated and embedded in paraffin. The 6 µm-thick sections were stained with haematoxylin-eosin or processed using the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981). Following deparaffinization, sections were hydrated, incubated for 20 min in 0.3 % H_2O_2 in PBS to reduce endogenous activity, and preincubated with 2 % goat serum to mask unspecific binding sites. Then the sections were incubated overnight with the monoclonal mouse anti-acetylated tubulin antibody, clone 6-11B-1 (Sigma), dilution 1:1000. Afterwards, the sections were washed twice in PBS and then incubated with the secondary antibody, goat anti-mouse biotinylated immunoglobulin. After 1 h of incubation the sections were incubated with ABC and developed with 0.05 % 3.3'-diaminobenzidine (DAB) and 0.03 % v/v H_2O_2. Some sections were counter-stained with Mayer's haematoxylin. Thereafter the sections were dehydrated in ethanol and mounted with synthetic resin (DPX; Fluka, Switzerland). Negative controls were performed by omitting the primary antibody.

RESULTS

More conspicuous acetylated tubulin positive nerve fibres are present in the area of the hilus, where the nerve trunks and their branches enter the spleen together with the *arteria lienalis* (Fig. 1). Positive nerve structures were seen also in the fibrous capsule (Fig. 2) and the trabeculae (Fig. 3) where the nerve fibres cut in the longitudinal and oblique sections appeared in the form of fine short-waved discontinuous profiles. Inside the spleen the acetylated tubulin positive nerve fibres were mainly found in the perivascular area of the trabecular arteries and their branches, including the central arteries of the lymphatic follicles. In all cases the nerve fibres were located in the periphery of the blood vessels, in their *adventitia* (Fig. 4). Exceptionally, the nerve fibres were seen to enter deeper into the *tunica media* layer of arteries. In no case were nerve fibres observed in the *intima* of blood vessels.

In the white pulp, the positive fine nerve fibres were seen at the periarteriolar lymphatic sheaths and the lymphatic follicle. Some fine nerves were seen to leave the *arteria centralis* and to insert themselves among the lymphocytes of the splenic follicles. A few fine nerve fibres are located around the lymphatic follicles making

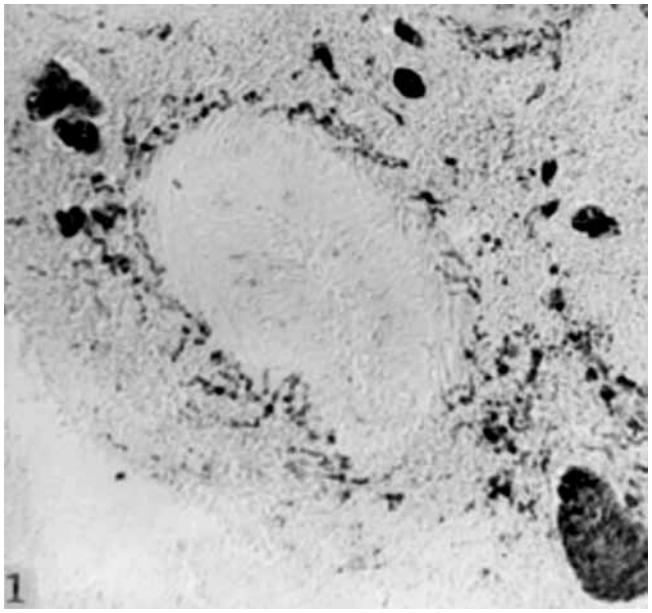

Fig. 1. Section of the spleen hilus. Large and medium sized nerves and plexus of fine nerve fibres are accompaning large blood vessels. ×190

Fig. 2. Section of the spleen capsule. Fine nerve fibres are running longitudinaly. ×280

Fig. 3. Section of the spleen trabeculae. Similar profiles of the fine nerve fibres as in the capsule are seen. ×260

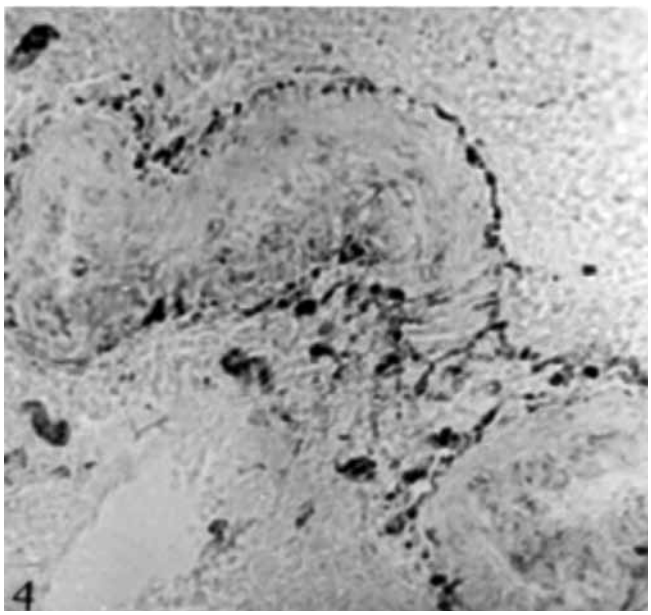

Fig. 4. Section of the splenic arteriole. Fine nerve fibres are located in the *adventitia* of the blood vessel. ×260

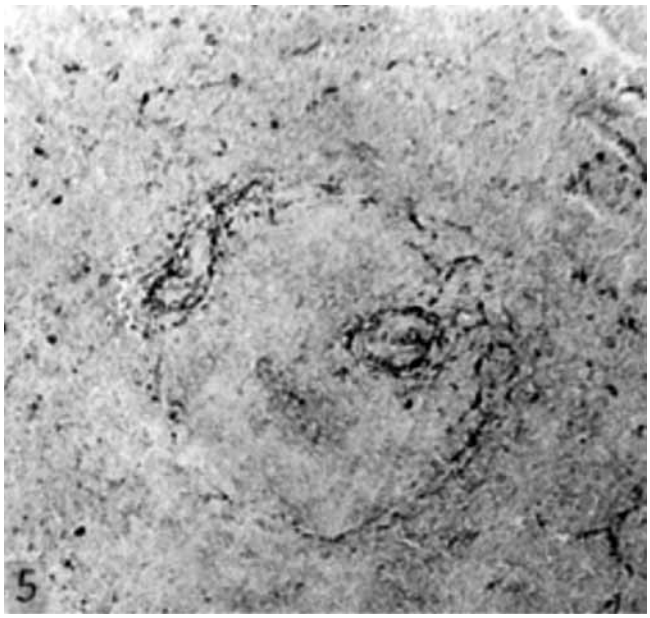

Fig. 5. Section of the lymphatic follicle and surrounding red pulp. Fine nerve fibres are surounding *arteria centralis* and its branch. Fine nerve fibres make a visible ring around the follicle. Punctate profiles of the nerve fibres are present in the red pulp. ×190

a fine annulus at its periphery (Fig. 5). In the red pulp, fine positive nerve fibres in cross and oblique sections were seen among the lymphocytes.

DISCUSSION

This study has demonstrated the distribution of acetylated tubulin in nerve fibres in the sheep spleen. We found rich nerve trunks at the hilus connective tissue and accompanying the splenic artery they enter the spleen. In the perivascular area of the trabecular arteries the nerve fibres penetrate the splenic parenchyma. Felten *et al.* (1985) noted that nerve fibres which travel with trabeculae are of noradrenergic nature in mice, rats, and rabbits. According to the authors, trabecular nerve fibres were present in the spleen of animals whose trabeculae are not associated with smooth muscle, suggesting a role other than the contraction of the spleen. The present study confirms the findings of these investigators of rich plexuses around arteries and arterioles of the splenic white pulp. This basic distribution of the nerve fibres described has also been in other mammalian species. In the mouse and rat spleens most innervation was distributed within the splenic vasculature (Williams and Felten, 1981; Felten *et al*., 1987; Schmidtová *et al*., 1995). The nerve plexus surrounding the trabecular arteries extended as the arteriolar branches that supply the parenchyma of the white pulp itself. Such distribution of the nerve fibres was confirmed in other studies made in the cat (Gillespie and Kirpekar, 1966) and the dog (Zetterström *et al*., 1973), in the mouse spleen (Williams and Felten, 1981) and we observed it also in the sheep spleen. Felten *et al*. (1987) found in rat spleen that sympathetic noradrenergic nerve fibres, stained with antiserum for tyrosine hydroxylase richly innervated the splenic white pulp. These fibres are distributed with the vascular systems, and associate mainly with the central artery and its branches and the periarteriolar lymphatic sheath. Reilly *et al*. (1979) presented in their electron-microscope study unmyelinated nerve fibres among the free cells in the white pulp of the mouse spleen. Our observations correspond to above mentioned authors. Moreover in the sheep spleen the nerve fibres were seen to form plexuses inside the periarteriolar lymphatic sheath and around the central artery of the lymphatic follicle visible in both longitudinal and cross-sectional views of the vessels. Additional fibres radiated away from the vasculature towards the marginal zone. Fibres of similar appearance ran along the parafollicular zone organized in an annular form. The close ultrastructural relationship between the naked adrenergic nerve terminals and immunocytes strongly suggests that there is an intimate relationship between the immune system and the sympathetic nervous system (Saito, 1990).

The occurrence of free nerve fibres, i.e., nerve fibres that are not associated with blood vessels in the red and white pulp is often a controversial finding. Filenz (1970); Reilly *et al.* (1976) had not be able to confirm such fibres in human, feline, or murine spleens. On the other hand, the delicate nerve profiles within a follicle were occasionally seen and described by Felten *et al*. (1987) in rats. In the sheep spleen single fibres which were not directly associated with vessels were detected inside the lymphocytic zone of the white pulp itself. Reilly *et al.* (1979) in the mouse spleen and Zetterstrom *et al*. (1973) in the dog spleen have shown a close association between unmyelinated nerve fibres and blood elements, but Felten *et al*. (1987) only rarely saw for tyrosine hydroxylase-positive nerve fibres in the red pulp of the rat. For the authors it was difficult to determine whether such a fibre was at the edge of an adjacent zone of the white pulp or actually was travelling freely in the red pulp. The red pulp of the pig and horse spleen is highly innervated (Ueda *et al*., 1991). However, Schmidtová *et al*. (1992) were not able to find nerve fibres in the red pulp in the rat spleen. Rather dense punctate profiles which we observed in the red pulp of the ovine spleen correspond to positive nerve fibres.

A few accounts relate the presence of the nerve fibres in the capsule and trabeculae. Schmidtová *et al*. (1992) in the rat and Reilly *et al*. (1976) in the mouse spleen did not find evidence of nerve fibres specifically associated with the splenic capsule, nor were there any subcapsular fibres. On the contrary, Felten *et al*. (1987) in the same species found within the trabecular and capsular system, tyrosine hydroxylase positive nerve fibres in the form of short discontinuous profiles. Using acetylated tubulin antibody we were able clearly to identify nerve fibres in the whole layer of the capsula and trabeculae

of the sheep spleen. Their presence in this area would be related to smooth muscle cells and their influence on the contractility of the capsulotrabecular system.

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THE CHOLINERGIC INNERVATION OF THE CAECUM IN CATS

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SUMMARY

The authors studied the innervation of the caecum in cats. Acetylcholinesterase (AChE)- and butyrylcholinesterase (BuChE)-positive nerve profiles were visualized by the direct tiocholine method. AChE- and BuChE-positive nerve components enter the caecum "from outside" in a common bundle with arteries and line these vessels in the form of plexiform bundles of nerve profiles as far as their terminal ramification in the organ wall. Nerve fibres, supplying both the longitudinal and circular layer of the wall of musculature, branch away from the periarterial AChE- and BuChE-positive nerve plexuses as well as nerve profiles entering the neuropil between neurocytes of myenteric and submucous plexuses. Moreover, they form loose nerve fibres in the *lamina muscularis mucosae* and penetrate also the *lamina propria mucosae* and the mucous crypts. AChE- and BuChE-positive nerve fibres run around lymphoid follicles or between them, and do not penetrate into the follicles, or B-dependent parts.

Key words: innervation; caecum; cat

INTRODUCTION

In the literary data detailing the nerve supply to different parts of the gastrointestinal tract (GIT), researchers have paid attention to the study of solitary and aggregated lymphoid follicles of the GIT (Gabella, 1994; Komuro and Hashimoto, 1990; Gershon *et al.,* 1994). It is known that the neuronal control of all the functional compartments of the GIT, from the morphological point of view, is performed by the autonomous system, which ensures outer interconnections of the digestive system with the CNS centres as well as with its own enteral nervous system (ENS) and can also influence the functions of lymphoid organs (Felten, 1993). Therefore, it is evident that the study of the appendix or caecum innervation is not the object of only marginal interest.

Results obtained by more authors (Felten *et al.,* 1981; Stopek *et al.,* 1996; Dorko *et al.,* 2001) indicate — in certain species — the specificities of the innervation of the lymphoid compartments of the appendix in some mammals.

Whereas in some animal species the appendix is entirely absent, in others e.g. cats it is an extremely small comma-shaped initial part of the *colon ascendens.*

Therefore, there is a question whether the analogous findings characterize AChE- and BuChE-positive components of nerve supply of this organ in cats, as representatives of laboratory animal species.

MATERIAL AND METHODS

Clinically healthy adult animals of both sexes were used in the study. The caeca of 20 cats (from the quarantine asylum, body mass 1.8—2.5 kg) were examined. All animals were anaesthetized with thiopental (50—60 mg.kg^{-1} i.p.). Constantly, multiple tissue specimens were collected from the caeca. AChE- and BuChE-positive nerve fibres were demonstrated by means of the direct thiocholine method of the cytochemical evidence of AChE (El Badawi and Schenk, 1967). The excisions were fixed in 4% formaldehyde at 4 °C for 2—10 hours. Sections of 16 µm were cut on a cryostat, incubated in acetylcholinesterase medium for 2—4 hours at 37 °C. As inhibitors of specific cholinesterases were used 62C47 and nonspecific cholinesterases iso OMPA. Individual sections were mounted on slides and examined under a light microscope Jenalumar 2.

RESULTS

AChE- and BuChE-positive nerve components enter the caecum in a common bundle with arteries typically carrying periarterial and so-called "adventitial" plexuses that line these vessels as far as their terminal compartments of the ramification in the organ wall. In the intramural topography, more AChE-positive and a few

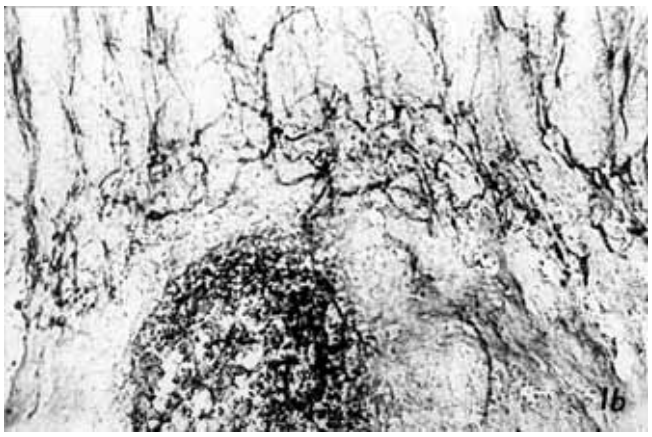

Fig. 1a,b. The densest deposits of BuChE-positive coloured reaction products are found in the *lamina muscularis mucosae*, which here mask thinner nerve fibres present (a). Characteristic appearance of AChE-positive nerve profiles lying in the same topography (b). Magn. ×120

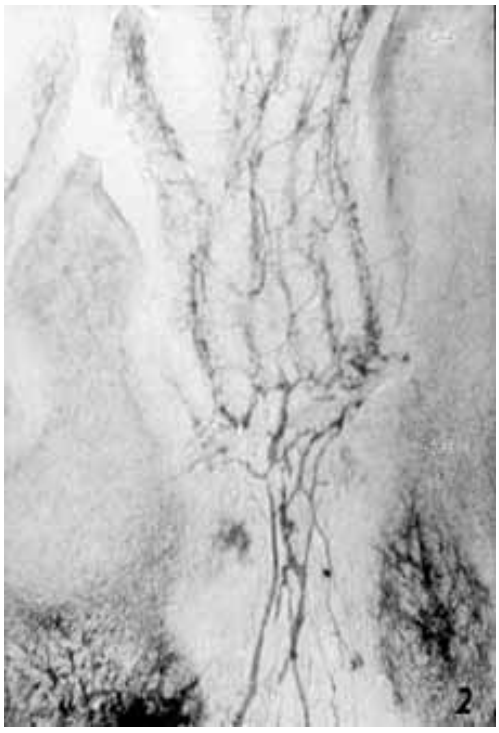

Fig. 2. Typical appearance of AChE-positive nerve profiles running between follicles from the submucous to the surface of the organ. Magn. ×120

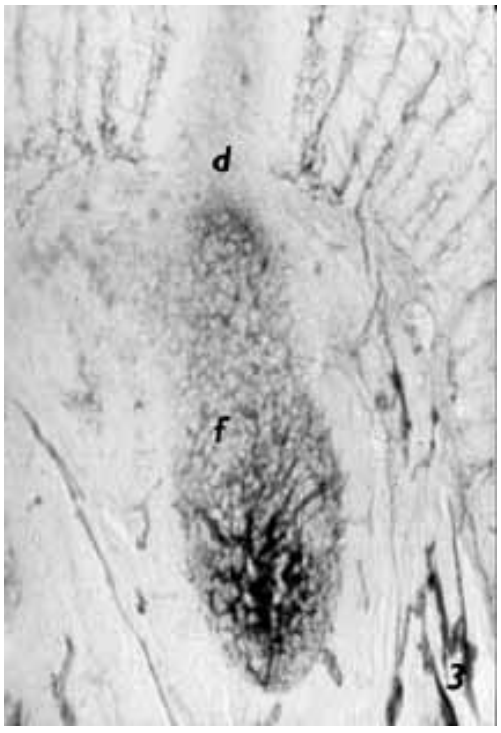

Fig. 3. Light diffuse AChE-positivity of the domain area (d) is in contrast with the massive, amorphous, and linear deposits of coloured reaction product in the extension of the germinative centre of a follicle (f). Evident AChE-positivity of the nerve profiles in *lamina propria mucosae* and the bundles of nerve fibres of the submucous plexus pushed aside. Magn. ×120

BuChE-positive nerve profiles branch away from the plexuses into the outer and inner muscular layers of the organ wall as the *plexus myentericus et submucosus*. The degree of intensity of the BuChE-positive-coloured reaction products is expressive in the longitudinal muscular layer and in the *lamina muscularis mucosae* (Fig. 1a,b).

The density of the AChE-positive nerves appears to be the greatest in submucous compartments, which lie outside the masses of abundantly-represented piriform formations of the lymphoid tissue. Nerve profiles, however, are reliably identified also in all the discarded and replaced periarterial plexuses in the neighbourhood of the lymphoid tissue. Large lymphoid follicles with their basal compartments often get close to the vicinity of the inner muscular layer, whereas all the structural components of submucosa, including vessels and nerves, are pressed away, partially aside, and partially toward the mucosal surface into narrow interfollicular septa (Fig. 2).

Penetration of AChE- and BuChE-positive nerve fibres into follicles was not found. They can be seen sometimes only in their marginal layers. Thin net-like bundles of AChE-positive nerve fibres are visualized in the *lamina muscularis mucosae*. Nerve fibres, however, are observed also in the area, where the *lamina muscularis mucosae* is interrupted, or pushed aside together with the interdomal area. Thin nerve fibres also penetrate the *lamina propria mucosae* and run in the stroma of mucous crypts (Fig. 3).

The findings of histochemical activity of AChE are manifest also in the non-neuronal topography in more forms and sites. There are follicles in which central zones are filled with granular and amorphous deposits of coloured reaction products; in others, the AChE-positivity is concentrated in the marginal or basal zones. Furthermore, there are linear forms, which by their appearence and course resemble a reticular skeleton.

The pictures of BuChE-positive lymphatic follicles in the materials examined are dull. The irregular density of linear deposits can be seen in the marginal layer or in the central zones of follicles. It is evident that AChE- and BuChE-positive nerve profiles innervate all the structural components of the caecum wall, whereas they lie predominantly beside or around lymphoid follicles, and they do not usually penetrate into the follicles; they enter only their marginal layers.

DISCUSSION

In the present study we report the presence and distribution of AChE- and BuChE-positive nerve structures in the cat's caecum and their specialities.

We found that AChE- and BuChE-positive preganglionic nerve bundles and fibres enter the caecum together with specifically fluorescent adrenergic postganglionic and afferent viscerosensitive components in the shape of typical periarterial nerve plexuses (Felten *et al.,* 1981; Stopek *et al.,* 1996). They represent a very conspicuous component of the histological findings because they contain a considerable amount of preterminal and terminal fibres that are considered for transformation of direct nerve impulses for certain quantities of a neurotransmitter released.

The role of AChE- and BuChE-positive nerve fibres, branching away from periarterial nerve plexuses and running among smooth muscular cells of longitudinal and circular layers of the musculature of the digestive tube wall as well as between gangliocytes of submucous and myenteric plexuses, is mainly that of influencing the gastrointestinal motility (Gabella, 1994; Gershon *et al.,* 1994). It is remarkable that conspicuous components of microscopic findings are usually relatively abundantly represented AChE-positive nerve fibres also lining very small thin arterial branches, which lie in the submucosa or penetrate through the *lamina muscularis mucosae* into the *lamina propria* as far as the mucous crypts (Schemann *et al.,* 1993).

Terminal compartments of the prevailing majority of AChE-positive and adrenergic enteral neurons lie in the close vicinity of the target effector components of the GIT, ensuring digestive processes (Costa *et al.,* 1987). Our findings that in the caecum as well as in other intestinal compartments, thin nerve branches and fibres run outside the solitary and aggregated lymphoid follicles and do not enter inside them confirm the findings of authors (Felten *et al.,* 1981; Schemann *et al.,* 1993; Stopek *et al.,* 1996). They are situated in the parafollicular or interdomal T-dependent compartments of the tissue. As the nerve fibres do not penetrate the germinal centres of follicles, the early stages of proliferation and differentiation of B-lymphocytes could not be directly influenced (Leon *et al.,* 1994).

It is evident that the compartmentation alone, i.e. the division of MALT (mucosa associated lymphoid tissue) into two functionally and morphologically different compartments, seems to be a significant agent of responses by the target cell, not only the entire complex of signal molecules in the given microenviroment, but often also to the same neurotransmitter (Felten, 1993). Both the expression and sensitivity of superficial cellular receptors depend on the local concentration of more signal molecules, whereas the effects of various cellular receptors getting into the microenvironment by the blood (Van Tits *et al.,* 1990) has to be taken into consideration.

The nerve fibres, lying in the T-dependent areas *lamina propria mucosae* and between mucous glands (outside the lymphoid tissue), reach the surrouding area or contact not only migrating lymphocytes, plasma cells, macrophages, and mast cells, but also thin-wall arterial components and the basal compartment of the mucous epithelial lining, including enterochromaffin cells (Sicard, 1986; Jepson *et al.,* 1993; Stopek *et al.,* 1996). As in the caecum in the same neuro-effector relations, there are not only adrenergic, cholinergic, but also peptidergic and purinergic nerve fibres. It is evident that the topography discussed has to take account the potential

direct and indirect effects of the diversity of chemical signal molecules, which in a relationship to the target cells act either synergically or antagonistically (Leon *et al.,* 1994). The neurohumoral regulation or control of local physiological processes, either during digestion or development and the maintenance of the appropriate state of the gastrointestinal immunity, is understood as a multifactorial way of regulating reciprocal intercellular communication (Felten, 1993; Schemann *et al.,* 1993).

Our findings of the nerve supply in the caecum in cats agree with those of other authors (Felten, 1993; Schemann *et al.,* 1993; Stopek *et al.,* 1996) regarding the fact that AChE- and BuChE-positive nerve profiles innervate all the structural components of the organ wall, whereas they predominantly lie beside or around lymphoid follicles, and they do not penetrate their inner B-dependent compartments.

As is known, the caecum in cats is a well-organized region of lymphoid tissue and its cholinergic innervation represents only one way among many possibilities for communication between the CNS and the immune system.

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CALCIUM EXCHANGE AND AXOPLASMIC TRANSPORT IN RABBITS

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SUMMARY

In rabbits subjected to a 40 min abdominal aorta ligation and then reperfused 24 h, the calcium binding and ultrastructural changes in myelinated axons of the lumbosacral segments were visualized. To assess the calcium accumulation, the binding agent pyroantimonate was used.

Ultrastructural results show that the nodal axon membrane is very well suitable to calcium exchange, and that the intra-axonal and interlamellar calcium accumulation may by influence negate the axoplasmic neurofilaments and their participation in axoplasmic transport.

Key words: rabbit; spinal cord; myelinated fibres; calcium

INTRODUCTION

The sequence of spinal cord cellular and neural membrane histopathological changes, including the white matter, was the subject of several light and electron microscopic studies using various experimental models (Wallace *et al*., 1987). It was found that shortly after the injury a cascade of pathological changes resulted, including oedema, vesicular disruption of myelin, demyelination, granular dissolution of the axoplasm, and neuronal and axonal necrosis (Azevedo *et al.,* 2000). Moreover, there is strong evidence that the early nervous tissue disturbance caused by ischaemic insult in itself is deeply aggravated during the reperfusion phase, when normoxaemic and mainly hyperoxaemic reoxygenation is instituted (Jalč *et al.,* 1995). With regard to the key role played by calcium as a second messenger, its binding to calmodulin and activation of Ca-Mg-ATPase present on the side arms of the neurotubules, makes the amount of calcium ions actually available in the axon a rate-limiting factor of the axoplasmic transport (Ochs and Jersild, 1984). It was confirmed that high intra-axonal concentrations (e.g., 60 mmol of calcium for longer than 6 h), or placing the peripheral nerve in a calcium-free medium, causes an irreversible block of axoplasmic transport — a phenomenon directly coupled to the destruction of axoplasmic structures identified by electron microscopy (Jalč *et al.,* 1992).

Since most of the results quoted were obtained in *in vitro* conditions, it was thought necessary to perform a series of experiments using a well defined and highly reliable ischaemic spinal cord model of the rabbit, and to describe the sites of calcium accumulation in the myelinated fibres in the spinal cord grey matter in a 40 min ischaemic period and after 24 h of normoxaemic reperfusion.

MATERIAL AND METHODS

Adult male rabbits weighing 2.5—3 kg were used in this study. The animals were anaesthetized with pentobarbital (30 mg. kg^{-1}, i.v.). A teflon cannula (Portex, 0.5—0.6) was introduced into the right femoral artery for monitoring the blood pressure. A small subcostal incision on the right side was performed, providing access to the abdominal aorta at the level of the renal arteries. A snare ligature was then placed around the aorta, distal to the left renal artery (Jalč, 2000). Shortly before tightening, a blood sample was taken from the central ear artery, followed by one taken immediately before release.

The animals were divided into two groups. Group A (n = 6): Nonischaemic controls in which no surgical intervention except a small subcostal incision on the right side was performed. In group B (n = 6) the animals underwent 40 min abdominal aorta ligation followed by 24 h of normoxaemic reperfusion.

All animals were reanaesthetized (50 mg.kg^{-1}, i.v.). L2—L6 laminectomy was made, the dura was longitudinally incised, and the spinal cord segment was irrigated by 2 % potassium pyroantimonate (Fluka AG). Small specimens were cut out from L4 segments and immersion-fixed in the same fixative. After postfixation in 1 % OsO_4, ultrathin sections were prepared, stained with 0.5 % uranyl acetate and 0.4 % lead citrate, and examined with a Tesla BS 500 electron microscope (Slocum and Roux, 1982).

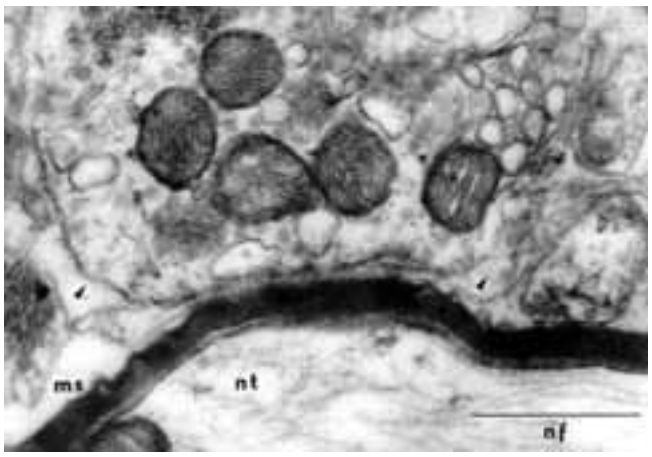

Fig. 1. The large myelinated axon with normally appearing neurotubules (nt) and neurofilaments (nf) is surrounded by a characteristic myelin sheath (ms). The Schwann cell cytoplasm around this axon appears normal. Nonischaemic control. Potassium pyroantimonate staining.
Magn. ×24000 (BAR 0.5 µm)

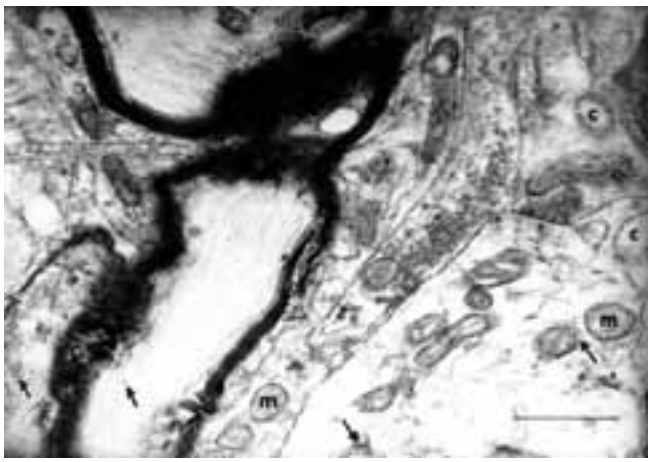

Fig. 2. Calcium induced myelopathy. The electron-dense Ca^{2+} deposits (arrows) are visualized in swollen mitochondria (m) with disrupted cristae (c), diffusely throughout the axoplasm, show neurofilament degradation, and in the large clefts of the myelin sheath, that formed as a result of interlamellar splitting. Forty min ischaemia, 24 h normoxaemic reperfusion. Potassium pyroantimonate staining.
Magn. ×13600 (BAR 0.5 µm)

RESULTS

In animals subjected to a 40 min abdominal aorta ligation and then reperfused for 24 h, an abundant accumulation of calcium pyroantimonate deposits was noted in relation to thick and middle-sized myelinated axons. The calcium deposits were found mainly in the large clefts of the myelin sheath that formed as a result of interlamellar splitting. The calcium deposits were seen as irregular patches of electron-dense material clearly contrasting with the light blackground of the axoplasm.

Moreover, the axoplasm of many middle-sized and thin myelinated axons was clearly affected. Swollen mitochondria with disrupted cristae could be found between slightly elongated axoplasmic vesicles. Longitudinally oriented interlamellar splits seen as light narrow spaces could be seen in the internodal region of the myelin sheath.

The paranodal region of the myelin sheath is the most strongly affected. Elaborate axon-oligodendroglial cell contacts in the paranodal region, seen as terminating myelin loops forming a spiral of paranodal glial cytoplasm and abutting successively on the outer surface of the axolemma adjacent to the node of Ranvier, are extremely enlarged, forming a row of saccular profiles of various sizes. Close to loosened and distended inner loops, variously shaped, light axoplasmic vacuoles appear covered by a smooth, thick, and dense membrane. Similar vesicles are often found in the nodal region (Figs. 1, 2).

DISCUSSION

In view of the fact that the long projection of spinal cord tracts, mainly comprising myelinated axons of various thicknesses, were demonstrably involved in the development of the ischaemia-induced delayed onset of paraplegia (Maršala *et al.,* 1989), the axonal and myelin sheath changes-resulting after ischaemia and reperfusion — with regard to calcium translocation and the possible accumulation of calcium cations in the myelinated fibres — were analysed. The location of calcium deposits clearly shows that the interlamellar clefts, often forming large distended vacuoles, are the main site of the calcium accumulation. Calcium influx into cells and fibres in white matter following spinal cord trauma was considered to be a possible factor causing the calcification of axons and calcium-induced spongiform and necrotizing myelopathy as well (Balentine, 1980). Intra-axonal and interlamellar calcium accumulation seen in this ischaemia-reperfusion model may negatively influence the axoplasmic neurofilaments and their participation in the axoplasmic transport. An immunoblot study of neurofilament degradation *in situ* and during calcium-activated proteolysis done on anterior and posterior spinal nerve roots, unsheathed sciatic nerve and spinal cord white matter, fully confirms this view (Schlaepfer *et al.,* 1985; Follis *et al.,* 1993). The occurrence of large calcium pyroantimonate deposits seen in the myelinated axons of lumbo-sacral segments clearly shows that ischaemia-reperfusion-induced neuronal damage is closely bound up with acute changes encompassing all the components of spinal cord neuronal circuits.

Our observations like the results of several studies, e.g. Simpson *et al.* (1990), Kusuoka *et al.* (1998), Lovblad *et al.* (1998), suggest that depolarization of neurons, calcium influx, excitatory amino-acid accumulation, increased activity of lipases and phospholipases, and oxygen free radical overproduction are the main factors influencing the development of the ischaemia-reperfusion-induced neuronal damage.

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THE SUSCEPTIBILITY OF COWS OF THE SLOVAK SPOTTED BREED AND ITS CROSSBREEDS TO INFECTION BY THE MICROSPORIDIA *Encephalitozoon cuniculi*

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SUMMARY

Our work focused on the diagnosis of a microsporidian species, *Encephalitozoon cuniculi* in the breeding of the Slovak spotted cow and its crossbreeds, as well as the evaluation of their susceptibility to infection during puerperal endometritis and/or *retentio secundinarum*. The diagnosis of *E. cuniculi* was performed in 54 dairy cattle aged 3 to 14 years in the spring period. From day 1 to day 14 after calving the dairy cattle were examined clinically, gynaecologically, and ultrasonographically (Aloka SSD 500, 5 MHz, a linear probe).

A sample from the *corpus uteri* of all the cows was taken by swabs in aseptic conditions for microbiological laboratory testing. *Encephalitozoon cuniculi* antibodies were diagnosed in the blood serum of the cows evaluated by the method of indirect immunofluorescence.

The *E. cuniculi* positive identification was recorded in 20 dairy cows (37.04 %). In 9 positive cows (16.67 %) no pathological findings on the genital organs were revealed ($P < 0.01$). In 6 dairy cows (11.11 %) puerperal endometritis was manifested and in 5 cows (9.26 %) *retentio secundinarum* was observed. The evaluation of dairy cattle infections with respect to their age proved that the highest percentage of positive cows (43.75 %) were among those aged from 5 to 8 years.

Key words: cows; puerperal diseases; microsporidia; *Encephalitozoon cuniculi*

INTRODUCTION

Some deliberations concerning protozoal invasions have recently appeared in the specialist journals when describing the aetiology of puerperal infections. Besides the known causative agents, such as *Trichomonas foetus* and *Neospora caninum* (Barr *et al*., 1993; Thornton *et al*., 1994; Bjorkman *et al*., 1996), the negative effects of less known microsporidia, e.g. *Encephalitozoon cuniculi*, have also been taken into account (Reetz, 1995). Some knowledge of the aetiology of puerperal infections, among which endometritis and *retentio secundinarum* occur most frequently (Choma *et al*., 2000), helps the veterinary surgeons apply an effective therapy. In addition, it helps breeders to lower the costs of breeding, of course, and thus it makes the breeding more profitable. Therefore, our work concentrates on the diagnosis of the microsporidian *Encephalitozoon cuniculi* in the breeding of dairy cows of Slovak spotted breed and its crossbreeds, as well as the evaluation of their infection rate by the above-mentioned parasite during puerperal infections and *retentio secundinarum*. In our conditions encephalitozoonosis *(E. cuniculi)* was serologically diagnosed in cows with papillomatosis (Lešník *et al*., 2000).

MATERIALS AND METHODS

Fifty-four dairy cattle aged 3 to 4 years were diagnosed with *E. cuniculi* during the breeding of the Slovak spotted cow and its crossbreeds in the spring. The cattle were housed. From day 1 to day 14 after calving the dairy cattle were examined clinically, gynaecologically, and ultrasonographically (Aloka SSD 500, 5 MHz, a linear probe, Figs. 1, 2). Based on these examinations, the cows were divided into three groups: the group of dairy cows with no pathological findings on the genital organs ($n = 20$), the group of dairy cows with *retentio secundinarum* ($n = 10$), and the group of dairy cows with puerperal endometritis ($n = 24$).

A sample from the *corpus uteri* of each cow was taken for microbiological laboratory testing by swabs in aseptic conditions. Subsequently, the samples were cultivated in nutrient culture medium, serum agar, and selective substrates (XLD-agar, Endo-agar, and CLED-agar) in laboratory conditions. Bacteria isolates were diagnosed on the basis of microbiological exami-

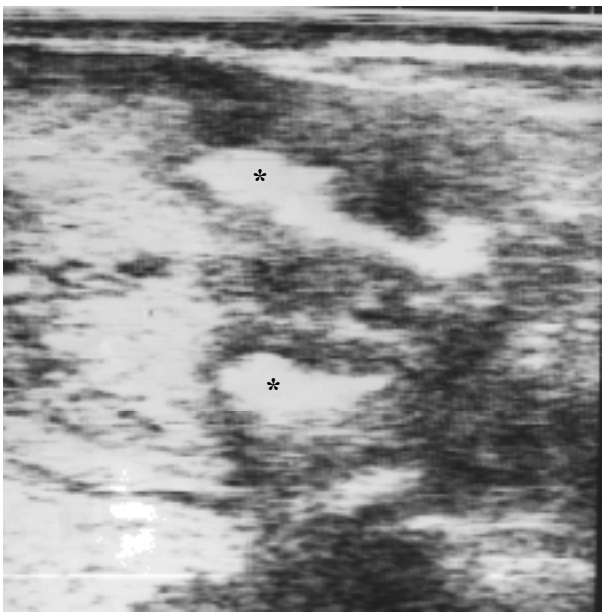

Fig. 1. Cross section through the uterine horns of the cows with *endometritis puerperalis* (at bifurcation). Hyperechogenic (white) regions (*) in the reflexive structure of dilated uterine horns scan purulent exsudate

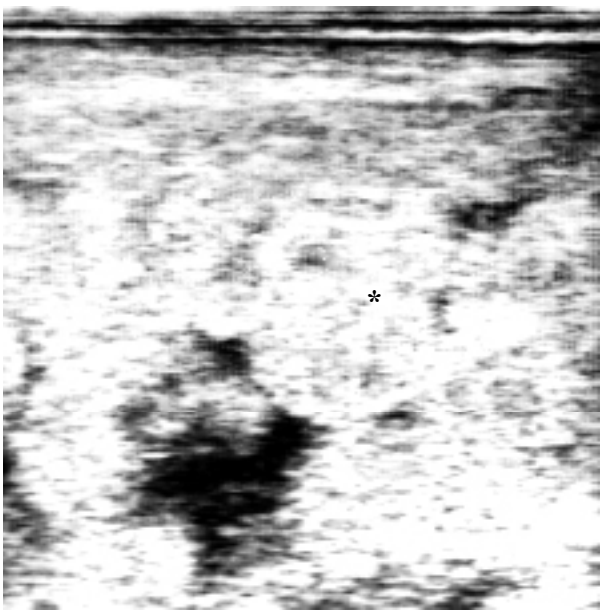

Fig. 2. Retained placenta (*). Hyperechogenic differentiation of caruncles, disintegrated products and cellular elements in the uterus

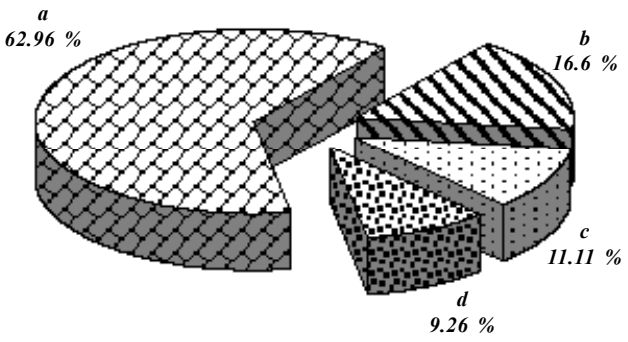

Fig. 3. Percentage of dairy cows evaluated according to the *E. cuniculi* positive diagnosis and the course of puerperium

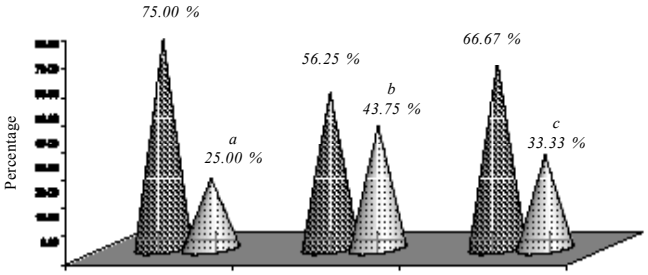

Fig. 4. Percentage of cows diagnosed with

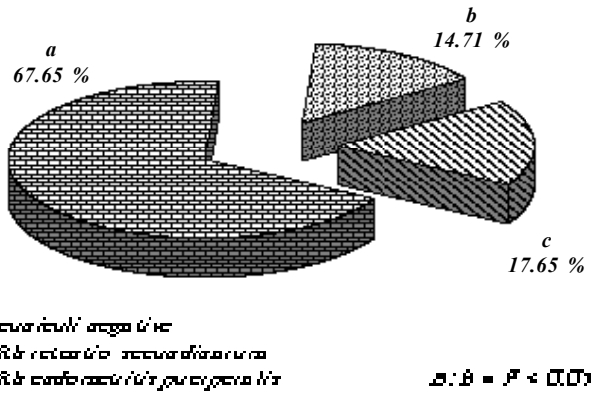

Fig. 5. Percentage of cows with puerperal infections diagnosed with *E. cuniculi*

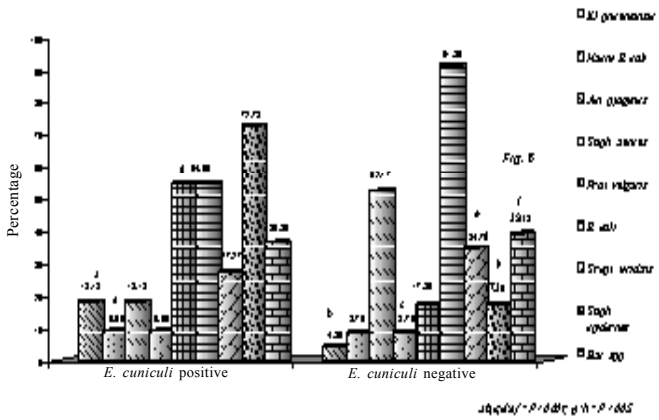

Fig. 6. Percentage of bacterial microflora in the uteri of cows with puerperal infections according to the *E. cuniculi* positive diagnosis

nation (native preparation, Gram's stain), cultural, biochemical (catalase reaction, oxidase test) and serological examinations (agglutinative reaction).

Encephalitozoon cuniculi was diagnosed in the blood sera of the cows evaluated by the method of indirect immunofluo-

rescence. The indirect immunofluorescence antibody test was used to determine specific *E. cuniculi* antibodies. The method was performed according to Chalupský *et al.* (1973). *E. cuniculi* antigen was obtained from E6 cells ("VERO" green monkey kidney cells) where the parasites of *E. cuniculi* were grown. The cells were cultivated in the modified RPMI 1640 medium supplemented with 5% foetal bovine serum and with the addition of antibiotics (penicillin, streptomycin and amphotericin B). After the cultivation, separation was conducted by centrifugation at $400 \times g$ for 20 minutes. The bovine serum tested was gradually diluted from the titre 1:16 up to the titre 1:512. The animals whose sera reacted at the titre of 1:64 and higher were evaluated as positive.

Statistical evaluation was conducted by the chi-square test.

RESULTS

The diagnosis carried out during the breeding of the Slovak spotted cow and its crossbreeds showed (Fig. 3) that, out of 54 dairy cows evaluated, *Encephalitozoon cuniculi* antibodies were present in 20 cows (37.04%). In 9 positive cows (16.67%) no pathological findings on the genital organs were recorded ($P < 0.01$). The course of their puerperium as well as the involutional processes on the genital organs corresponded with physiological criteria. In 6 dairy cows (11.11%) puerperal endometritis was manifested and in 5 cows (9.26%) *retentio secundinarum* was observed. The evaluation of the susceptibility of dairy cattle to infection with respect to their age proved (Fig. 4) that the highest percentage of positive cows (43.75%) were among those aged between 5 and 8 years. In the cattle aged 3 to 4 years 25% of dairy cows were positive, and in the cattle of 9 to 14 years of age 33.33% of cows were evaluated positive ($P < 0.01$).

The analysis of the dairy cows with puerperal infections (Fig. 5) implies that the positive indications of *Encephalitozoon cuniculi* antibodies occurred in 32.36% of the infected cows, out of which there were 17.65% in the cows with puerperal endometritis and 14.71% in those with retained placentas. Bacterial microflora findings in positive cows with puerperal endometritis (Fig. 6) proved that, in the majority of infected cows, the presence of no other pathogen, apart from the diagnosed *E. cuniculi,* which might have aroused the disease was recorded. *Klebsiella pneumoniae* was diagnosed in 18.18% of the positive dairy cows, beta-haemolytic *E. coli* in 9.09%, *Actinomyces pyogenes* in 18.18%, *Staphylococcus aureus* in 9.09%, *Proteus vulgaris* and non-haemolytic *E. coli* in 54.55% of the cows, *Streptococcus viridans* in 27.27%, *Staphylococcus epidermidis* in 72.73%, and *Bacillus* spp. in 36.36% of the positive cows.

DISCUSSION

The parasite is supposed to participate in various organ diseases. Nevertheless, the course of disease is usually asymptomatic, therefore, the serological methods are those predetermined for its diagnosis. One of the most important among them is the immunofluoroscence method. It has succeeded in detecting antibodies against antigens *E. cuniculi* in rabbits (Cox *et al.*, 1979), goats, pigs, and horses (Waller *et al.*, 1983). In Slovakia, microsporidiosis was for the first time detected in a rabbit (Levkut *et al.*, 1996), in dogs (Ševkovič *et al.*, 1997) and in cows (Halánová *et al.*, 1999; Lešník *et al.,* 2000).

In 1995 a case of abortion in two heifers in their 7th month of gravidity was described by Reetz (1995). In the microbiological examination of the foeti and the placental examination of these abortions, none of the usual bacterial or mycotic agents of abortion were discovered. The presence of microsporidia *Encephalitozoon* was detected by the immunohistochemical examination.

During our observations, positive indications of *Encephalitozoon cuniculi* antibodies were recorded in 32.36% of the cows with puerperal infections. The majority of infected cows diagnosed with *E. cuniculi* showed no other pathogen that could potentially arouse the disease. *Klebsiella pneumoniae* was diagnosed in 18.18% of the positive dairy cows, beta-haemolytic *E. coli* in 9.09%, *Actinomyces pyogenes* in 18.18%, *Staphylococcus aureus* in 9.09%, *Proteus vulgaris* and non-haemolytic *E. coli* in 54.55% of the cows, *Streptococcus viridans* in 27.27%, *Staphylococcus epidermidis* in 72.73%, and *Bacillus* spp. in 36.36% of the positive cows.

In the aetiology of puerperal cow infections that cause breeders in practice significant economic losses (Hav- rila *et al.*, 1999; Choma *et al.*, 1999), mainly bacterial and viral causative agents are described. Metabolic diseases are frequently described as the main cause of these problems (Wright *et al.*, 1992). However, parasite infections, which may play an important role particularly in non-specific diseases of the reproductive cow organs, are neglected.

The unknown aetiology of the diseases, as well as the fact that abortion was registered in the anamnesis of 20% of positive dairy cows imply that either a facultative pathogen or *E. cuniculi* might have participated in the puerperal infections of the dairy cows observed in the study.

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A STUDY ON ANTIBODY PREVALENCE DUE TO MICROSPORIDIAN *Encephalitozoon cuniculi* IN DOGS (*Canis familiaris*) USING INDIRECT IMMUNOFLUORESCENCE

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SUMMARY

The presence of specific serum antibodies to *Encephalitozoon cuniculi* was studied in a group of 178 dogs from Eastern Slovakia. The indirect fluorescence of antibodies (IFA) was chosen as the diagnostic method. The entire cell corpuscular antigen of the *in vitro* microsporidia grown and swine anti-dog FITC-conjugated immunoglobulin was used in the IFA test.

The animals whose sera reacted by immunofluorescence in a titre of at least 1:20 were considered positive. Specific anti-*E. cuniculi* antibodies were found in 53 out of 178 dogs investigated (29.8 %). Two thirds of the serum positive samples (37 out of 53) were in the lower titre range of 1:20 to 1:40. Sixteen dogs were seropositive at a dilution of from 1:80 to 1:320. The other 125 dogs (70.2 %) showed seronegative reactions.

Our screening results indicated, that the IFA test is a very sensitive method for the detection of early microsporidian infection in dogs and for the indication of very small amounts of specific post-infection antibodies.

Key words: microsporidia; *Encephalitozoon cuniculi;* dogs; serological survey; indirect immunofluorescence

INTRODUCTION

Encephalitozoon cuniculi (phylum MICROSPORA) is an obligate intracellular protozoan parasite invading a variety of cell types in a broad range of mammalian hosts including man. The parasite is well known as an agent causing encephalitozoonosis, a chronic disease mostly with latent progression. From the latent course the disease may progress into an active form of the infection that is usually influenced by suppression of the host immune system (Levkut *et al*., 1997).

Kantorowicz and Lewy (1923) in Germany first reported microsporidiosis in the dog (*Canis familiaris*). Since that time, canine encephalitozoonosis has also been diagnosed in numerous countries including France, England, Tanzania, the Republic of South Africa, Czechoslovakia, Zimbabwe, as well as in the USA (Van Dellen *et al*., 1989).

In susceptible individuals *E. cuniculi* prefers to affect the central and peripheral nervous and renal tissues. The infection in dogs is occasionally connected with clinical signs of nonsuppurative encephalitis combined with weight loss in puppies (Plowright, 1952) and recurrent nephropathies in young and geriatric individuals (Stewart *et al*., 1986), well known as an *encephalitis-nephritis* syndrome. Subsequent uraemia and death may also occur. Moreover, various ophthalmic lesions such as cataract, retinitis, and superficial or deep keratitis have been reported in blue foxes — *Alopex lagopus* (Arnesen, Nordstoga, 1977) and domestic cats — *Felis catus* (Buyukmihci *et al*., 1977).

Moreover, encephalitozoonosis has been confirmed as a zoonosis (Eckert, 1989; Deplazes *et al*., 1996; Kučerová-Pospíšilová and Ditrich, 1998). Some species of mammals including the dog seem eligible to be potential animal sources of the microsporidial infection for humans. For this reason, we decided to obtain the real serological status in a group of our usual clinical patients using indirect immunofluorescence of antibodies detection as a diagnostic method.

MATERIAL AND METHODS

Serum samples. The presence of specific antibodies to *E. cuniculi* was studied in a group of 178 dogs that came from the East Slovakia region. Blood samples were taken from the dogs with various symptoms and diagnoses at the Clinic of the Department of Internal Medicine of Small Animals, Solipeds and Birds at the UVM in Košice. Blood was withdrawn from the antebrachial vein (*vena antebrachii*) in the standard way. After allowing the clot to form, the blood was spun in a centrifuge at 600 × *g* for 15 minutes. Decanted sera were stored frozen at –20 °C until used in the serological assay, however, not longer than six months.

The prevalence of specific antibodies to *Encephalitozoon cuniculi* was investigated using IFAT as the orientating di-

agnostic method. In the case of a positive immunological reaction, as well as in the control direct fluorescent staining with Calcofluor White M2R (Fig. 1), spores were observed as oval fluorescent formations of 1.5—2.5 mm in size. The titre of each serum sample was defined as the reciprocal value of the highest serum dilution showing strong peripheral fluorescence of 50% spores (Fig. 2). Both positive and negative control sera as determined by ELISA (Štefkovič *et al*., 1999) were included in the examinations for comparison of the serological reactions.

All canine serum samples were first tested at an initial dilution 1:20. Sera that positively reacted at this screening titre were subsequently examined also at dilutions of from 1:40 to 1:320. The animals whose sera reacted by immunofluorescence in titre at least 1:20 were considered positive according to the study of Stewart *et al*. (1979).

E. cuniculi organisms. An entire cell corpuscular antigen of the microsporidia was used in the IFA test. The spores of murine origin (Vávra *et al.,* 1972) were grown within E6 cells of VERO — Green Monkey Kidney Culture. The infected cell culture was cultivated in RPMI 1640 media (SIGMA, Germany) supplemented with 5% of Foetal Bovine Serum (SIGMA, Germany) with an addition of Antibiotic-Antimycotic solution containing penicillin, streptomycin and amphotericin B (SIGMA, USA).

Spore isolation. After the bursting of the infected cells, mature spores were released into the media. The organisms were collected from the culture supernatants after centrifugation at 450 × *g* for 20 minutes. Sediment was resuspended in non-buffered Percoll (density 1.30 g/ml, pH 8.8) and, following centrifugation at 750 × *g* for 20 minutes, separated from the spores. Subsequently, the organisms were processed according to Koudela *et al.* (1993).

The indirect fluorescent antibody test (IFAT). The IFAT method was used to determine specific antibodies to *E. cuniculi*. The test was done as described in detail by Chalupský *et al*. (1973). Both a positive and negative serum was included in all the tests as well as the controls. To control antigen preparation quality, some slides were unspecifically stained with optical brightener Calcofluor White M2R (SIGMA) and observed under blue fluorescence (Fig. 1).

The antigen spots were flooded with 10 ml of dilutions from 1:20 to 1:320 of sera tested and then incubated for 30 min at 37 °C, washed and covered with 20 ml of fluorescein isothiocyanate-conjugated swine anti-dog immunoglobulin (SwAD/FITC; SEVAC a.s., Czech Republic) in a dilution of 1:160. Dry slides were counter-stained with Evans blue and coverslips were mounted with buffered glycerine. The immunofluorescent reaction was evaluated in the fluorescent microscope ZEISS JENALUMAR using 510 nm colour light, filters of 405—409 nm, and a barrier filter 550 nm by use of immersion objective ZEISS JENA 100× and cedarwood Immersion Oil (OLYMPUS, Great Britain).

RESULTS

Out of 178 dogs investigated, 53 were found positive by serological IFAT examination (29.8%). Blood serum samples of 25 dogs reacted positively at a dilution 1:20, another 28 were positive at a dilution of from 1:40 to 1:320. The other 125 dogs showed seronegative results (Tab. 1).

Table 1. Serological positivity to microsporidian *Encephalitozoon cuniculi* in the dogs tested by the IFAT

Animals	Serological evaluation		Reciprocal IFA titres to *E. cuniculi*				
	negative	positive	1:20	1:40	1:80	1:160	1:320
Number (n)	125	53	25	12	5	8	3
Percentage (%)	70.2	29.8	14.1	6.7	2.8	4.5	1.7

Control staining of *E. cuniculi* spores is demonstrated in Fig. 1 and a positive IFA result in Fig. 2.

DISCUSSION

Encephalitozoonosis is an opportunistic infection of mammals usually with an asymptomatic course in immunocompetent hosts (Koudela *et al*., 1993). Clinical

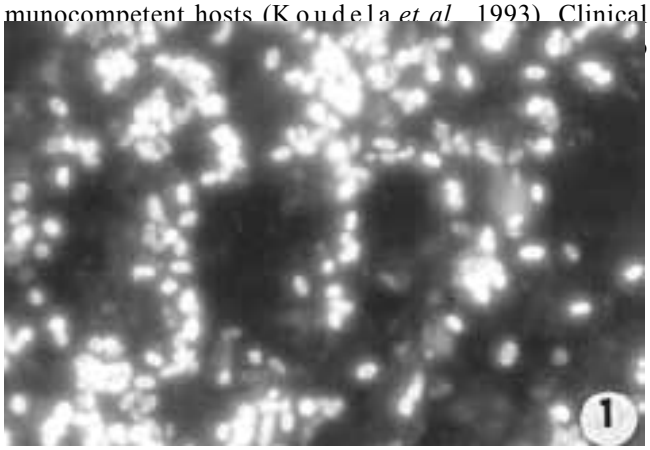

Fig. 1. Control staining of *E. cuniculi* spores without addition of the sera tested, using optical brightener Calcofluor White M2R (SIGMA), ×1000. Blue fluorescence, 450 nm colour light, 390—420 nm excited filters and 470 nm barrier filter

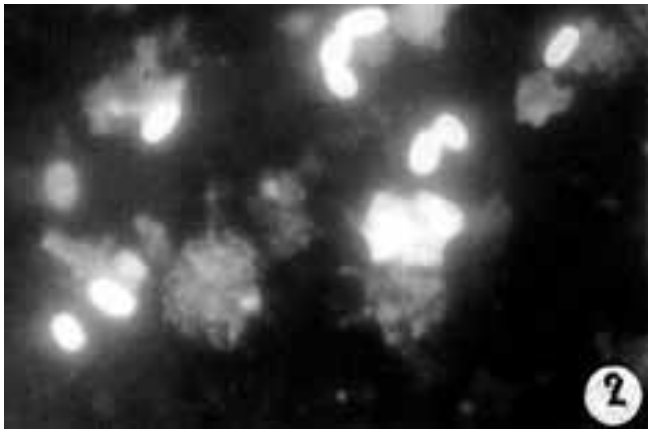

Fig. 2. A positive IFA result — fluorescence of the parasite spores after the binding of specific antibodies present in the sera of an examined animal, x 1200. Green fluorescence, 510 nm colour light, 405—490 nm excited filters and 550 nm barrier filter

practically impossible due to the marked similarity of some of the signs to rabies or other diseases affecting the nervous system (Bálent *et al.,* 1995). *In vivo* diagnosis is therefore mostly based upon the results of serological examination. Several methods have been developed for this purpose, such as complement fixation test, india immuno-ink reaction, immunoenzymatic assay, the method of indirect microagglutination, the method of radial haemolysis and the western blotting (Kučerová-Pospíšilová and Ditrich, 1998). However, the most frequently employed method for the evaluation of anti-*Encephalitozoon cuniculi* antibody prevalence is the indirect immunofluorescent test because of its reliability and sensitivity.

In Slovakia, animal encephalitozoonosis was first reported in farmed (Hipíková *et al*., 1995) and laboratory rabbits (Levkut *et al*., 1996) using IFAT as the diagnostic method. Later, the disease was also diagnosed in mice following natural as well as experimental infection (Štefkovič *et al*., 1997; El Naas *et al*., 1998). The seropositivity to *E. cuniculi*, moreover, was also confirmed in an HIV-positive man (Čisláková *et al*., 1998) and finally the first finding of IFA anti-*E. cuniculi* antibodies in cows was just recently recorded in Slovakia (Halánová *et al*., 1999).

The humoral immune response to the agent causing canine microsporidiosis was first studied by means of the IFA test in South Africa (Stewart *et al.*, 1979). As the minimal antibody titre which would represent positive infection with *E. cuniculi* was not known they assumed, by their experience with experimentally infected dogs, that titres as low as 1:20 may be significant. Non-specific fluorescence was sometimes detected at a dilution of 1:10 so that this titre was considered negative (Stewart *et al.*, 1979). Because of these facts the serum positivity in our study was evaluated on the basis of the previous findings.

Our results have shown a 29.8 % prevalence of specific antibodies to *E. cuniculi* in the dogs examined. The majority of the serum positive samples (37 out of 53) were in the titre range of 1:20 to 1:40. Similarly, Stewart *et al*. (1979) obtained results that indicated an 18 % antibody IFA prevalence in canine serum samples submitted for various clinical pathological examinations in the Pretoria and Durban areas. Most of these positive samples were also in the lower titre range. However, a 70 % seropositivity was detected in a group of dogs from previously proved *Encephalitozoon* infected kennels with more than a half positivity in the titre range of 1 : 160 to 1 : 320. Such a high prevalence can be expected after outbreaks of the encephalitozoonosis-related clinical signs and the high titres obtained would suggest recent infection.

However, it is necessary to note that in our study there we were not dealing with an average sample of the dog population in the area observed. All our dog patients were admitted to the clinic with the aim of solving their health problems. The actual seropositivity in the general canine population in Eastern Slovakia would be much lower.

Another study done in stray dogs determined a 13.3 % prevalence of antibodies to *E. cuniculi* (Hollister *et al*., 1989). In this survey 33 sera out of 248 examined reacted positively in the ELISA test at titres of from 1:1000 to 1:3200 and were classified as low, moderate or strongly positive. Comparison of total IgG and specific IgG showed that specific IgG was greatly increased in the moderately and strongly positive sera. This finding confirms our previous assumption about the lower general prevalence of anti-*E. cuniculi* antibodies in dogs.

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THE EFFECT OF OPPORTUNISTIC MICROSPORIDIAN INFECTIONN ON THE IMMUNE SYSTEM IN MICE (An Outline)

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SUMMARY

Opportunistic microsporidian infections introduce considerable risks, especially for patients with reduced immunity. Encephalitozoonosis as a microsporidian infection of mammals was unknown before 1995 in Slovakia. It is caused by opportunistic pathogens of *Encephalitozoon* spp. Active macrophages and related cells play an important role in the pathogenesis of encephalitozoonosis. Natural immunity is not based on previous individual experience of the pathogen or similar non-pathogenic microorganisms. Specific immunity is based upon the clone principle — only those cells with clones which have specific receptors react with antigens. The $CD8^+$ lymphocyte population is soon quickly activated and increases during encephalitozoonosis. The immunosuppression during bovine papillomatosis can be coupled with the appearance of opportunistic encephalitozoonosis. In the human population microsporidia have been described as opportunistic pathogens in immunocompromised patients after organ transplantations.

Key words: opportunistic infection; encephalitozoonosis; $CD8^+$ lymphocytes

INTRODUCTION

The body of the host organism activates its immune system during parasitic diseases, which restricts and eliminates parasites and their toxic metabolic residues. Immunological mechanisms have a multifactorial nature during parasitic infections. Some parasites become resistant to macrophages during phylogenesis and are adapted to intracellular parasitism. Some protozoa are inhibited and killed by phagocytic cells. In contrast, for example leishmania, cryptosporidia, *Encephalitozoon* spp., *T. gondii* or *P. carinii* reproduce and still survive the active immune response of the host. They are described as opportunistic pathogens in immunocompromised individuals. Opportunistic infections introduce considerable risks, especially for patients with reduced immunity. The concomitant incidence of protozoan infection often reduces immunity and suppresses the host's immune response (immunosuppression). Immunosuppression makes an organism receptive to infections of other aetiologies.

Microsporidian infection

Encephalitozoonosis as a microsporidian infection of mammals was unknown before 1995 in Slovakia. Clinical signs appeared by accident. Consequently *Encephalitozoon* spp. were described as opportunistic pathogens. The confirmation of microsporidian origin was the impulse for the study of this problem. Taxonomically, they are classified in the phylum Microspora (Sprague *et al.*, 1992).These parasites infect hosts in every animal class of vertebrates and invertebrates, especially laboratory animals, rabbits, carnivores, and primates, including man (Weiss and Wossbrinck, 1998). They infect different types host cells, mostly the epithelial and endothelial cells, fibroblasts, macrophages, and possibly other types of cells (Vávra, 1996).

The active macrophages and related cells play an important role in the pathogenesis of encephalitozoonosis. There are the leukocytes, which are differentiated early from pluripotent tribal cells into the progenitors of cell lines. Their development continues through monoblasts and promonocytes in bone marrow and monocytes in the blood stream, which enter tissues and mature into macrophages. NK cells (Nature Killer Cells) are described as a heterogeneous population of cytotoxic cells, which do not have a modal symbol either T- or B-lymphocytes (Pospíšil, 1994).

They are active in the early stage of parasitic infection, namely by the production of IFN γ (interpheron gamma). This stimulates macrophages and NK cell activity too. During the infection elements of natural (non-specific) and specific immunity are active. Natural immunity is not based on previous individual experience of the pathogen and similar non-pathogenic microorganisms (Korych, 1993).

Specific immunity is based upon the clone principle (Ho- rejší, 1993). T-lymphocytes are the most effective ele-

ments of specific cellular response. The adoptive transfer of sensitized syngeneic T-enriched spleen cells from mice with chronic infection protected SCID mice from lethal disease (Didier and Shadduck, 1997). The experimental infection of immunocompetent BALB/c mice suggests that the destruction of spores by macrophages depends on T-cellular factors (Schmidt and Shadduck, 1983) and consequently on IFN γ (Didier *et al.,* 1994). Some studies have demonstrated that spleen cells from chronically infected mice release cytokines after microsporidian inoculation. They activate macrophages to kill spores *in vitro* (Diddier, 1995).

However, no study has defined the accurate role of the subtypes of T-lymphocytes in immunocompromised, infected animals. One experiment observed that protection depended on the lytic effect of these cells on the infected target cells (Khan *et al*., 1999). Possibly, the number of NK cells increases irrespective of the number of T-lymphocytes during early infection. It is known that $CD8^+$ lymphocytes have an important role in the course of various intracellular infections. The $CD8^+$ population is soon quickly activated and increases during encephalitozoonosis (Khan *et al.,* 1999). $CD8^+$ lymphocytes may depend primarily on $CD4^+$ cells during infection. $CD8^+$ lymphocytes are transformed into $CD4^+$ by additional mechanisms. The existence of these mechanisms has been observed during some microbial infections. $CD8^+$ CTLs were created during infection. $CD8^+$ CTLs mediated the lysis of infected target cells. Scientists consider on the basis of these discoveries, that

— encephalitozoonosis results in the rapid activation and proliferation of $CD8^+$ CTLs;

— antigen specific CTLs show cytolytic activity against infected target cells;

— the decrease in $CD8^+$ lymphocyte cytotoxicity induces a substantial increase in the number of parasites, which kill the host in the long run (Khan *et al*., 1999).

Other studies suggest the importance of IFN γ during encephalitozoonosis. Cytokines appear to be constant, regulating molecules of the MHC 1 class, which therefore should play an important role in the induction of $CD8^+$ lymphocyte- mediated immunity. The interactions between T-lymphocytes and IFN γ during encephalitozoonosis are the subject of investigation at present.

The different production of cytokines by $CD4^+$ lymphocytes has an important regulating effect on the immune response and prevents the separation of these cells into two groups:

— Th1 cells participating in late delay over sensitive reactions;

— Th2 cells participating in a protective response (Tučková, 1994).

Th2 cells stimulate a protective response especially through IFN γ, IL-2 (interleukin 2) and IL-12 (interleukin 12) cytokines. IFN γ is an immunomodulator produced by T-lymphocytes and NK cells. The ability of B-lymphocytes present in antigens is increased after IFN γ bonds on the membrane receptors. IFN γ stimulates the cytokine activity of NK cells also. IL-2 is activated by monocytes and B lymphocytes. The importance of humoral response was observed in animals, which were experimentally infected with microsporidian *Encephalitozoon* spp. Opsonized and complement-fixed antibodies were detected in infected mice. They probably contribute to the protection of the immunocompetent organism (Didier and Shadduck, 1997). No controllable antibody response supports the development of infection in some individuals. Hypergammaglobulinaemia based on a hypersensitive response was generated in blue foxes and dogs with prolonged infection. This later results in the formation of immunocomplexes with kidney failure (Mohn and Nordstoga, 1975). IgM responses appeared in the course two weeks after infection in immunocompetent animals. Generally, the highest level of IgG is attained around 5—6 weeks and persists over a lifetime.

There are many antigen elements during parasitic infections (specific antibodies sometimes introduce only 20 %), not all antibodies generated are specific or have a protective effect. Long-lasting high levels of specific anti-bodies are exploited in the intravital diagnosis of infected animals. This makes possible the isolation of seropositive individuals from the breeds of origin.

A characteristic of encephalitozoonosis is its chronic latent course in immunocompetent individuals. The difficult clinical forms of renal failure and vascular disease sometimes with fatal ends develop during encephalitozoonosis in young carnivores. Occasionally, some cases of infection are described in naturally-infected laboratory animals. In contrast, the lethal disease has been generated after experimental infection in immunodeficient laboratory animals (e.g. athymic or SCID mice) and mice have been killed by disseminated microsporidiosis (Koudela *et al*., 1993).

The immunosuppression during bovine papillomatosis can be coupled with the appearance of opportunistic encephalitozoonosis too (Lešník *et al.,* 2000). Bovine papillomatosis is known as a benign carcinoma disease. It is related to T-cell immunosuppression following some co-factors, environmental immunosuppressants respectively, for example arsenic, lead, and cadmium. The results recorded in their experiment indicate that *Encephalitozoon cuniculi* may play an important role as opportunistic pathogens during various bovine diseases connected with immunosuppression. It is known from immunological studies of bovine papillomatosis, that infection is coupled with a significantly higher number of $CD2^+$ and $CD4^+$ lymphocytes and significantly higher number of B-lymphocytes generating molecules of IgM (Levkut *et al.,* 1997). In particular, T-cell activity is suppressed.

Opportunistic infections in the human population

In the human population Microsporidia are described as opportunistic pathogens in immunocompromised patients after organ transplantations. The appearance of microsporidian infection can be observed in HIV-infected or other immunodeficient individuals. Pneumonia due to the opportunistic protozoan parasite *Pneumocystis carinii* can be a grave life-threatening complication also (Groß *et al*., 1997). Complications due to candidosis and toxoplasmosis are often described in men with reduced immunity. Serological results have demonstrated the prevalence of *P. carinii* up to 80 % in AIDS patients in the USA and 7 % in Africa.

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THE ANTIMICROSPORIDIAL EFFECT OF ALBENDAZOLE IN RABBITS EXPERIMENTALLY INFECTED BY *Encephalitozoon cuniculi*

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SUMMARY

The development of experimentally induced encephalitozoonosis (*Encephalitozoon cuniculi*) was observed in 12 adult female rabbits of the New Zealand white breed. Animals from the negative control group were not infected by microsporidia. Animals from the positive control group were infected by *Encephalitozoon cuniculi* microsporidia with a single intraperitoneal dose of 5.10^7 ml^{-1}. Animals from the experimental group after inoculation with *Encephalitozoon cuniculi* in the same dose and way were subsequently administered albendazole — in the form of Aldifal 2.5 % susp. a.u.v. (MEVAK — SR) in a dose 5 mg.kg^{-1} of body weight (in a volume of 0.2 ml *per os* by a tube) from day 7 p.i., twice a week for 11 weeks and overall this was applied 21 times.

During the experiment the increase in body weight and the development of the clinical symptomatology of the disease were observed in relation to the serological prevalence.

Key words: *Encephalitozoon cuniculi;* rabbits; encephalitozoonosis; therapy; albendazole

INTRODUCTION

From the epizootiological and epidemiological points of view *Encephalitozoon cuniculi* (*E. cuniculi*) and *Encephalitozoon hellem* have an important role within the *Encephalitozoon* spp. *E. cuniculi* is the only known microsporidian which is an obligate intracellular parasite of mammals (rodents, rabbits, carnivores, primates) and birds. The typical mammal hosts of *E. cuniculi* are rodents and rabbits.

However, in the large-scale breeding of rabbits encephalitozoonosis takes a chronic asymptomatic-latent form. It can cause significant economic losses because of the loss of body weight (Flatt and Jackson, 1970; Pattison *et al*., 1971). As a result of various immunosuppressive factors, the latent form can develop into the clinical one (Bálent *et al*., 1995). In that case the economic losses are multiplied. Because of this and since encephalitozoonosis has a zoonotic character, prevention and therapy are the most appropriate courses.

The rules for the preventive measures proposed by Cox *et al*. (1977) and Bywater and Kellett (1978) for the breeding of rabbits are generally accepted. Works concerning the problems of pharmaceutical studies of *Encephalitozoon cuniculi in vitro* conditions demonstrate that the drugs examined had a higher or lower antimicrosporidial effect, but that *in vivo* they were not very effective or had a high toxicity (Shadduck, 1980; Pakes and Gerrity, 1989). *In vivo* albendazole seems to be the most efficacious. This is supported by the studies of Van Gool *et al*. (1993) on people suffering from AIDS with disseminated encephalitozoonosis.

Taking all this into account, we present the development of encephalitozoonosis in rabbits experimentally induced with *E. cuniculi*, after the therapeutic influence of albendazole in comparison with a untreated positive control group. The degree of development of the encephalitozoonosis, we assessed on the basis of the development of the clinical symptomatology of the disease in relation to serological prevalence of indirect immunofluorescent antibodies.

MATERIALS AND METHODS

The experiment was carried out on a group of 18 adult, female, New Zealand-white rabbits. The experimental animals were divided into two control and one experimental groups. There were six animals in each group.

The first control group, body weight 2.45—3.19 kg, represented a negative control group — the animals were not infected by microsporidia. The other control group, body weight 2.63—3.19 kg, represented a positive control group — the animals were infected with *Encephalitozoon cuniculi* microsporidia by a single intraperitoneal dose of $5 \times 10^7 ml^{-1}$.

After inoculation with *Encephalitozoon cuniculi* in the same dose and way as in the positive control group, albendazole was subsequently applied — to animals from the experimental group, weighing 2.41—3.15 kg.

Animals from the negative control group were housed separately from animals of the positive control and experimental groups. Animals of all three groups were kept in cages under standard zoohygienic conditions according to the rules of the *European Convention on the Protection of Animals* (1989). They were fed a standard feed mixture KK. Animals from the experimental group had albendazole administered in the form of Aldifal 2.5% susp. a.u.v. preparation (MEVAK, SR) in a dose of 5 $mg.kg^{-1}$ of body weight (in a volume of 0.2 ml *per os* by tube). Albendazole was administered from day 7 after inoculation with *Encephalitozoon cuniculi* twice a week for 11 weeks (in total).

The titre of antibodies was determined by the IFAT method (test of indirect immunofluorescence of antibodies) according to Chalupský *et al*. (1971). In both the positive control and experimental groups, the titre of antibodies was observed on day 0 (before infecting the animals), and after infection, on days 7, 14, 21, 30, 45, 60, 90, and 120 of the experiment. The titre of antibodies in the negative control group was observed on days 0, 30, and 120. During the experiment, food intake and general health status were observed twice a week with the aim of recording the degree of clinical symptomatology. Body weight was recorded before, in the middle, and at the end of the experiment. Values of body weight were evaluated by Student's *t* test.

RESULTS

In Table 1 we present the numerical values of antibody titres at certain intervals in the experimental and control groups.

The animals from the negative control group show the absence of encephalitozoonosis. On day 7 after infecting the animals, antibody response to *E. cuniculi* antigen was observed in four animals from the experimental group (66.66%) and in five animals from the positive control group (83.33%; apart from titre of one animal 1:64). Low titres (from 1:16 to 1:32) were not considered to be positive reactions.

In the experimental group on day 14 after inoculation (after two prior doses of albendazole) there was only an inadequate antibody response in five animals (88.33%) and the same low titre remained in one rabbit (1:32), whereas in the positive untreated control group positive serum samples were registered in all animals but two rabbits.

In the positive control group at later time intervals (days 21, 30, 45, 60, 90, and 120) in all animals positive serum samples were registered from 1:32 to a minimum of 1:64 (apart from one rabbit on day 21).

Tab. 1. The titres of antibodies in rabbits of the experimental, positive and negative control groups in the course of encephalitozoonosis

	Rabbit No	Inoculation day 0	Titre of antibodies							
			day 7	day 14	day 21	day 30	day 45	day 60	day 90	day 120
Experimental group	A1	0	1:32	0	1:128	0	1:64	1:64	1:64	1:64
	A2	0	0	0	0	0	0	1:64	1:64	1:64
	A3	0	0	0	0	1:64	1:64	0	0	1:32
	A4	0	1:32	0	0	0	1:64	1:64	0	0
	A5	0	1:32	0	1:64	1:64	1:64	1:64	1:64	1:64
	A6	0	1:32	1:32	1:64	1:64	1:64	1:64	1:64	1:64
	x	0	21.33	5.33	42.66	32.0	53.33	53.33	42.66	48.0
Positive control group	B1	0	1:16	1:32	1:32	1:64	1:64	1:64	1:64	1:64
	B2	0	1:64	1:32	1:64	1:128	1:512	1:256	1:256	1:128
	B3	0	0	1:64	1:64	1:128	1:64	1:128	1:64	1:64
	B4	0	1:32	1:128	1:128	1:256	1:128	1:64	1:64	1:128
	B5	0	1:16	1:64	1:64	1:256	1:128	1:128	1:64	1:64
	B6	0	1:32	1:128	1:128	1:128	1:64	1:64	1:128	1:64
	x	0	26.66	74.66	80.0	160.0	160.0	117.33	106.66	85.33
NCG	C1-C6	0	NT	NT	NT	0	NT	NT	NT	0

NCG — negative control group **NT — not tested** **0 — negative result**

In the experimentally-treated group from day 21 untill the completion of experiment, positive serum samples were also registered, but not in all animals. The titres did not exceed the value of 1:64 (apart from one rabbit on experiment day 21).

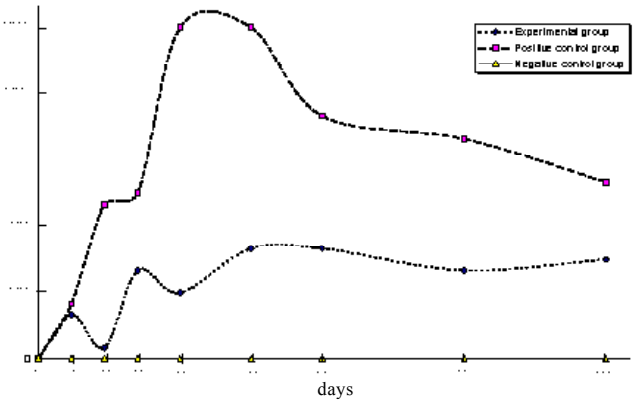

Fig. 1. The average value of the titre of antibodies in rabbits of the experimental, positive and negative control groups in the course of encephalitozoonosis

Albendazole *in vitro* inhibits the growth of various protozoal parasites, reduces the number of microsporidia, and causes growth deformities in *Encephalitozoon* spp. spores (Canning and Hollister, 1992). Koudela *et al.* (1994) studied the microsporidial effect of albendazole in immunodeficient mice, which were experimentally infected by *E. cuniculi*. Neither histopathological changes nor *E. cuniculi* were found after albendazole administra-

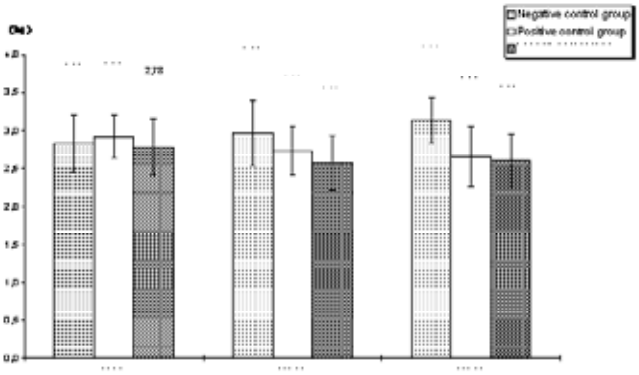

Fig. 2. The body weight of rabbits in the experimental, positive and negative control groups in the course of encephalitozoonosis

tion in doses of 5 and 50 mg.kg^{-1} body weight for 21 days. After two weeks administration, *E. cuniculi* signs were not observed, but when the therapy was interrupted, exacerbation of the disease occurred resulting in the loss of the animals within three weeks.

Orenstein (1991) explains the antimicrosporidial effect of albendazole by the way that it binds to colchicine-sensitive areas of tubulin resulting in the inhibition of its polymerization in microtubules, which leads to the blocking of cell division.

Fig. 1 demonstrates that there are significant quantitative differences in the dynamics of the changes in the average antibody titre values between the experimental and the positive control groups. Significantly higher values in the positive control group, when compared with the experimental group, were registered from day 14 until the end of the experiment. The biggest differences were observed on days 30 and 45.

The body weight of rabbits in the experimental, positive and negative control groups is presented in Fig. 2.

The Table 1 shows that the body weight in the positive control and experimental groups, at the end of the experiment, decreased, compared with their initial body weight, whereas in the negative control group this increased ($P > 0.05$). However, in the untreated group a higher growth depression is registered compared with the treated group. Statistically significant differences in body weight were not registered even between the negative control group and the treated experimental group.

During the experiment, in the experimental treated group, no symptoms of the clinical form of encephalitozoonosis were registered. Bilateral, or unilateral *conjunctivitis* and lacrimation were recorded in half of the animals from about the middle of the experimant. For 2—3 weeks later mild hair loss was recorded in rabbits.

In the positive untreated control group more significant symptoms of the encephalitozoonosis was recorded in 4 animals: In rabbit No. B1: bilateral purulent *keratoconjunctivitis,* slight incoordination of movements. In rabbit No. B2: spontaneous *alopecia* on the back area and lateral side of thigh, *incoordination* of movements, *myoclonus* in the scapula and back areas, mild *torticollis* in the last third of the experiment, seromucinous *keratoconjunctivitis,* great loss of weight. In rabbit No. B5: spontaneous *alopecia* on the vertex, back and neck areas, *myoclonus* in the scapula and back areas. In rabbit No. B6: seromucinous *keratoconjunctivitis,* mucopurulent *rhinitis*.

DISCUSSION

Latent asymptomatic infection lasts while microsporidia multiplication and the immune responses of the host are in balance. From this aspect, it is evident that pharmacotherapy can change this balance by reducing microsporidia multiplication. Fumagilin (Shadduck, 1980), chlorochin and oxytetracycline (Pakes and Gerrity, 1989) had a considerable effect in suppressing the growth and multiplication of microsporidia in cell cultures. Sulphafurazole potentiated by trimethotrim also had a certain antiprotozoal effect (Waller, 1979).

Other studies suggest that albendazole may be the most advantageous preparation *in vitro* and *in vivo*. Thus, an antimicrosporidial effect of albendazole *in vitro* against *E. cuniculi* was recorded at a concentration of 0.015 mg.ml^{-1} (Ditrich *et al.,* 1994). A concentration of 0.005 mg.ml^{-1}, led to a 90% growth inhibition of *E. cuniculi* (Beauvais *et al.*, 1994).

Our results suggest that albendazole decreases antibody response to *E. cuniculi* antigens (probably by reducing microsporidia multiplication). This is in indicated by the fact that the antibody response (titre of antibodies 1:32) found in four animals from the experimental group on day 7 after inoculation with *E. cuniculi* was so weak that after two doses of albendazole (on day 14), further specific antibodies were not detected, apart from one animal. This can also be seen from the finding that positive samples of serum with a low titre of antibodies (1:64) were subsequently recorded from day 21 up to the end of the experiment, and from the fact that we detected two negative serum samples on days 90 and 120 of the experiment.

On the other hand, in the untreated positive control group antibody response was detected in all animals on day 14 with recorded positive serum samples in four animals. Positive serum samples (apart from one animal on day 21) were registered in the untreated group up to the end of experiment and, when compared with the animals from the experimentally-treated group, the average values of antibody titres were significantly higher. The low titre of specific serum antibodies detected in the treated group corresponded well with the course of the disease.

The latent course of the disease lasted until the end of the experiment. From the middle of the experiment, bilateral, or unilateral *conjunctivitis* and lacrimation were recorded in 50 % of the animals, furthermore there was a mild, temporary increased hair loss in rabbits, when handled, lasting for approx. two weeks. These signs do not indicate the clinical form of the disease.

Some of the typical signs of the clinical form of the disease in rabbits (motor paresis particularly of the hind quarters, *torticollis, convulsions,* tremors, and *coma*), as described by Innes *et al*. (1962), Wright and Craighead (1922), were recorded in the untreated group in 50 % of the animals. The fact that along with the marked spontaneous alopecia, central nervous system signs were also registered in our animals simultaneously (*incoordination* of movements, *myoclonus* in the back and scapula areas, or even mild *torticollis* in one rabbit) suggests that the course of the disease proceeded in the clinical form. In the other three animals the disease proceeded in the latent form.

The ophthalmic finding is interesting in connection with the observation of Bjerkas (1990), who found lesions in the cornea and lens with a gradual deterioration of sight or even blindness in mink bred on farms in the course of chronic encephalitozoonosis (induced by *E. cuniculi*). The ophthalmic changes detected were most probably induced by contamination with spores from the environment. We do not suppose that it was an allergic reaction, or contamination of the inoculum e.g. by mycoplasmas and the like. This is also indicated by the that typical eye infections detected in people (*E. cuniculi, E. hellem*) result from direct contamination by microsporidia from the environment.

In the untreated group, the statistically insignificant decrease in body weights registered in the middle and at the end of the experiment, compared to initial body weights, or to the weights from the negative control group, is directly linked with the degree of disease development.

In the treated group, the mild decrease, registered in body weight has probably no connection with the disease. We suppose that this mild growth depression was induced by oesophagus irritation — long-lasting and relatively frequentoral intubation.

Our results concerning the influence of albendazole on the development of encephalitozoonosis in rabbits indicate its inhibitory effect on the development of the disease, and suggest that it is very well tolerated by rabbits. Since our results, indicating the suppressant influence of albendazole on encephalitozoonosis development in rabbits, are a positive sign that the possible medication of feed mixtures by albendazole in rabbit-breeding would be well-founded.

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THE EXCRETION OF TYLOSIN RESIDUES IN EWES' MILK AFTER ITS EXPERIMENTAL ADMINISTRATION

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SUMMARY

Seven clinically healthy ewes (Slovak merino) were used in the experiment. The ewes were treated intramuscularly with tylosin base 0.05 g.ml^{-1} (Ivatyl 50 inj. ad us. vet. Bioveta, The Czech Republic) at a dosage of 100 mg.10 kg^{-1} for five consecutive days. The milk samples were collected before treatment, and at 12-h intervals for 7 days during and after the treatment. The samples were analysed by a high-performance liquid chromatography method with solid phase extraction.

The mean values of tylosin residues in the milk were 631.6 µg.l^{-1} 12 hours after the first administration. The highest mean concentrations were detected 12 hours after the second injection (1821.6 µg.l^{-1}). After the end of the drug administration, the residue concentrations decreased, and 36 hours after the last administration the tylosin residues were found below the maximum residual limit (50 µg.kg^{-1}) and the mean concentration was 30.9 µg.l^{-1}. The residues were not detected in the ewes' milk 48 hours after the completion of the drug administration.

Key words: residues; tylosin; ewes' milk

INTRODUCTION

Macrolide antibiotics have an important role in the treatment and prevention of diseases, especially those caused by mycoplasma and gram-positive bacteria (Villemin *et al.*, 1984).

Tylosin is a macrolide antibiotic composed of a 16-membered oxygenated ring with several sugars attached and is used in veterinary medicine both as a feed additive for growth promotion and therapeutically (Delpine *et al.,* 1994). Tylosin is a broad-spectrum macrolide antibiotic which is produced by *Streptomyces fradiae* and has been developed specifically for veterinary use in the form of phosphate and wine salts. It is effective against gram-positive bacteria (e.g. *Erysipelothrix insidiosa*), less effective against some gram-negative bacteria (*Haemophilus, Moraxella, Bordetella*) and against corynebacteria and spirochaetacea (especially *Treponema hyodysenteriae*). When therapeutic doses are applied, the effect of tylosin is bacteriostatic (Vodrážka *et al.,* 1986). Ivatyl 50 inj. ad us. vet. contains a tylosin base, and is used for the treatment of contagious agalactia and pneumonia of ewes and goats.

This antibiotic may leave residues in edible animals and such drug residues can have toxic effects or cause allergic reactions in consumers (Kanjuka and Šutiak, 1990) or the induction of resistant bacteria (Delpine *et al.,* 1994).

Data from intramuscular injections of tylosin have shown that tylosin is rapidly distributed in tissue; and that it is found in lung tissue or milk at substantially higher levels than in blood serum (Moats, 1983). Therefore to ensure human food safety, the conditions of the use in animals must be established and the maximum residue limits (MRL) have been set for food products. A provisional EC (1994) maximum residue limit of 50 µg.kg^{-1} was laid down for milk. *The Codex Alimentarius of The Slovak Republic* set the same MRL (1996). It is very important in the HACCP system to take into consideration the possibility of the presence of drug residues as hazards in raw materials used for food processing (Bytrický *et al.,* 2000).

In the present study, the tylosin residues in the milk of ewes treated by intramuscular injections have been followed.

MATERIAL AND METHODS

Seven clinically healthy ewes (Slovak merino) were used in the experiment. The ewes were treated with tylosin base 0.05 g.ml^{-1} (Ivatyl 50 inj. ad us. vet. Bioveta, The Czech Republic). Tylosin was administered to the animals in a dose of 100 mg.10 kg^{-1} of live weight, immediately after the morning milking, for five consecutive days. The milk samples were collected from the morning and evening milking at intervals of 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, and 144 hours after the start of the experiment. The determination of tylosin residues was performed by the HPLC method with solid phase extraxtion (Sokol *et al.,* 1996). The method consists of

Table 1. Tylosin residues in ewes' milk (μg.l^{-1}) after the intramuscular administration of Ivatyl 50 inj. ad us. vet.

Time (hours p.i.)	Ewe No. 1	2	3	4	5	6	7	Mean ($\bar{x}$)	±SD
0*	0	0	0	0	0	0	0	0	0
12	769	–	355.1	583	1096	236	751.4	631.9	311.2
24*	0	336	190.2	0	268.5	0	102.9	128.2	139.4
36	2936	2004.4	963	1034.7	2121.3	1870	–	1821.6	738.1
48*	597.8	0	1396.9	659	323	191.7	126.1	470.6	474.2
60	2520.5	1209.6	925.4	1571	2566.9	1325.8	1411.3	1647.2	643.7
72*	160.7	229.7	205.3	185.3	450.4	260.7	351.3	263.3	103.2
84	1032.3	833.4	646.3	1188	1606	583	1432	1045.9	387.8
96*	145	190.6	170.4	88.6	310.7	99.7	120.8	160.8	75.3
108	1313.5	1275.5	271.7	475	962.1	867.1	1134	899.8	397.3
120	150.5	216.1	141.9	116.2	72.3	216.7	81.6	142.2	58.2
132	27.4	0	72.9	76.9	22.9	16.3	0	30.9	31.8
144	0	0	0	0	0	0	0	0	0

* — intramuscular administration; – no sample analysed

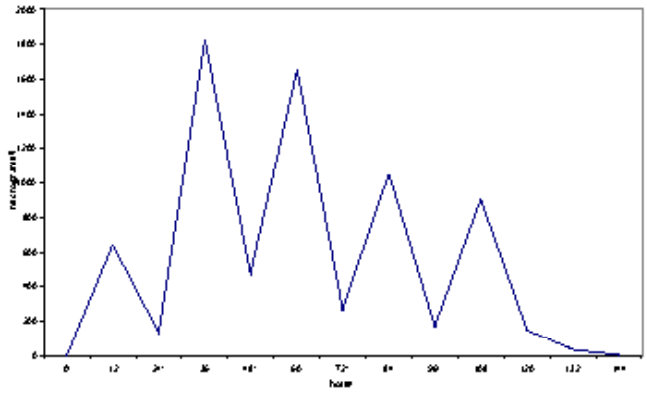

Fig. 1. Mean tylosin concentrations in ewes' milk after the experimental administration of Ivatyl 50 inj. ad us. vet.

two steps — processing the sample based on the solid phase extraction and liquid-liquid extraction, and the HPLC analysis on reversed phase column.

RESULTS AND DISCUSSION

According to our results, tylosin residues were detected in the milk samples from the evening milking (12 hours after the first drug administration). The concentrations in the individual samples were in the range from 236 to 1096 μg.l^{-1} and the mean concentration was 631.9 μg.l^{-1} (Table 1, Fig. 1). After the first drug administration the milk samples of three ewes contained no tylosin residues. These results could be caused by lowered resorption followed by lowered biological availability, increased biotransformation of the drug, or by higher milk yield in comparison with other ewes. The common antimicrobials used by veterinarians (tetracyclines, sulphonamides) have shown limited udder distribution after intravenous or intramuscular administration. Weak basic lipophilic macrolides (e.g. tylosin) showed extensive penetration into milk after intravenous treatment. Tylosin also showed good potential distribution through the udder after parenteral and intramammary administrations (Saran, 1995). The highest mean concentrations were measured 12 hours after the second drug administration (1821.6 μg.l^{-1}). After the final drug administration, we followed the rapid decrease in the mean concentrations.

Dudriková and Lehotský (1998) used the same antibiotic preparation for the treatment of heathy cows. Their results showed that at the highest recommended therapeutic dose, the withdrawal time of 72 hours after treatment is not sufficient to ensure that the milk is completely free of residues.

The residues were in detectable concentrations up to and including 132 hours after the first drug administration (36 hours after the final drug administration). However, the tylosin residues were found below the maximum residual limit (50 μg.kg^{-1}), and the mean concentration was 30.9 μg.l^{-1} (ranging from 0 to 76.9 μg.l^{-1}). The milk of two ewes contained higher levels of tylosin residues (72.9 μg.l^{-1} and 76.9 μg.l^{-1}). The withdrawal time laid down by the drug producer for ewes' and goats' milk is 48 hours. According to these results we can state, that from the food-residue standpoint, the use of Ivatyl inj. ad us. vet. is safe.

According to our results, it would be interesting to evaluate the doses of drug administered, which will secure adequate microbial inhibitory concentrations in blood serum and milk, mainly during first two days of the treatment.

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THE INFLUENCES OF *Rhaponticum carthamoides* Willd. ON THE QUALITY OF THE FINAL PRODUCTS OF FATTENED PIGS

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SUMMARY

The results of a feeding experiment on fattening cross-breed swine (landrace × Slovak domestic breed), which were genetically balanced, and of equal age and equal weight (cca 75) kg are presented. The experiment lasted 33 days. The control group A animals were fed with a classical diet which was composed of the complete feed mixture VUL-OŠ-06 for fattening pigs from 40 to 120 kg. Animals in group B (the experimental group) were fed with a diet composed of 90 % of diet A and 10 % of hay meal of *Rhaponticum carthamoides* Willd. After completing the feeding experiment the animals were slaughtered to find out the quality of the pork. The values of N-substances measured were as follows: group. A 18.37 %, group B 23.17 %; pH: group A 5.76 group B 6.08; fat: group A 3.92 %, group B 7.43 %; thickness of bacon: group A 2.5 mm, group B 2.6 mm and dry matter group A: 27.59, group B 29.83 %.

The results of the parameters measured show a better quality of pork from animals fed with diet (B) compared to the animals fed with classical diet (A).

Key words: swine; pork quality; *Rhaponticum carthamoides* Willd.; pig; hog

INTRODUCTION

At the present time the Slovak market is filled with domestic and foreign feed additives for the feed of farm animals, which aim to improve the quality of their products. Most of these additives are prepared on the basis of synthetic mixtures. In human and veterinary nutrition, nowadays, food mixtures made of natural, mainly plant sources are preferred. *Rhaponticum carthamoides* Willd. (RcW) is a medicinal plant, which has not been known in our country until recently.

This plant is easy to grow in our climate and agricultural conditions. A lot of authors have written about the positive influence of this plant on the healing of defects of the nervous system, psychological and body weakness, its anti-stress effect and its support and improvement of the immune system. These and a number of other specific effects of the RcW were found in human medicine and worked out by Opletal and Opletalová (1990).

In a number of our experiments, we followed the influence of the shoot (over-ground bio-mass) of this plant on selected types of laboratory and farm animals. In the experiment on mice, we found that a 10 % content of hay meal prepared from the shoot of RcW was the optimal diet for the growth and physiological markers of laboratory animals (Šelepcová, 1989, 1993, 1995). Higher dosages caused a slackening in the growth of the internal organs and also in the total growth of the mice (Šelepcová *et al.,* 1995). In their experiment conducted on an artificial rumen Rusitec, Šelepcová *et al.,* (1993) confirmed this result. In an experiment on rabbits, a 20 % content of the hay meal prepared from RcW in the feed influenced the increase in the fat depot in both sexes (Šelepcová, 1993).

In other experiments, we found the positive influence of the addition of the hay meal from RcW in the feed of laying hens, an increase in the weight gain and quality of mutton in sheep (Šelepcová *et al.,* 1999), and in the overall health condition and improvement in the adaptability to the stress factors of laboratory animals (Šelepcová, 1993). RcW is a perennial plant, which is able to yield up to 16—25 t/ha of the shoot. This truth forced us to test and observe it as a plant with a view to adding it to the feed of farm animals.

One of the test criteria either in new breeds, feeding technology, or new products is to find out their nutritional characteristics and the method of farming them. Besides this, the production and the quality of the product from the farm animals has to be considered.

In our experiments we found that the nutritional value of RcW is very similar to that of alfa alfa. The content of N-substances, protein, and digestible protein are equal. It is even better in the content of amino acids and the lower amount of fibre (Šelepcová, 1999). The biologically active substances, of which RcW is composed are markedly higher among all the

Table 1. Quality of the butcher's meat control group A and B

Sample No.	Bacon thickness in mm		pH		dry matter in %		Water in %		Fat in %		N- substances in g	
	A	B	A	B	A	B	A	B	A	B	A	B
1	2.4	2.7	5.74	6.00	27.55	29.36	72.45	70.64	4.09	6.20	19.00	23.32
2	2.7	2.5	5.78	6.16	27.38	29.20	72.62	70.80	3.75	8.51	17.70	23.04
3	2.2	2.3	5.80	6.24	27.67	26.87	72.33	73.13	4.15	3.28	22.39	20.36
4	2.5	2.4	5.59	5.94	27.45	31.72	72.55	68.28	3.76	8.30	16.69	24.37
5	2.6	3.1	5.67	6.06	27.50	31.43	72.50	68.57	3.98	9.37	17.41	24.50
6	2.6	2.6	6.01	6.08	27.25	30.40	72.75	69.60	3.80	8.94	17.01	23.51
Average	2.5	2.6	5.76	6.08	27.46	29.83	72.53	70.17	3.92	7.43	18.37	23.17
SD	0.18	0.28	0.14	0.11	0.36	1.75	0.14	1.97	0.18	2.31	2.13	0.60
V	0.03	0.08	0.02	0.01	0.13	3.07	0.02	3.86	0.03	5.34	4.52	0.36

SD — standard deviation; **V** — variation coefficient

fodder plants grown in Slovakia (Sláma, 1980; Šelepcová, 1993, 1999). Ekdysteroides are among several specific active substances, which carry the characteristics of this plant.

In a model experiment on fattening swine we found that the pleasant influence of a diet with 10% content of hay meal of RcW on the health condition, weight gain, and adaptability of animals under the experimental conditions (Šelep- cová, 1989). These were confirmed by our production experiments (Šelepcová, 1993).

The aim of this experiment was to determine the influence of feeding the experimental feed mixture with a 10% content of hay meal of RcW on the qualitative indicators of the meat of the fattened swine.

MATERIALS AND METHODS

The feeding experiment on the fattening swine was carried out in a sty housing. Forty animals of a cross breed (landrace × Slovak domestic breed) were chosen from 300 pigs for our experiment. They were genetically balanced, of equal age and weight of (cca 75 kg). On the first day of the experiment, we registered the weight of the animals and divided them into two groups of 20 each. Both groups were put in the same sty housing to have all the animals in the same experimental conditions.

We carried out the experiments in two phases

The 1st phase lasted seven days. It was the time for adaptation. The animals were fed with a complete feed mixture VUL-OŠ-06 for fattening pigs from 40 to 120 kg live weight. The feed dosage was according to the norm.

The 2nd phase lasted 33 days. It was the experiment proper. In this phase the animals were fed with two different diets as follows:

Group A: 20 animals were further fed with the original feed mixture and were used as a control group.

Group B: 20 animals were fed with a diet, which was composed of 90% of the diet for group A and 10% of a hay meal of RcW.

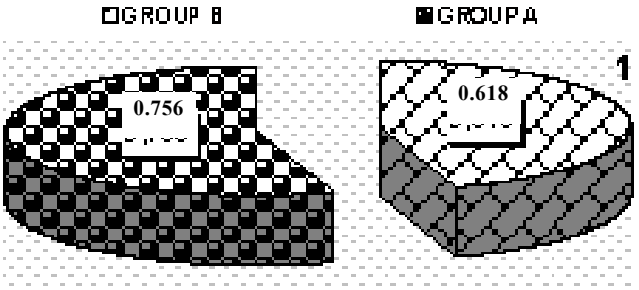

Fig. 1. Average values of the weight gain of the control group A and experimental group B

The feed mixture fed to both groups of animals was formulated in a granule form. All animals of both groups were weighed on the 26th and 33rd days of experiment to assess their weight gain.

After finishing the 33 days of the feeding experiment, six animals from each group were killed to ascertain the parameters, which were the subject of this experiment. The average weight of the animals killed was 102 kg, and they were killed in a slaughterhouse under standard conditions to assess the quality of the meat.

Samples for analysis were taken from the muscles of the MLD *(musculus longissimus dorsi)*.

The data were obtained as follows:

— pH was measured 45—60 min after slaughter in the muscle of the MLD at the last thoracic vertebrae with a piercing pH metre (Microprocessor pH Meter pH 95 (WTW)),

— dry matter content was determined by the method according to ČSN 57 0154,

— the fat content was determined by method 57 0168,

— the content of N-substances was determined by the Kjeldahl method on the KJELTEC auto-analyser 1030,

— the thickness of bacon was determined in mm and was measured by directly inserting the measure.

For statistical evaluation we used F-test and Smirn-Kolmogor test.

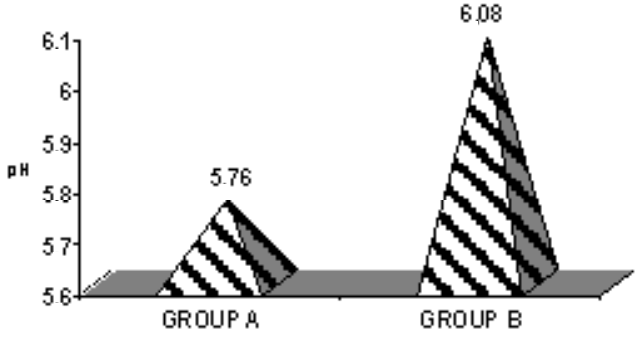

Fig. 2. Average pH values of the experimental group A and control group B

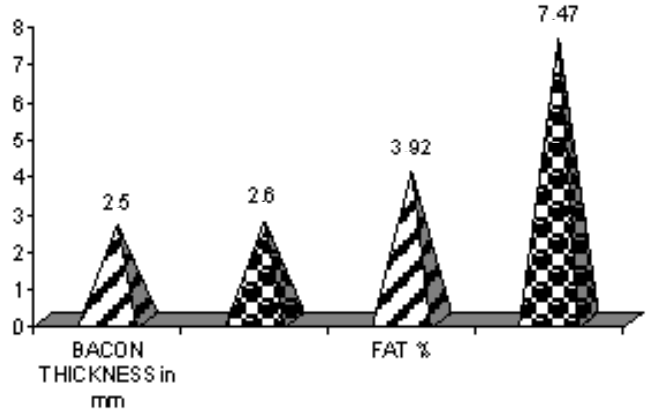

Fig. 4. The thickness of the bacon in mm and the amount of fat in percentage of the experimental group A and the control group B

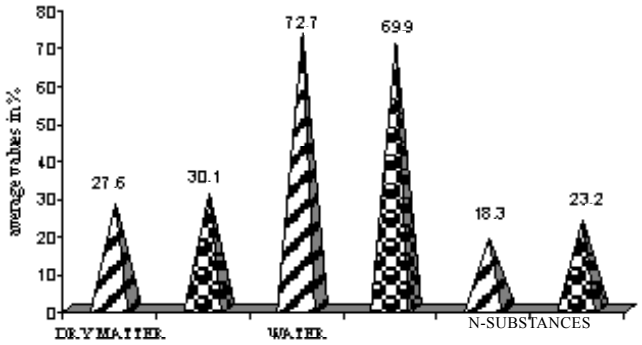

Fig. 3. The quality of the slaughter product of the experimental group A and control group B

RESULTS AND DISCUSSION

At the end of the feeding experiment the animals had an average weight of 102 kg. The average weight gain in the control group A was 0.618 kg/pig/day and in the experimental group B 0.756 kg/pig/day (Fig. 1).

Table 1 shows the values observed and percentages of the parameters measured. We have observed no statistically significant differences, despite the fact that different values were found in the two groups.

Even though the observed indicators of the quality of pork obtained from the experimental animals in group B, fed with a diet of 10 % content of hay meal of RcW, have not shown statistically significant differences compared to the meat from the control animals in group A, the results show the advantages of feeding diet B. Our substantiation is as follows.

The value of pH is one of the basic indicators of the qualitative changes of meat. pH 5.76, which is the average value measured in the meat from animals group A, is the value for a qualitative deviation of PSE (pale-soft-exsudative). These qualitative changes of meat used to happen mainly in animals, which are highly sensitive to stress burdens. Some of the stress burdens are pre-slaughter activities, transportation, loading and unloading of the animals and also their stay in the slaughtering shed. A typical mark of stress is the abnormal process of *post mortem* changes, which are manifested by the fast decomposition of ATP, by the intensive process of glycolysis with the deposition of lactate in the muscles, which later causes the decrease in the pH (Pleva *et al.,* 1985).

Krasnov *et al.* (1976) and Šelepcová (1993) describe the significant psychostimulating effect of RcW, on the laboratory and farm animals. Similarly Saratikov (1970) described the increased performance of mice and rabbits after applying an extract obtained from RcW.

The average value of pH measured in the meat of experimental animals group B, was 6.08. From this, it is possible to assume that the experimental diet in the meat experiment favourably influenced the resistance of the animals to the stress burdens. In no cases did the value of pH in this group decrease below 5.8, which is the critical level for meat PSE (Fig. 2, Tab. 1)

The values of N-substances measured were different between the two groups (Fig. 3, Tab. 1). Davidek *et al.* (1983) consider the referential value of pork N-substances to be in the range of 9.1—20.2 %. The content of N-substances in the meat of our experimental animals in group B was 4.83 % higher than in the control group A.

Feoktistova *et al.* (1967) described the favourable influence of *ekdysteroides* for the metabolism of N-substances. Watson (1982) describes the stimulating effect of *ekdysteroides* on the synthesis of RNA. Its activity depends on the direct bond of highly specified protein-receptors. The transcription of DNA following the synthesis of RNA and the formation of a specific protein is primarily stimulated. Japanese authors Seiichi *et al.* (1969) found that ekdysteroids speed up proteo-synthesis in the liver and partially the synthesis of RNA. Zalabák *et al.* (1980) confirm that oestrogens decrease the de-amination of amino acids, which leads to their better use for the synthesis of protein, which positively influences the nitrogen balance. It seems that steroids contained in RcW, reacted to the increasing content of the N-substances in the meat of the experimental animals.

The next qualitative indicator of meat is the ratio of the content of water to the dry matter. Jedlička (1988) states that the referential value of water in the muscles is 34.4—77.7%. The values obtained in the samples of our experiments are within the range of these referential values. However, under this heading, we have observed a lower content of water and a higher content of dry matter in the samples of the experimental group compared with the control (Fig. 3, Tab. 1). This fact led us to conclude that the meat obtained from animals fed with a 10 % hay meal of RcW, is of a higher quality from the point of view of energy content.

Comparing the thickness of the bacon with the fat extracted, we found an almost equal thickness in both groups. The content of the fat extracted from the muscles of the experimental animals is more than that of the control group (Fig. 4, Tab. 1). The values shown in this indicator were also within the range of the referential values given by Jedlíček (1988). Bambujeva and Salnik (1967) evaluated the metabolism of lipids on mice, to which they had applied an extract made from RcW before the burden. It was found that the content of the non-esterified fatty acids and phospho-lipids in the blood and the total content of lipids in the liver were higher than in the control group.

The authors deduce that the extract caused the interconnection between the muscle activity and the energy source of oxidation reactions, where fats can be used. On the basis of these investigations, we can deduce that in the experimental group the inter-muscular fat but not the depot fat had increased, which improves the technological and cooking characteristics of the meat.

We have learnt from accessible literature sources, that an extract made from the root of RcW is applied in human medicine for only 30 days (Opletal *et al.* 1990). After this period the stimulating effect will change to retardation. In view of this fact we have chosen 30 days for the length of feeding experiment. In the experiment on lambs, the most significant rise from days 45—60 of the experiment was measured (Šelepcová *et al.,* 1999). Therefore it would be very important to repeat and lengthen the duration of the experiment up to 60 days to achieve a new perception.

CONCLUSION

The results of our experiment show the better quality meat from animals fed a diet with a 10% content of hay meal from *Rhaponticum carthamoides* Willd. As this is our first experiment with this type of problem of observing the qualitative indicator of meat of swine fed with hay meal of RcW, we cannot arrive at a definite conclusion, even though we have found similar results in the experiment on lambs (Šelepcová *et al*., 1999) and on poultry (unpublished). However, we can confirm that the higher weight gain of the pigs in group B (0.756 kg/pig/day) against group A (0.618 kg/pig/day) was not caused by lowering the quality of the meat.

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THE RESULTS OF INTER-LABORATORY HISTOLOGICAL EXAMINATION OF FOOD

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SUMMARY

Reliability, accuracy, and comparability of results are the principal requirements for all measurement and testing procedures. Inter-laboratory tests are an important part of managing the quality of laboratory work. The guaranted quality of analytical data is the basic condition for decisions on the usability of food and certification of food products. The importance of histological methods in the analysis of food is indisputable. One reason why these methods are not used sufficiently, is that there is very little information on analytical quality of these tests. This contribution brings experiences and results from the first phase of the circle test, which was organised for routinely working laboratories, and also for veterinary educational workplaces. It is a histological qualitative evaluation of meat products, which is completed with a quantitative evaluation. Currently, classical methods together with computerized processing of received picture material can be used to make histological diagnosis more objective.

Key words: inter-laboratory examination; histological methods; food; meat products

INTRODUCTION

Reliability, accuracy, precision and comparability of results are the principal parameters for chemical, biological, and physical measurements and for examination procedures. The ways of evaluating the suitability and reliability of the methods used and the quality of work in the laboratory have been developed by using common studies to determine a set of criteria to evaluate systems of quality management. The general procedure for ensuring the quality of laboratory work includes determining the minimum criteria of quality, using reference materials for examinations, manuals of methods and work seminars for educating staff and achieving unified work procedures and evaluations of results. It is important to accept such standards, which are internationally acknowledged, e.g. documents produced by international organisations of the ISO (International Standards Organisation), Codex Alimentarius, or CEN (Comittée Européen de Normalization). The most frequently used and acknowledged systems of quality management are instructions for good laboratory practices (GLP – Good Laboratory Practice) and the European standard EN 45001. Inter-laboratory examinations are an important part of quality management as well. Participation in inter-laboratory examinations eliminates errors in the work and it is usually necessary for the accreditation of analytical laboratories (Bol- ton, 1998).

Moreover, the production and inspection of food production needs reliable analytical methods to evaluate, whether the product complies with the prescribed requirements. The guaranteed quality of analytical data is a basic condition for the certification of food products. The examination procedures also include histological methods; their importance in the analysis of food is indisputable. In practice, the supervision of food has not been utilized sufficiently for a long time now. There are several reasons for this. However, one of them is the fact that the result of histological examination depends on the experience of the examiner and cannot be expressed in exact numbers.

Histological diagnosis can be carried out at present in combination with classical methods of food histology and the possibilities of using a programme for the analysis of the picture. With the histometric evaluation of the quality of meat products, an interesting alternative could be found to chemical analysis, which also is not free of problems. New diagnostic possibilities for food histology are offered utilizing of rarely used or unused immunohisto- and cytochemical examinations (Lücker *et al*., 1999).

The problem with the routine utilization of methods, which classical and modern food histology offers, is the fact that only sporadic pieces of information on the analytical quality of these examinations are available. Histological methods are used as targeted examinations in European countries (e.g. in Germany and Austria) in the execution of veterinary supervision over food. Also, the implementation of quality manage-

ment measures in the histology of food is needed to obtain certification within the accreditation proceedings for official examination laboratories. Creation of a corresponding examination of ability in the field of histological examinations of food for laboratories in Germany was initiated at the 52nd session of veterinary hygienists in Berlin (1999). A project "*Examinations of ability in histology of food*" was elaborated. The preparation phase included planning, production, examinations, and the distribution of reference material. Besides routine-working laboratories, also a veterinary educational workplace participated in the circle examination (Lücker *et al.*, 2000).

Histological examination of food is very rarely practised in the Czech Republic. Our laboratory is the only workplace, which systematically follows the problems of the histological examination of meat products. Therefore we welcome the chance to participate in this evaluation. This contribution brings experiences and results from the course of the first phase of the circle examination.

MATERIAL AND METHODS

The laboratory examination itself includes three phases:

1st phase — preparation of histological reference material, qualitative evaluations, reporting (back) of results,

2nd phase — semi-quantitative evaluation, reporting (back) of results,

3rd phase — histometric evaluation of selected parts, reporting (back) of results.

Each laboratory received 6 reference samples which were produced in the Institute for the Nourishment of Animals at the University of Giessen, according to a common standard, basic composition (Lücker *et al.*, 1999). The samples contained finely-crushed and thermally treated mixture, with the addition of other special components, according to Table 2. The procedures for processing material samples and preparing of histological specimens are given in the *Official Collection of Examination Methods*, according § 35 of LMBG, 06.00.13. Common samples were prepared for evaluation.

Our laboratory was number 13 of those participating, and received samples for examination, numbered A 021, B 013, C 008, D 016, E 009, and F 023. The samples were processed using a classical technique of preparation such as paraffin sections. Carnoy's liquid was used for the fixation of samples. The preparations were scanning-coloured (haematoxylin-eosin) and target-coloured, according to Calleja (on collagen tissue). We have examined 6 sections of each sample in a light microscope, with magnifications 40× and 100×, respectively.

RESULTS

Results of the qualitative examination of model samples of meat products are given in the Table 1 and in picture illustrations (Fig. 1 to Fig. 5). Table 2 gives a survey of targeted components in the samples and a comparison with our results.

Table 1. Results of qualitative examination of model samples

Contents	Findings in individual samples					
	A 021	B 013	C 008	D 016	E 009	F 023
Skeletal muscle	+	+	+	+	+	+
Cardiac muscle	-	-	-	-	-	-
Smooth muscle	+	-	-	+	-	-
Collagen tissue	+	+	+	+	+	+
Elastic tissue	+	-	-	-	-	+
Parts of skin	-	-	-	+	-	+
Fat tissue	+	+	+	+	+	+
Cartilage	+	+	+	+	-	+
Bone	+	+	+	-	+	-
Udder	-	-	-	-	-	-
Lungs	-	-	-	+	-	-
Salivary gland	-	+	-	-	-	-
Nervous tissue	+	-	-	-	-	-
Liver	-	-	-	-	-	+
Kidneys	+	-	-	-	-	-
Spleen	-	-	-	-	-	-
Lymphatic tissue	-	+	-	-	+	-
Spice	+	+	+	+	+	+
Others	-	-	Re-processed	-	flour	-

Presence of individual components is marked in the table as a positive result, regardless to whether the finding occurred in one or in more examined sections.

Table 2. Targets of examination — added components (Lücke, 2000) and comparison of results

Sample	Added component — target of examination	Results — Determined/ not determined
A	Heart	–
	Kidney	+
B	Lymphatic knots	+ as lymphatic tissue
	Salivary gland	+
C	Re-processed product (mixture with cover)	+
D	Skin	+
	Fowl skin	–
E	Lungs	–
	Tonsils	+ as lymphatic tissue
	Spleen	+ as lymphatic tissue
	Brain	–
F	Fowl skin	+ as parts of skin (not specified)

Table 2 includes a survey of components, which were intentionally added to the samples and also gives a comparison with our results. However, the total evaluation of individual laboratories was targeted not only to correct, positively-determined components, but also to correct, negative evaluations of samples.

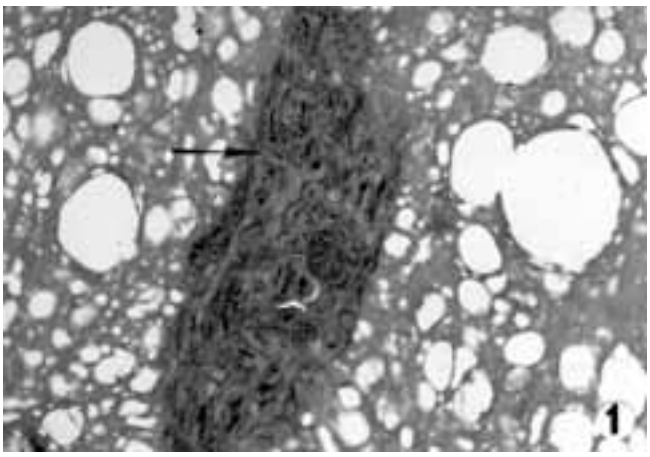

Fig. 1. Sample A 021: haematoxylin-eosin, magnification: 100×
Kidney

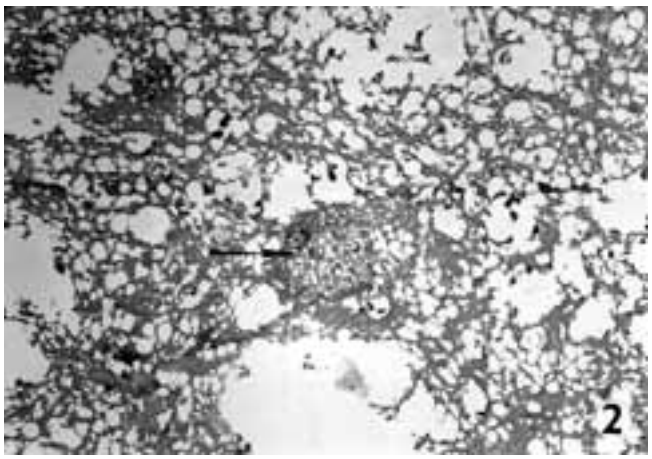

Fig. 2. Sample B 013: haematoxylin-eosin, magnification: 40×
Salivary gland

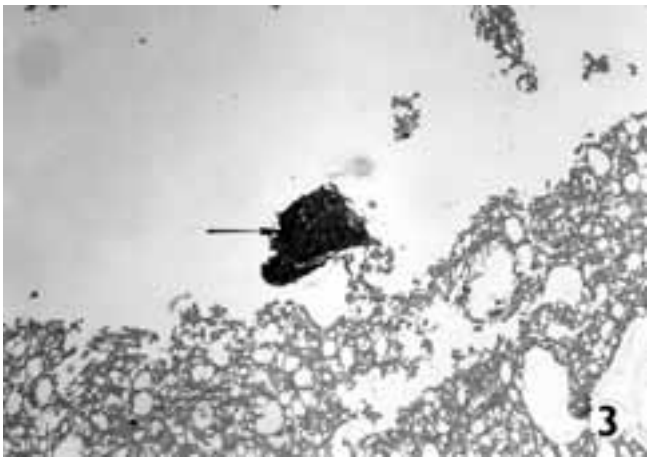

Fig. 3. Sample B 013: haematoxylin-eosin, magnification: 40×
Lymphatic tissue

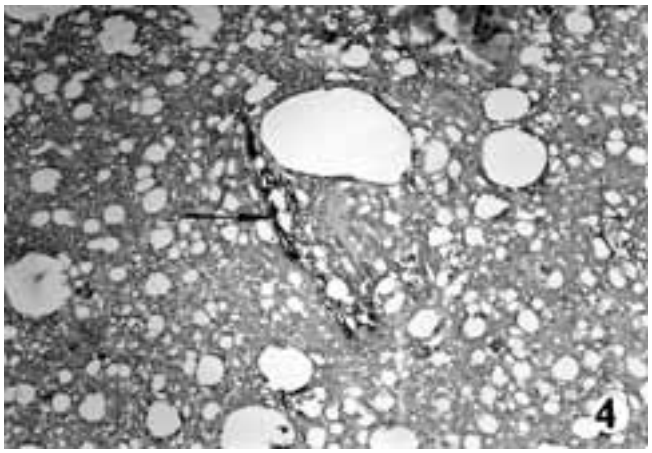

Fig. 4. Sample C 008: Calleja, magnification: 40×
Re-processed product with cover

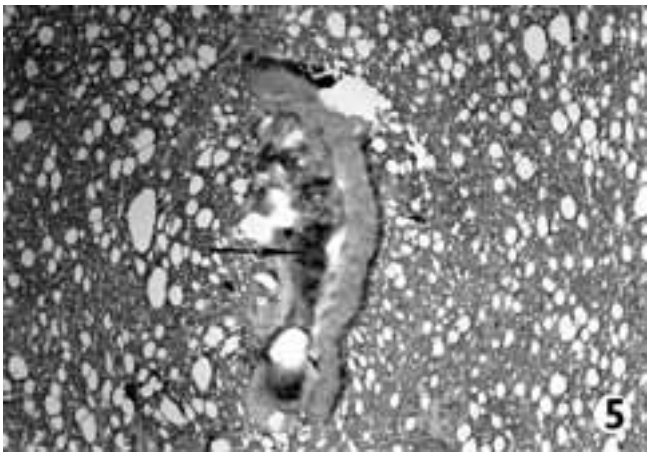

Fig. 5. Sample F 023: Calleja, magnification: 40×
Skin

DISCUSSION

The inter-laboratory tests are organized with the purpose of comparing performance and specialization of analytical laboratories of various types and specialization — microbiological, chemical, and other examinations of various materials (Berg *et al*., 1994; Boenke, 1998; Hund, 2000). For example, Horwitz (1992) gives in a general description, the performance and interpretation of results for methods of inter-laboratory studies. The measures, ensuring quality of the work in laboratories for the histological examination of food, have been discussed in literature only marginally (Lücker *et al*., 2000).

Materials were processed in the participating laboratories in the ways mentioned in the *Official Collection of Examination Methods*, according to § 35 of LMBG, 06.00.13. Our procedure was different in the phase of sample fixation. Usually, we use Carnoy's fixation liquid when processing samples from meat products. We had no

problems with processing samples compared with some other workplaces, as mentioned by Lücker *et al.* (2000).

The number of slices chosen for examinations and colouring procedures also complies with the recommendations. The procedure with scanning-colouring (haematoxylin-eosin) a target-colouring (Calleja) is the same as the procedure given in the *Official Collection of Examination Methods*. Colouring according to Calleja, which most participants used, makes collagen tissue outstanding in a way suitable for diagnosis (this tissue develops in organs, and especially in skin). Also in the case of diagnosis of re-processed products, this helps to differentiate surroudings easily.

Parts of the heart muscle were not sufficiently conclusive for us. Parts of the nerve tissue (except sections through peripheral nerve fibres) can be distinguished with difficulty in the samples; this was shown in the control examination by the authors of the project (Lücker *et al.*, 2000). We have determined lymphatic organs such as lymphatic tissue, without particular differentiation; very small pieces do not enable other differential diagnosis. The material examined was very finely-crushed and this made the evaluation difficult and reduced the chances of catching the specified material. A similar situation occurred with very low additions (fowl skin). Moreover, the authors of this project came to this conclusion, as they summarized that the data of the findings were not consistent, in relation to evaluation of presence some normal parts of meat products, such as elastic fibres. These conclusions have shown the need for the correct classification of the parts which have to be found (Lücker *et al.*, 2000).

According to Lücker *et al.* (2000), 56 examiners from 25 workplaces participated in the circle test. Evaluations were performed using a calculation of the percentage share of correctly positive and correctly negative results and a percentage share of the maximum available points. 29 participants out of 56 (52 %) correctly determined 80 % of samples. In the total evaluation, 46 participants (82 %) reached at least 80 % and 13 participants (23 %) reached at least 90 % of the maximum possible points.

Our workplace determined 79.8 % of findings correctly positively and correctly negatively and reached 87.5 % of maximum possible points. These results can be classified above the average values of both the evaluation of correctly determined findings and the total evaluation of results (Lücker *et al.*, 2000). We consider this as positive result. The participation in the circle test was the first opportunity for us to compare the level of quality of the results produced, as we are the only specialized laboratory which performs similar examinations in the Czech Republic.

CONCLUSIONS

The work gives the results of the first part of an inter-laboratory test for the histological methods of examining of meat products – qualitative evaluation. With regard to the difficulties with the material examined (high degree of crushing), not fully standardized procedures, and time pressure, the results can be considered positive. It would be good to define other standardization of examination conditions such as the precise description of techniques, improvement of other documentation, and less crushing of reference material.

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THE ISOENZYME SPECTRUM OF SERUM LACTATE DEHYDROGENASE IN ENZOOTIC BOVINE LEUKOSIS

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SUMMARY

In our work the results of enzymological analyses of the isoenzyme spectrum of lactate dehydrogenase in the blood serum of cattle are presented. The occurrence of enzootic bovine leukosis evoke interest of using electrophoretical analyses of lactate dehydrogenase isoenzymes as one possible marker of the manifestation of leukaemogenesis, or the onset of pre-leukosis initiation. Using a densitometrical evaluation of electrophoretograms of the blood serum of cattle, a decreasing trend in the individual fractions LDH_1 to LDH_5 were noticed, as well as a significantly low occurrence of the minor fractions LDH_4 and LDH_5. Serological examinations of ELISA tests were done in parallel. Significant subfractions of LDH_1 isoenzyme in the electrophoretical analysis of EBL positive animals were observed.

Key words: cattle; enzootic bovine leukosis; lactate dehydrogenase; isoenzymes

INTRODUCTION

Enzootic bovine leukosis (EBL) is caused by an exogenous retrovirus — bovine leukaemia virus (BLV, OIE 2001). History of EBL occurrence has began in the 19th century, but its enzootic character was known only in the forties of the last century, and in this time it was known in almost all European countries.

In the Slovakia, the occurrence of EBL on the basis of haematological and clinical examination was first described by Slanina *et al.* (1968) in imported dairy cows of Brown Latvian cattle. Nowadays, the diagnosis of EBL consists of the principle of the evidence of antibodies against BLV found by serological methods. Antibody evidence confirms the so-called "pre-leukosis status", which relatively rarly progresses to the clinical form of leukosis, patho-morphologically lymphosarcomatosis, resp. With regard to the pre-leukosis state, which probably consists of the initial phase of leukaemogenesis, there is not enough information about the genesis of this process. It was the reason for our decision to observe the isoenzyme spectrum of lactate dehydrogenase (LDH) in the blood serum of BLV serum-positive animals, as one possible marker of the manifestation of leukaemogenesis, pre-leukosis initiation, resp. The marker observed presents the final enzyme of the anaerobic part of glucose metabolism, and catalyses pyruvate reduction to lactate. In relationship to individual organs, it is the ubiquitous enzyme with cytoplasmic localisation. Increase in LDH activity has been described in progressive muscular dystrophia, myocardial infarcts, hepatopathias, haemolytic anaemias and in malignant tumour diseases.

MATERIAL AND METHODS

In connection with the occurrence of EBL in the East Slovak region, samples of the blood serum of Holstein-Friesian cows examined from three to six years of age (n = 12 + 12) were analysed in cooperation with The State Veterinary Institute in Košice. Blood samples were taken from the *v. coccygica media* before feeding and processed the same day. For serological examination we used the enzyme immunosorbtion test (ELISA test LINE, IBVL Ab ELISA, CZ), in which viral p 24 antigen is used.

The values of total LDH activity were determined by the Lachema test (The Czech Republic). Isoenzyme LDH_1 to LDH_5 were separated by vertical electrophoresis in 5.5 % polyacrylamide gel (Dietz and Lubrano, 1967). The limitation of electrophoretogram fractions and the extinction curve specification were evaluated densitometrically with operative and evaluative programme DensitoScan (densitometer DS 90 MikroLaAp Co. Ltd.). From the area of the densitometrical curve, the concentration of the isoenzymes spectrum was directly determined. The results obtained were a variation statistically processed, with the calculation of average values and variability characteristics. The significance of differences between the files was evaluated by the Student's *t*-test.

RESULTS AND DISCUSSION

The results of the densitometrical evaluation of the electrophoretograms of the LDH isoenzyme spectrum are listed in Table 1. When comparising the total LDH activity in both groups examined, significant differences in favour of the serum-positive group of animals (21.88 and 11.35 μkat.l^{-1}) were found. With regard to the isoenzyme spectrum, the major fractions LDH_1, LDH_2, and LDH_3 were detected in EBL a serum-negative group of cows, in EBL serum-positive cows only the major fractions LDH_1 and LDH_2 were found. However, the fraction LDH_1 in serum-positive animals was widened by a strong subfraction, and the LDH_3 fraction showed only a very low activity.

The investigation mentioned differs in its results from Blahovec and Šlesárová (1991), who found maximal concentration of isoenzyme LDH_3 in malignantly transformed cells, and the activity of which changes according to kind of malignancy. Reference values for the total activity of LDH in blood serum of cattle are declared in the scale 11.1—21.4 μkat.l^{-1}.

The characteristics of the isoenzyme spectrum in individual species of domestic animals, including fishes and pheasants has been described by Heinová *et al.* (1996). In domestic animals, specifically in Holstein-Friesian cattle, the densitometrical characteristics of the isoenzyme spectrum of LDH were identified by Wright *et al.* (1980). They mentioned the same data, the major fractions LDH_1, LDH_2, and LDH_3 and the minor fractions LDH_4 and LDH_5 in the normal physiological state of the body.

Kubešová and Vorlíček (2000) have confirmed the important position of enzyme lactate dehydrogenase in oncological diagnosis and in the symptomatology of tumoral diseases. The metabolism of tumoral cells takes place held mostly in anaerobic conditions, and it limits the increased activity of this enzyme. More studies have shown the correlation of LDH activity to tumoral disease activity in longterm observations. In isoenzyme analysis for human diagnosis, the importance of the isoenzyme fractions LDH_3 and LDH_4 is also indicated.

The study of enzyme polymorphism has shown, that there exist more enzymes, which are present in organisms in two or more isoenzyme forms. Isoenzymes of dehydrogenases, oxidases, transaminases, phosphatases and proteolytic enzymes are known. Their diagnostic use has mostly been in clinical practice in human medicine — especially in the diagnosis of myocardial infarct (LDH_1, LDH_2), chronic hepatitis (LDH_5). Racek and Slabý (1980) have declared an increase in the total activity of LDH in the human leukaemic population, but they have not registered changes in the isoenzyme spectrum yet. From damaged tissue is LDH washed up from the cells and the serum isoenzyme spectrum is equivalent to the isoenzyme profile of LDH in damaged tissue. Changes in the serum isoenzyme spectrum (higher values) in organ damage are as follows: myocard LDH_1, LDH_2, skeletal muscles (dystrophic) LDH_4, LDH_5, liver LDH_5, kidneys LDH_2, pancreas LDH_3, lungs LDH_4 and LDH_5, in shock all values are increased, mainly LDH_4 and LDH_5. In isoenzymes LDH_1 and LDH_2, 30 to 60 % of total LDH value are regarded as physiological.

Otsu *et al.* (1985) analysed the LDH isoenzyme spectrum of the patient with a tumoral disease — neuroblastoma. They identified a higher activity of total LDH and abnormal isoenzyme LDH_2 before the operation. Three days after the operation those values return back to the normal scale of reference intervals. They stated, that tumoral proliferation causes an increase in the total activity of LDH and the occurrence of isoenzyme LDH_2 subfraction, which differs from former data.

In connection with the influence of negative environmental factors, Šutiaková *et al.* (1993) described atypical zones in the electrophoretograms of isoenzymes LDH_2, LDH_3, and LDH_4 in the risk area of emission fall-out. The authors mentioned presented information about the variability of values of the LDH isoenzyme spectrum in domestic animals, which are comparable with our data.

Results of our densitometrical investigations confirm that the clinical-enzymological analysis of isoenzymes LDH_1 to LDH_5 can be used to complement the diagnosis of EBL as a possible marker of leukaemogenesis, preleukosis in cattle, respectively.

Table 1. Distribution of serum LDH in EBL positive cattle

Parameter	1st group (experimental)				2nd group (control)			
	x̄	s	x max.	x min.	x̄	s	x max.	x min.
Tot. LDH (μkat.l^{-1})	21.88	1.62	24.31	19.75	11.35	1.39	13.31	9.76
LDH1 (%)	73.31	2.03	76.42	70.29	41.61	2.25	45.05	37.73
LDH2 (%)	23.44	2.53	26.96	19.55	32.32	1.28	33.34	30.23
LDH3 (%)	2.58	1.24	4.4	0.62	21.49	2.36	25.91	19.28
LDH4 (%)	0.66	0.45	1.26	0.11	4.14	1.22	6.07	1.95
LDH5 (%)	0.44	0.32	0.99	0.06	0.61	0.68	2.00	0.06

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THE QUICK FREEZING AND VACUUM THAWING EFFECT IN ADVANCE OF TRANSITORY RIGIDITY OF MUSCLES ON THE TECHNOLOGICAL QUALITY OF RAINBOW TROUT (*Oncorhynchus mykiss*)

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SUMMARY

The quick freezing and vacuum thawing effect in advance of transitory rigitidy of muscles was tested on the pH of the muscles and texture of whole rainbow trout (*Oncorhynchus mykiss*). The increased lactic acid production on thawing brought an appreciable toughening and a significant decrease in *post mortem* pH in comparison with the control group. The vacuum thawing was without a substantial loss of fluid and any cracking of the flesh. The pH values in control group were influenced by the weight (148—226 g) of the fishes: the bigger trout had lower *post mortem* pH.

Key words: lactic acid; texture; rainbow trout; quick freezing; vacuum

INTRODUCTION

Since muscular activity uses glucose as its source of energy, active fish maintain higher levels of glucose in their blood than do sluggish fish (Miligan *et al.,* 2000). There is more glycogen in the red muscle of Atlantic salmon (*Salmo salar*) reared in a swimming raceway than in that of inactive salmon from a cage. This has important consequences for the texture of cultured fish. Raceways or the natural environment of wild fish seem to confer a more elastic texture to the flesh. The observations appear to relate to variations in the pH of the flesh, originating in variations in the glycogen content (Totland *et al*., 1987).

The physical activity converts muscle glycogen into lactic acid, and the pH of the muscle declines. In mammals, such lactic acid is rapidly removed and transported to the liver for reprocessing. After death, a proportion of any residual muscle glycogen is likewise converted to lactic acid, which lowers the pH further. If the fish have spawned recently the carbohydrate reserves are very low, the *post mortem* pH is neutral or higher, and the texture is unacceptably sloppy. In this case, it can be improved by a period of cold storage, which firms the product. Fish with muscle of relatively low pH are tough and so are unsuitable for cold storage, which toughens them further. The observations again appear to relate to variations in the pH of the flesh, originating in variations in the glycogen content (Kelly, 1969).

The "cold-shortening" can be revealed in the course of the rapid freezing of muscles before the onset of transitory rigidity of muscles. This toughening, once it has occurred, cannot be suppressed by any form of technological treatment. Hence it is preferable to freeze only meat containing negligible amounts of adenosine threephosphate (ATP) (Valin, 1982).

Once the fish are frozen, bacterial spoilage caused by the exogenous enzymes of the bacteria ceases, and autolytic changes caused by the breakdown of the chemical constituents of the fish by the intrinsic enzymes tend to be relatively slow and contribute little to any loss of quality. The major causes of quality loss during frozen storage and on thawing are dehydration, drip loss, protein denaturation and discoloration and cold-store odour. Weight loss by dehydration is directly proportional to the exposed surface area and can be reduced by two methods, covering the surface with packaging material, or surrounding the product with a layer of ice. The use of vacuum packing may be employed (Hall, 1992).

MATERIAL AND METHODS

Preparation of the fishes. The fresh fishes after capture were dissected and divided into two groups. A control group (6 fishes) was stored in the refrigerator at 3.5 ± 1 °C aerobically. A second group (6 fishes) was packed in a vacuum, weighed and quickly frozen to –18 °C. After 8 hours thawing at 18 °C packing was opened up, fishes were took out, weighed together and stored at 3.5 ± 1 °C in the refrigerator aerobically. Two samples (see down) from each fish in both groups were analysed at 12, 24, 48, 72, 96, 120, and 144 hours after death, and two samples from each fish in the control group were tested at 6 hours after death. Before the first sampling, each fish in the control group was weighed.

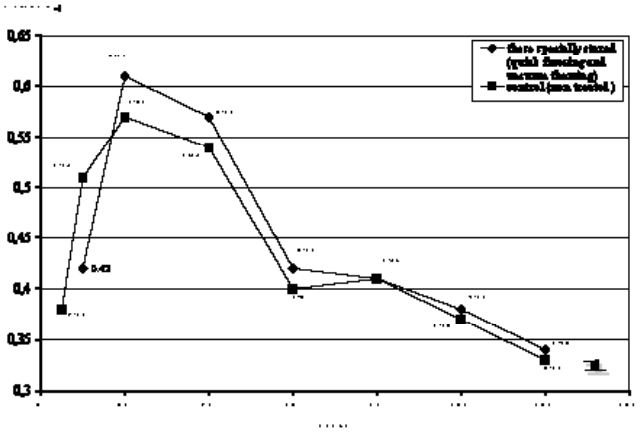

Fig. 1. The lactic acid production in trout muscles during 7 days storage of both groups

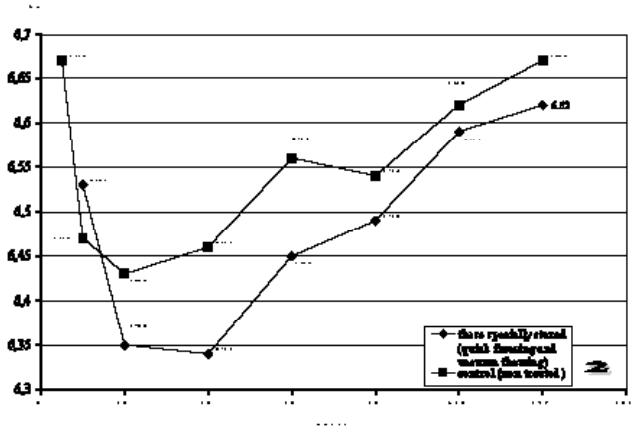

Fig. 2. The changes in pH of the trout muscles during 7 days storage in both groups

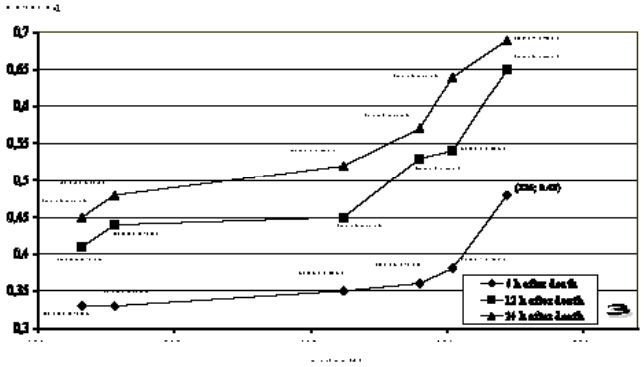

Fig. 3. The influence of body weight on average lactic acid concentrations in the muscles of control trout

Lactic acid determination. Lactic acid was determined 12, 24, 48, 72, 96, 120, and 144 h after death in both groups and 6 hours after death in the control group. Two samples (10 g) from each fish were diluted up to 100 ml with distilled water and mixed for 2 min in a mixer (BRAUN. Type: 4169). Samples were filtered with Whatman No. 1 paper. One millilitre of filtrate was diluted up to 10 ml with distilled water and tested by capillary isotachophoresis analyser (ZKI-001, Labeco, Spišská Nová Ves, Slovakia). Each sample (30 µl) was injected into the sampler of the analyser. Isotachophoretic separation was carried out under the following conditions:

— leading electrolyte (LE): 10^{-2} mol HCl + 1.5×10^{-2} β-alanine + 0.1 % MHEC (methyl hydroxyetylcelulose), pH = 3.0;

— terminating electrolyte (TE): 5×10^{-3} mol acetic acid, pH = 3—4;

— conductivity detector.

The pH value was measured at the same time intervals as the lactic acid by a pH meter (WTW mod. PH 90), and the glass electrode was applied directly to the fish muscles.

Mathematical and statistical analyses. The analytical lactic acid data were processed and evaluated by the method of linear regression (according to the program GraphPad Prism). The average values of lactic acid concentrations and pH were calculated by statistical analyse. *T*-test was used to determine the significance of the differences between the groups.

RESULTS

The changes in the average values of lactic acid concentrations in the rainbow-trout muscles during storage under aerobic conditions at 3.5 ± 1 °C are shown in Fig. 1. It needs to be noted that there was no statistically significant difference between average lactic acid concentration values of the specially stored and control fishes. The lactic acid production was found to be later but higher in the specially stored fishes compared with the control, and thus it influenced the pH changes of the fish muscles during storage. The lower *post mortem* pH of the specially stored fish muscles retarded the autolytic changes, and the pH profile of average values in both groups of fishes (Fig. 2) differed significantly ($P < 0.05$).

The experiment showed that the body weight (148—226 g) of the control trout had little effect on the lactic acid production in the muscles during the 24 h after death. The influence of body weight on average values of lactic acid concentrations in the rainbow-trout muscles was greater after 24 h than after 6 h or 12 h storage at 3.5 ± 1 °C aerobically (Fig. 3).

The lactic acid production showed that bigger fishes toughened more rapidly than smaller, but for the most important factor influencing texture, we must look to the pH of the muscle. The flesh with the pH values between 6.3 and 6.5 represented the most acceptable (normal texture); that with pH above 6.55 was unacceptably sloppy.

The vacuum thawing was without a substantial loss of fluid or any fragmentation of the flesh. Moreover, the gases released from the fish as thawing proceeded did not cause any rupturing of the muscles.

DISCUSSION

"Biological condition" is a general term referring to the state of the energy reserves of a fish, either replete from a period of feeding or impoverished from starvation, spawning, or a combination of the two. Carbohydrate reserves usually start to decrease at the outset of depletion, but in some species they can be maintained by synthesis within the fish from protein or lipid precursors (Hall, 1992). A detailed study of the effects of starvation and the resumption of feeding on the carbohydrates of rainbow trout shows that starvation reduces the glycogen level in the liver, but in both red and white muscle the level of glycogen is maintained. Since the glycogen has decreased in the livers, these findings alone are insufficient to demonstrate gluconeogenesis in starving rainbow trout (Black, 1983).

The specific activity of phosphoenolpyruvate-carboxykinase (PEPCK) and fructose 1-6 diphosphatase (FDPase) activity increased significantly in the liver of trout during starvation (Black, 1983). The starvation leads to visible emaciation, but this is not so common in non-fatty species (Love, 1988). The experiment demonstrated that larger fishes were considerably tougher than smaller fishes. The muscles of larger fish are intrinsically tougher and after death tend to stabilize at a lower pH than that of smaller fish.

The pH of the flesh is of great importance in fish technology. It is the most important factor governing the texture of the cooked flesh. The pH of the flesh exerts a big influence on the strength of the connective tissue that holds the muscles together. Connective tissue is strong at neutral pH but greatly weakened at more acid values, so that muscles crack or fall to pieces (Love, 1980; Lavéty *et al*., 1988). Consequently, they cannot be mechanically skinned, hung on a tenter for smoking, or sliced. The fragmentation appears not to be affected by the method of thawing, rate of freezing, or the length of cold storage (Love, 1988). There are, however, important species differences that stem from intrinsic differences in the mechanical strengths of the connective tissues involved (Yamaguchi *et al*., 1976). The cold connective tissue buffered at pH 7.1 is more that four times as strong as that at pH 6.2: it is the rupture of connective tissue that underlies the fragmentation (Love *et al*., 1972). The experiment demonstrated that rainbow-trout muscles with pH values between 6.3 and 6.5 were most distinctive and formed an advantageous texture without any cracking.

When fishes were frozed to –18 °C, the lactic acid production was stopped, but during thawing it was activated and increased. Rapid freezing can therefore be used to good effect on fish, that are unpleasantly sloppy through having a high pH: the texture can be made to firm up and improve acceptability. In the case of fish with a low pH, the texture will get to much firm, therefore it will be unacceptable; the act of vacuum freezing and thawing increases lactic acid production, significantly deacreases pH values, and alone toughens the texture appreciably.

In fact, during the thawing of any fish product there is a loss of fluid from the flesh, which is explained by the denaturation of the protein during the freezing process. Of course, as the temperature falls, more of the water is converted to ice and the concentration of enzymes in solution increases, therefore below the freezing point of water, the concentration and temperature are very closely related. The optimum temperature range for the denaturation of the protein is –2 to –1 °C. Thus, in order to reduce thaw-drip to minimum, the time spent in this temperature zone during freezing must be as short as possible (Hall, 1992).

The experiment showed that the effect of quick freezing and vacuum thawing in advance of *post mortem* transitory rigidity in rainbow trout is of considerable importance in the maintenance of fish quality. The characteristic negligible amounts of ATP in trout muscles did not give rise to marked contractions during thawing, what could bring about not merely an appreciable toughening but also a sustantial loss of fluid. We suppose that quick and deep freezing reduces the increase in the concentration of enzymes and other compounds, and thus may cause denaturation of the protein (with loss of its water-binding capacity). The large numbers of small ice crystals reduced the possibility of shrinkage and rupture in the cell walls. The fluid lost was absorbed by the fish, and vacuum thawing did not lead to a substantial loss of fluid or any rupturing of the flesh.

The quick freezing and vacuum thawing in advance of postmortal rigidity of muscles extends the high-quality life of the fish. The rates, at which the autolytic processes take place, is dependent upon the lactic acid production and temperature during storage. The autolytic and deteriorative processes were retarded in the group of trouts after quick freezing in comparation with the control.

Temperatures below –10 °C or –12 °C inhibit the growth of all bacteria and of the majority of yeasts and moulds, respectively. Storage at –18 °C can thus be regarded as an ideal method for stabilizing the microbial flora. Lowering of the temperature reduces reaction rates. Furthermore, as the water in the fish freezes it becomes bound, thus reducing the water activity (a_w) and hence bacterial growth (Rosset, 1982).

Thus it may be said that quick freezing and vacuum thawing in advance of postmortal rigidity preserve fish by a combination of reduction of temperature, lowering in pH values, and water activity. This effect ensures a better quality of fish, which can be readily acceptance by the consumer.

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VETERINARY DRUG RESIDUES AND THE SAFETY OF FOODS OF ANIMAL ORIGIN (Current Issues)

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SUMMARY

The current agricultural practice and rearing of animals for food depends heavily on the use of pharmacologically active compounds — veterinary drugs. Such drugs are beneficial to animal health, animal welfare, and the economic returns on animal rearing. The use of veterinary drugs has helped to increase the food supply, however, their negative consequences, such as the presence of drug residues in foods, cannot be ignored. The presence of drug residues in foods can be a health hazard to consumers.

The safety of food is a cause for growing consumer concern on a worldwide scale. Today's consumers require the safest possible food in sufficient quantities at a reasonable price. In order to protect the health of consumers of foods of animal origin, it is necessary to lay down effective and unified official monitoring and testing programmes for the control of veterinary drug residues in foods of animal origin. National reference and control laboratories should carry out these programmes in accordance with the accepted veterinary-hygiene legislation.

Key words: veterinary drugs; residues; legislation; monitoring

INTRODUCTION

The presence of veterinary drug residues in foods of animal origin intended for human consumption has become a subject of continuous concern on the part of health and veterinary authorities, food hygiene and research programmes and the public in general. Veterinary drug residues occur in foods of animal origin as an unwanted concomitant consequence of the use of drugs in animal production. In all the animal industries, veterinary drugs are used for three primary reasons: (1) therapeutically, for treating existing disease conditions; (2) prophylactically, at subtherapeutic concentrations; and (3) subtherapeutically for production enhancement (increased growth rate and feed efficiency). Residues can occur in all types of foods of animal origin including milk, meat, internal organs (liver, kidney), skin, fat, eggs and honey (Nogueira and Silva, 1989; Brady and Katz, 1992).

The health of food-producing animals is intrinsically linked to the health of humans, the consumers of the products. The logic is, if you improve the health of animals, the health of the human population should not be compromised. Public attention today focuses primarily on the beneficial and adverse effects on human health of veterinary drugs administered to food-producing animals. The beneficial effects are derived largely from the maintenance of good health in animals and, therefore, the reduction or elimination of chances that disease will spread from animals to humans. Thus veterinary drugs considerably help to increase the food supply. However, the drugs administered to food-producing animals and the residues of these drugs could enter the human food chain and increase the risk to humans (Coffman *et al*., 1999).

The negative effects of veterinary drug residues on the health of humans depend on a number of factors. The main factor is the *human organism* invaded by the unwanted residuum, an exogenous substance. No less important factor is the very *substance* and its *quantity*. According to Paracelsus, any substance, including one, which has been considered harmless, can cause damage to health provided that it was consumed in excessive amounts. The negative effect of a veterinary medicinal product depends also on the *pharmacokinetics* of the drug. The aim of pharmacokinetic studies is to obtain information regarding the consumer's safety about the pathways and movement of the active substance in the animal body by observing its uptake, i.e. absorption, distribution, chemical conversion, i.e. biotransformation or metabolism, elimination, and excretion.

By studying the pharmacokinetic properties of a veterinary drug, one can obtain information about the persistence of its unwanted residues in edible tissues and determine the withdrawal period for the veterinary drug (Gracey and Collins, 1992; Hera and Billová, 1994; Wild, 1997; Šutiak and Šutiaková, 1998).

Veterinary drugs are divided generally into systemic substances (with low molecular weight) and non-systemic substances (with high molecular weight). Systemic substances are absorbed by the body within the gastrointestinal tract (stomach and

intestines) and distributed throughout the body by circulating blood in substantial amounts. The non-systemic substances are not absorbed through the gastrointestinal tract or only in trace amounts. If the drugs are supplied to the animals in water or feed, only those, which are absorbed by the gastrointestinal tract, may induce residues in edible tissues of food-producing animals. With these drugs, the determination and observation of a withdrawal period is necessary (Coffman *et al*., 1999).

Residues of veterinary drugs, their metabolites or products of degradation may exhibit acute or chronic toxicity in man. It is the chronic toxicity, which is more important in practice, i.e. the toxic effects observed after the long-term administration of veterinary drugs in small amounts or subtherapeutic doses. The possible clinical implications of consuming residues of veterinary drugs on man are carcinogenicity, genotoxicity, mutagenicity, teratogenicity, embryotoxicity, hypersensitivity, etc. (Wild, 1997; Coffman *et al*., 1999).

LEGISLATIVE BACKGROUND TO TESTS FOR RESIDUES OF VETERINARY DRUGS

The control of residues of veterinary drugs in food-producing animals has been a cornerstone of the European Union's (EU) agricultural policies to ensure consumer protection and promote even competition for markets for many years. One of the basic principles anchored in the EU legislation concerning marketing authorization of veterinary drugs is that foods of animal origin produced by animals treated with veterinary medicinal products must not contain residues that might pose a risk to the health of consumers. Residues of veterinary drugs are all pharmacologically active substances of prescribed purity or complying with requirements on purity specified by the criteria of Food and Agriculture Organization (FAO), World Health Organization (WHO), or the European Union) regardless of whether they are active principles, excipients, or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal products in question has been administered (Council Regulation (EEC) No 2377/90; Co-dex Alimentarius of the SR, 1996).

Before a veterinary medicinal product intended for food-producing animals can be authorized in the EU, in accordance with Council Directive 81/851/EEC, all pharmacologically active substances contained in the product have to undergo a safety and residue evaluation (the establishment of a legally binding maximum residue limit of all pharmacologically active substances) under Council Regulation (EEC) No 2377/90, and have to be included in Annexes I, II or III of the Regulation (Grein, 2000). **The Maximum residue limit** (MRL) is the maximum concentration of residue resulting from the use of veterinary medicinal products (expressed in $mg.kg^{-1}$ or $\mu g.kg^{-1}$ on a fresh weight basis) which is legally permitted by the Community as acceptable in or on a food (Council Regulation (EEC) No. 2377/90). The MRL is the highest acceptable quantity of pharmacologically active substances that poses no risk to human health and may be present in an animal product as a result of the approved use of a veterinary drug complying with the requirements on withdrawal period before obtaining the product from the treated animal (Codex Alimentarius of the SR, 1996).

For so-called "old" substances, i.e. substances that were included in veterinary medicines on the market in the EU on the day Regulation 2377/90 came into force on January 1st, 1992, a transition period was garanted to allow for the evaluation of these substances. This transition period, which originally expired on December 31st, 1997, was extended until December 31st, 1999 for those old substances, for which a MRL application was submitted by producers before the fixed deadline. Any substance not included in Annexes I, II or III by December 31st, 1999 resulted in the marketing authorisation of products containing this substance being withdrawn.

With effect from January 1st, 2000, only those substances listed in Annexes I, II or III of Council Regulation (EEC) No 2377/90 can be used in food-producing animals. Annexe I of Regulation No 2377/90 lists substances for which definitive MRLs have been established, Annexe II comprises substances which are considered as generally safe and therefore it appears unnecessary to establish MRLs for them, and Annexe III presents a list of substances for which only provisional MRLs have been established. A safety and residue evaluation may also result in a recommendation to include a substance in Annexe IV of the latter Regulation which means that the substance is prohibited from use in veterinary medicinal products for food producing animals.

The safety and residue evaluation in accordance with Regulation 2377/90 is carried out by the Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA), supported by safety and residue experts, upon receipt of a valid application for the establishment of maximum residue limits (Gre- in, 2000). The MRLs' values are based on the pharmacokinetics and toxicological testing of pharmacological substances, their metabolites and products of their degradation, on studies of their acute and chronic effects including genotoxicity, carcinogenicity, mutagenicity, teratogenicity, embryotoxicity, hypersensitivity, etc. (Gracey and Collins, 1992; Wild, 1997) (Fig. 1).

Maximum residue limits are based on Acceptable Daily Intake (ADI), mean weight of a person (60 kg), and daily consumption of foods of animal origin. Since accurate consumption figures are difficult to obtain, and there are, in any case, substantial variations between individual consumers and the foods eaten in different countries, to ensure the protection of the majority of consumers it is assumed that the average person consumes, on a daily basis, a market basket consisting of 500 g of meat from large slaughter animals (made up of 300 g of muscle, 100 g of liver, 50 g of kidneys and 50 g of fat) or from poultry (made up of 300 g of muscle, 100 g of liver, 10 g of kidneys, 30 g of fat and 60 g of skin) together with 1.5 litres of milk, 100 g of eggs or egg products, 10 g of honey and 300 g of fish muscle. The total amount of residues present in this daily food package is not allowed to exceed the ADI (Hera and Billová, 1994; Grein, 2000).

Once MRLs have been allocated, it is then necessary in the context of granting a marketing authorisation for a veterinary medicinal product to determine the withdrawal period. This is the period after the administration of the product during which

Fig. 1. The procedure for establishing limits for residues (Wild, 1997)

PHARMACOLOGICALLY ACTIVE SUBSTANCE
⇩
Toxicological testing
absorption, distribution, biotransformation, excretion;
acute and chronic toxicity;
genotoxicity, carcinogenicity, mutagenicity, embryotoxicity, hypersensivity, etc.
⇩
NOAEL
⇩
safety factor
⇩
ADI for humans
⇩
MRL
(maximum residue limit in foodstuffs)

— NOAEL (no observable adverse effect level) — maximum dose without observable adverse effects in animals, expressed in mg per kg body weight and day
— SF (safety factor) — NOAEL:100
— ADI (acceptable daily intake) — acceptable daily intake for humans that can be ingested daily over a lifetime without a health risk to the consumer, expressed in mg per kg body weight and day — ADI = NOAEL:SF

it can adversely affect the harmlessness of animal products, or the time between its last administration and obtaining the product from the treated animal (Hera and Billová, 1994; Codex Alimentarius of the SR, 1996). The withdrawal period corresponds to the number of days (or hours) that must elapse from the last administration before the food products from the treated animal can be used for human consumption. After the elapse of the withdrawal period the concentration of veterinary medicine present in the food product should not pose a risk to the consumer's health, i.e. it should correspond to the MRL or remain below this limit (Codex Alimentarius of the SR, 1996). It is interesting that although the MRL values are obligatory for all the EU Member States, the withdrawal periods are set individually by each state according to its respective specialists (Elliot *et al.,* 1998).

Maximum residue limits of veterinary medicinal products specified by the Codex Alimentarius of the SR (part 14, 1996) are in agreement with MRLs included in Annexe I Council Regulation (EEC) No 2377/90.

The maximum residue limits are only of value if backed up with good residue control programmes. With regard to the increased interest of the public in the protection of human health, the need for a unified monitoring system for the control of veterinary drug residues in animal products carried out by national reference and control laboratories, in agreement with the approved veterinary hygiene legislation, comes into the foreground (Council Directive 96/23/EC).

THE MONITORING OF RESIDUES OF VETERINARY MEDICINAL PRODUCTS

The monitoring of residues of veterinary drugs in animal products should give the consumer a guarantee of the safety and wholesomeness of foods. National reference laboratories carry out the control of residues of veterinary medicines in animal products in accordance with Council Directive 96/23/EC to ensure that MRLs established by the EU are not exceeded. Council Directive 96/23/EC requires that the routine analytical methods accepted during the MRL procedure are made available to the national reference laboratories for residue monitoring.

Once the CVMP has evaluated the MRL application and recommended the inclusion of a substance in Annexes I or III of Regulation 2377/90, the EMEA, together with the scientific opinion for the establishment of (provisional) MRLs and the CVMP Summary (Assessment) Report, submits a substance-specific analytical method to the European Commission. The analytical method submitted comprises a complete description of the procedure as well as validation data.

On the basis of the scientific recommendation, the Commission prepares a draft Commission regulation amending the respective annexes of Regulation 2377/90. Any such regulation requires the adoption by the EU Member States before the European Commission can adopt it. The Commission, when circulating the draft of any Regulation amending Annexes I or III of Regulation 2377/90 to Member States, submits the accompanying analytical methods which were approved by the CVMP via their permanent representatives.

The provision of the regulatory methods approved by the CVMP to reference laboratories has been facilitated with the latest amendment to Regulation 2377/90, which is Council Regulation (EC) No 1309/99. This Regulation lays down that the EMEA provides the competent authorities and the Commission with appropriate methods for identifying pharmacologically active substances for which the MRLs have been determined, once the MRLs have been adopted and published in the Official Journal of the European Communities. However, these provisions only apply for new MRL applications submitted after coming into force in Regulation 1309/99 (Grein, 2000).

EU legislation regarding the control of residues of veterinary medicinal products (Directive 96/23/EC) came into force on 1st July, 1997. This Directive intends to ensure the quality and safety of foods of animal origin for human consumption and lays down measures to monitor the substances and groups of residues in live animals and animal products listed in Annexe 1:

Council Directive 96/23/EC (ANNEXE 1)

Group A — Substances having anabolic effect and unauthorized substances

(1) Stilbenes, stilbene derivates, and their salts and esters
(2) Antithyroid agents
(3) Steroids
(4) Resorcylic acid lactones including zeranol
(5) Beta-agonists
(6) Compounds included in Annexe IV to Council Regulation (EEC) No 2377/90

Group B — Veterinary drugs and contaminants

(1) Antibacterial substances, including sulphonamides, quinolines
(2) Other veterinary drugs
  (a) Anthelmintics
  (b) Anticoccidials, including nitroimidazoles
  (c) Carbamates and pyrethroids
  (d) Sedatives
  (e) Non-steroidal anti-inflammatory drugs (NSAIDs)
  (f) Other pharmacologically active substances
(3) Other substances and environmental contaminants
  (a) Organochlorine compounds including PcBs
  (b) Organophosphorus compounds
  (c) Chemical elements
  (d) Mycotoxins
  (e) Dyes
  (f) Others

In the control of residues, it is necessary to have a good internal system of regulatory control of drugs. National programmes of residue testing have adopted a two-tier testing system consisting of screening and confirmatory tests (Decision 93/256/CEE and Decision 93/257/CEE). Determination of the presence or absence of veterinary drug residues in animal products depends fully on the sensitivity of the analytical method used. Screening tests (with a qualitative design allowing the detection of compounds at the level of the MRL) are rapid, high-volume, low-cost tests that allow one to classify a large number of samples as being either "negative" or "potentially positive". All potentially positive samples are then subjected to a confirmatory test (with a quantitative or qualitative design allowing the detection of compounds at or below the level of the MRL). These are low-volume, high-cost tests geared to produce no false positives and a minimal rate of false negatives. This combination of screening and confirmatory tests provides an efficient and cost-effective means of controlling veterinary drug residues in animal products (Elliot *et al*., 1998; Abjean *et al*., 2000).

The development of individual analytical methods with appropriately low detection limits, complying with complex and mutually interlinked requirements for screening procedures (rapid, reliable, broad-spectrum, sensitive, low-cost), constitute an impressive challenge. The methods already developed are related to the specific needs of the final consumer and analytical technologies available at the time. Investigation of veterinary drug residues was initiated in the early fifties. The veterinary profession has occupied an essential role in monitoring the occurrence of intolerable concentrations of veterinary drugs in animal products. The criteria for the assessment of the negative effects of veterinary drug residues have grown stricter. The massive administration of these substances in the rearing of farm animals must be subjected to continuous and uncompromising control. Foods are a basic, everyday commodity, therefore the food and health aspects of administering of these pharmacologically active substances are very important.

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CHRONICLE

Prof. MVDr. Tomáš Gdovin (1921—1969)

Prof. MVDr. Tomáš Gdovin — an important personality in Slovak veterinary circles, the head of the Department of internal diseases of even-toed ungulates, vice-rector of the College of Veterinary Medicine, dean of the Veterinary faculty of the Agricultural College in Košice, member of SAPV, corresponding member of CSAPV.

Prof. Gdovin was a native of the East Slovakian village Porubka, district Bardejov. He was born to a large peasant family on the 8th April, 1921 as one of 8 children. The village and peasant environment in which he grew up decided the direction of his university studies and the other activities in his life.

After completing the secondary education at the Collegiate Secondary School in Prešov in 1942 he decided to study veterinary medicine. In the years 1939—1945 it was not easy in Slovakia to make one's way in this discipline. In November 1939 when the Czech protectorate closed all Czech universities the students of the College of Veterinary Medicine in Brno and those who were interested in this study found themselves in a critical situation. Although the universities and colleges in Bratislava were able to function, there was no veterinary faculty there and its establishment was unrealistic despite the efforts of the Veterinary Chamber, particularly its chairman Dr. P. Opluštil. The Ministry of foreign affairs of the Slovak State arranged in the first months of 1940 the opportunity for Slovak students to complete or start their veterinary studies in Vienna, Zagreb, or Budapest.

This opportunity was taken also by Tomáš Gdovin who applied to the Ministry of Economy for a veterinary scholarship. He was successful so he was able to enroll in the College of Veterinary Medicine in Vienna in 1942. There he completed 5 semesters of study. After the end of the World War II he changed to the College of Veterinary Medicine in Brno. He completed his veterinary studies in Brno by obtaining a veterinary diploma on the 9th July, 1946. He graduated on the 15th February, 1947, on the basis of successful defence of the thesis: *"Trypanosoma equiperdum infection and the occurrence of parasites in the peripheral blood of rats"*.

After obtaining a teaching qualification he joined the staff of the Buiatrics Clinical Department headed by Prof. MVDr. A. Klobouk as an assistant. His work at this department, although for relatively short time (from the 1st September, 1946, till the 31st October, 1947) affected his further personal and professional development. He considered Prof. Klobouk his teacher. From the 1st December, 1947, he was an employee of the Office of the Commisioner for Agriculture and Land Reform in Bratislava and from the 28th March, 1949, a district veterinarian in Sereï. Both periods of employment significantly affected his professional development. As a practical veterinarian he had remarkable success in prophylactic and curative activities and in establishing intensive animal production units. These were the posts that allowed him to obtain a wide practical knowledge of facts and abundant experience, which he could use later at the university.

On the 17th July, 1951, he returned again to academia, only this time to Košice, to the newly established College of Veterinary Medicine. He was appointed the head of the 2nd Clinical Internal Disease Department. From 1953 to 1957 he headed the Department of Infectious and Invasive Diseases and in 1957 was appointed the head of the Clinical Department of Internal Diseases II. In order to stress the orientation of the department in the process of education its name was changed to the Department of Internal Diseases of Even-toed Ungulates. He deserves the biggest credit for both establishing and building up the department's personnel and material equipment.

Originally, the department was located in the same building the Clinical Department of Clinical Diagnosis and Internal Diseases I (headed by Prof. Dr. Fried) so the facilities for education and the out-patient's clinic and hospital were shared. In the beginning, the department had to operate in modest but very difficult conditions in the facilities of the former Comenius Institute which were adapted by the members of staff themselves who worked in self-organized teams. Prof. Gdovin built up the clinic together with young assistants who had just graduated from the College of Veterinary Medicine in Brno.

By overcoming organizational, personnel, material, and spatial problems, he started up the clinic with great initiative and success. The internal and external conditions were gradually resolved. The building up of a clinical block intended for education and in-patient and out-patient operation marked a change for the better. In 1976 the Clinic was relocated to a new clinical pavilion with suitable conditions for clinical activities. Starting from the 1st January, 1955, Dr. Gdovin was appointed deputy assistant professor, from the 1st January, 1957, assistant professor and from June 1, 1963, full professor of internal diseases of even-toed ungulates.

After his joining the College of Veterinary Medicine in Košice, Prof. Dr. T. Gdovin was one of the prominent authori-

ties of the college. He was a very active in diverse areas. This was witnessed by a range of important academic and social-professional functions: the head of a cathedral department, a member of the scientific council, a vice-rector of the CVM, for 7 years the dean of the faculty of Agricultural College (1953—1959), a member of the Scientific-technical Council of the minister and commissioner for agriculture, a member of editorial boards of specialist journals, a member of the Scientific Council of the Research Institute of Veterinary Medicine in Brno. He was also a member of several inter- and extra-university commissions. In 1959 he became a corresponding a member of CSAPV and the chairman of the veterinary branch of this academy. Three days before his death he was elected a regular member of SPA and the chairman of its veterinary branch.

The clinic headed by Prof. Gdovin successfully fulfilled its pedagogical objectives, reached important research results and, in addition to that, was acknowledged as successful and widely known for its great help to practising veterinarians.

Prof. Gdovin ensured the high standard of teaching of the subject, which was very important in intensive animal rearing with regard to the fact that it concentrated on the economically most important animal species (cattle, pigs and sheep). The extent of this clinical subject, as a special branch of science, widened considerably because the health problems of productive animals under the conditions of intensive husbandry with new technologies of rearing and nutrition, increased the concentration of animals, and the constant effort to increase their productivity, changed and extended too.

The relationships between students and Prof. Gdovin were friendly, and the practical aspects included in his lectures drew their attention. His teaching experience was used also within the postgraduate education of practical veterinarians, particularly in the form of numerous lectures held at the Institute for the Postgradual Education of Veterinarians, Košice.

His almost 20-years of teaching and organizational activities were closely related to his diverse and fruitful research and publication activities. He looked for solutions to pressing problems, particularly those related to intensive animal production. He directed his attention to the study of ethology, diagnosis and the prevention of diseases of the young, and gastrointestinal diseases of ruminants and pigs. He started with the topical problems of practice at that time: cattle tuberculosis, anaemia of piglets, disorders of mineral metabolism, and epididymitis of rams. Later on he gradually switched over to the field of clinical biochemistry and physiology and in the last stage also to the study of prophylactic and therapeutic effectiveness of domestic and foreign medicines and biopreparates. The research topics and the extent of investigative activities of Prof. Gdovin were best reflected in many papers published by him dealing with the most of the important branches of internal medicine. The knowledge obtained was summarized in 130 publications and many presentations on conferences at home and abroad. He was the head of the collectives of authors who published two books: *"Internal diseases of cattle, sheep, goats and pigs" (1964) and "Diseases of pigs"* (1966). He was the co-author of a further 3 books.

Prof. Gdovin paid great attention to the scientific and professional development of his staff. Seven of his co-workers attained the degree *candidatus scientiarum* (CSc.) and three the title of assistant professor. His characteristic feature was the unselfish passing of his theoretical knowledge and practical experience to his students and co-workers.

The achievements of the College of Veterinary Medicine in Košice during the first two decades of its existence were closely related to the person and work of Prof. Dr. T. Gdovin. After his joining the College of Veterinary Medicine, he laid stress on close connection between the school activities and practice. This orientation positively and significantly affected the education and research activities of the College and its staff.

Prof. Dr. T. Gdovin was a dean of the faculty in the fifties (1952—1959), the period which was politically complicated and very difficult for universities and colleges. The responsibility taken by him was great in the eventful early years. It was necessary to develop the faculty with regard to the personnel, space and its position. As a prominent academic functionary, he also had to decide about some troublesome cases and problems affecting the destiny of students and members of the staff. When evaluating his role in the history of the university and his contribution to its development and success one must consider the atmosphere of that period, economic possibilities and conditions and particularly the determining political influences. Together with the management of the faculty he had to make an enormous effort to prevent the relocation of the school to Nitra and not long after that even its abolition. He was a person full of energy, enthusiasm, and optimism, a person with set rules and a sense of purpose. He was extremely diligent and hard-working and kept working up to his unexpected death.

He died suddenly and unexpectedly without parting from his closest — full of life and work — in the evening of the 26th April, 1969, in his garden, at the age of 48 years.

His death surprised his closest relatives but also teachers and students, wide veterinary circles, and professionals of different disciplines. The news of his death caused grief among his friends, teachers and scientists at the University as well as practical veterinarians who had been in working and friendly contact with him for many years.

On the occasion of the 65th anniversary of his birth, on the 48th April, 1986, in the presence of his closest family members, representatives of the College, teachers, students and social organizations, the rector of the College of Veterinary Medicine, academician O. J. Vrtiak, unveiled a commemorative plaque with his head in profile in the foyer of the clinical pavilion.

This year we commemorate the 80-th anniversary of the birth of Prof. Dr. T. Gdovin, one of the important representatives of the University of Veterinary Medicine in Košice. On the 14th November 2001, a commemorative plaque was unveiled in the birthplace of Prof. Gdovin, on the occasion of the anniversary of his 80th birthday.

Both plaques (1986 and 2001) wil remined present and future generations the life and work of Professor Gdovin — this prominent university teacher, scientist and honest person who with his life's work made an unforgettable contribution to the history of veterinary education and agricultural practice.

Doc. MVDr. Ján Jantošovič, PhD.
Prof. MVDr. Rudolf Cabadaj, PhD.

Doc. MVDr. et JUDr. Andrej Bugarský, PhD.
Doc. MVDr. Marián Kozák, PhD.

Prof. MVDr. Ján Rosocha, CSc. (1921—1993)

Prof. MVDr. Ján Rosocha, CSc., an promintent university teacher and scientist, the head of a cathedral department, a long-serving academic of the College of Veterinary Medicine in Košice, the founder of Slovak animal hygiene and an honorary member of the International Society for Animal Hygiene.

He was born on the 17th September, 1921, to a large peasant family, in the village Negrovec (district Mezhorie) in the former Carpathian Russia. He completed his secondary education at the state Russian school in Uzhorod where he passed the final examination on the 21st April, 1943. After that he enrolled in an Agricultural Institute in Cluj. However, after a short period of study he had to join the Hungarian army. In a short time, he became seriously ill and found himself in Budapest. He had to muche a great effort to get back home. After an operation he spent more than one year in a Tatra spa.

In 1945 he enrolled in the College of Veterinary Medicine in Brno. There he obtained a veterinary diploma (MVDr.) on the basis of dissertation thesis *"The study of the decelerating effect of pig-specific antireticular-cytotoxic serum on the production of lymphocytic infiltrations of the CNS and the development of poliomyelitis in general"* which he prepared at the College of Veterinary Medicine in Košice.

Despite his serious long illness, from which he successfully recovered, he graduated from the College of Veterinary Medicine in Brno and in September 1950 joined the staff of the newly established College of Veterinary Medicine in Košice, first as an assistant at the Department of Microbiology and General Hygiene and later as an assistant-specialist.

With regard to Dr. Rosocha work experience and scientific qualification, the rector of the College of Veterinary Medicine in Košice appointed him the head of the Department of Animal Hygiene, established on 15th May, 1952. The education at this department started in the academic year 1953/1954. In the beginning special animal hygiene was taught by Dr. Z. Koppel, the director of the branch of the State Scientific and Veterinary Institute in Košice. However, from the academic year 1954/1955 general and special hygiene was taught by Dr. Rosocha.

He was appointed a deputy associate professor starting from the 1th January, 1957, associate professor from 1st December and university professor from the 1st September, 1980.

On the 23rd April, 1960, he defended his habilitation thesis on the theme: "The microclimate in typical pig styes in the Košice district, reflected in the hygiene of housing and the study of infectious potential of enzootic bronchopneumonia virus in the conditions of the outer environment", and on September 5th, 1964, PhD. his thesis on the theme *"An erpizootiological and virological survey of influenza in some pig herds in the Košice district"*.

He made full use of his capabilities also as an academic administrator: as a vice-dean in the period from 1959 to 1964 and a vice-rector for scientific-research activities from 1969 to 1974. He performed these functions with a responsible attitude and a good knowledge of facts.

From 1980 till 1987 he was the head of the Department of Microbiology, Immunology and Animal Hygiene. He helped to establish friendly relationships and collaboration with universities in Budapest, Bucurest, Moscow, Hannover, Vienna, and others. From the academic year 1987/1988 until the end of summer semester of 1990 he was the head of the Department for Animal Hygiene.

His research activities were also abundant. He contributed to a considerable degree to the introduction of new animal hygiene elements into animal housing in Slovak intensive animal production. In the first stage, in agreement with the orientation of research of his department, he concentrated on collaborating with other scientists on resolving the immunological and epizootiological problems arising from infectious diseases: infectious poliomyelitis (research of immunogenesis and immunization), swine erysipelas (improvement of vaccination possibilities), swine brucellosis (effectiveness of allergy-diagnosis), swine influenza (biological properties of viral strains isolated in Slovakia).

From 1965 he concentrated on the topics of animal hygiene: the influence of microclimate and airborne microbial contamination on the typical animal housing (for cows, calves, pigs and sheep) and layer halls; the infectious potential of aerosolized swine influenza virus in weaned piglets under different housing conditions; the incidence of swine leptospirosis in Eastern Slovakia; the study of the effects of some disinfectants against microbes in intensive animal housing (search for preparations that are inexpensive, highly soluble in water, effective, and relatively harmless to the equipment). From 1970 to 1980 the effect of some new disinfectants was tested under different external conditions; research was carried out into the microclimate in intensive housing for gallinaceous fowl particularly with regard to the harmful effect of air ammonia. Between the years 1981 and 1990 he studied the effectiveness of sanitation preparations and interventions in intensive animal production; the microclimate and animal hygiene in large-capacity sheep housing, and the influence of bioclimate and environment on the health of sheep.

His involvement in research was reflected in extensive publication activities. He was an author and co-author of more than 80 scientific and specialist studies published at home and abroad.

He was the co-author of the following books: Vrzgula, L. *et al.*: *"Diseases of Sheep:",* Publ. House Príroda, Bratislava, 1974; Slanina, Ľ. *et al.*: *"Health and Morbidity of Calves in Intensive Rearing",* Publ. House Príroda, Bratislava, 1977.

He was the author of the following education texts: Roso-

cha, J. *et al.*: *"Instructions for Practical Lessons on Animal Hygiene",* Publ. House SVPL, Bratislava, 1967; Rosocha, J. *et al.*: *"Animal Hygiene"* (for the Hygiene of food branch of study), Publ. House Príroda, Bratislava, 1982; Rosocha, J. *et al.*: *"Animal Hygiene — Special Part",* Publ. House Príroda, Bratislava, 1983.

He was a consultant to 6 PhD. students and 2 specialist assistants who became assistant professors.

From 1975 he was the head of the Laboratory for Animal Hygiene of the Institute of Experimental Veterinary Medicine in Košice (Department of Animal Hygiene since 1980). He cooperated with animal hygiene establishments in Brno, Nitra and Trnava.

As a university teacher he gained the respect and recognition of students and teachers. His lectures were based on profound theoretical and practical knowledge and experience.

He held several professional and scientific posts: member of the Subcomission for Animal Hygiene for the Council of Mutual Economical Help; chairman of the Commission for Animal Hygiene of the State Veterinary Administration of The Slovak Republic; honorary member of the Society of Rumanian Veterinarians; between 1974 and 1978 the chairman of the Veterinary Section of the Slovak Society for Agricultural and Food Sciences and Forestry of the Slovak Academy of Scien-ces.

He was one of the founders of the International Society for Animal Hygiene (founded in 1970), a member of its executive committee, president for the period of 2 years (1979—1982) and vice-president thereafter. Together with the staff of his Department he organised the IVth International Congress of Animal Hygiene in the High Tatras with the participation of many guests from abroad.

His meritorious work was endorsed on several occasions: he was awarded distinctions of the Ministry of Agriculture and Nutrition *"Excellent Worker of Agriculture and Nutrition"* (1969) and of the Ministry of Education *"Merited Teacher"* (1976). In 1976 he was awarded the conmemorative medal of the State Veterinary Administration of SSR. In 1971 the rector of the College of Veterinary Medicine in Košice awarded Dr. Rosocha the gold medal of Dr. P. Adámi and later on also the medal of L. Pasteur. From the rector of College of Veterinary Medicine in Brno he received the Medal of Prof. Dr. I. J. Pìšina and in 1980 the conmemorative medal of the College of Veterinary Medicine in Brno. In 1977 the rector of the University of Veterinary Sciences in Budapest awarded him the medal of Prof. Dr. J. Marek. In the eighties he was awarded the state distinction and medal *"A Certificate of Merit for the Development of the East Slovakian Region"*. In 1991 he was awarded a gold medal of the Slovak Academy of Sciences and was elected an honorary member of the International Society for Animal Hygiene.

Prof. Rosocha was a very friendly and selfless person. He was known for his humanity, spontaneous friendliness, tolerance, deliberation and objectivity. Among his characteristics were also mental composure and a sense of fairness. A peaceful family background provided by his wife and daughter allowed him to concentrate successfully on his teaching and scientific activities.

He retired on 1st September, 1990. He played an active part at the University almost to the last day of his life. He passed away in Košice on 25th October, 1993, at the age of 72. He was buried in Košice.

This year we commemorate the 80th anniversary of his birth. On this occasion we honour him as a noble-minded person and devoted teacher of the University of Veterinary Medicine in Košice.

Doc. MVDr. Ján Jantošovič, PhD.
Prof. MVDr. Rudolf Cabadaj, PhD.
Doc. MVDr. et JUDr. Andrej Bugarský, PhD.
Doc. MVDr. Marián Kozák, PhD.

The 80th birthday of Prof. MVDr. Michal Bartík, DrSc.

This year in September Prof. MVDr. Michal Bartík, DrSc., the prominent Czechoslovak biochemist and toxicologist, for many years a university teacher, head of the department, and academic administrator celebrated his important birthday.

He was born on the 29th September, 1921, in Nitrianske Sučany, district Prievidza. He completed his secondary education at the State Secondary School in Prievidza where he successfully passed his school-leaving examination in 1941. After leaving he decided to study veterinary medicine.

In 1941, after obtaining a scholarship, he enrolled in the College of Veterinary Medicine in Vienna. There he successfully completed only 7 semesters (with regard to the approaching front-line). After the World War II he changed to the Veterinary College in Brno. He completed his study of veterinary medicine by obtaining a diploma on the 5th May, 1946, and was awarded the title MVDr. by defending a thesis on the theme *"Intoxication with spray preparations containing dinitro-o-cresol"* on 2nd February, 1947. After that he joined the staff of the Veterinary College in Brno, first as an assistant and later as an assistant-specialist at the Chemical Department (1945—1947) and the Buiatric Clinic (1947—1949). Already his thesis, dealing with biochemical-toxicological problems, indicated his life-long orientation. After August 1947 he became an employee of the Veterinary Diagnostic Institute in Bratislava.

In 1949 a College of Veterinary Medicine was established in Košice. Even the first meeting of representatives of the Comissioner for Education (Dr. Clementis) with Prof. Hovorka from the 2nd to 3th March, 1949, and the dignitaries of the College of Veterinary Medicine in Brno dealt besides other aspects also with the potential composition of teachers of the newly established school. It is interesting that the first proposal envisaged Dr. M. Bartík as the head of the Buiatric Clinic (with regard to the fact that he was at that time on the staff of such clinic headed by Prof. Klobouk). However, in the later proposal for the teachers in the first year of study

Dr. M. Bartík was supposed to be the head of the Chemistry Department.

Dr. M. Bartík joined the staff of the College of Veterinary Medicine, the Chemistry Department on the 1st January, 1950, and remained there till the end of June 1990. He was the third veterinarian on the staff of the new veterinary college, the head of an department and therefore was one of the first few teachers who participated from the beginning in the development of their particular department and the organization of the education process. In the winter semester of the academic year 1949/1950 the lectures on chemistry were read by his compatriot MUDr. A. Neuwirth from the Medical Faculty. Immediately after his arrival in Košice Dr. Bartík helped with great enthusiasm to build up the Department for Veterinary Chemistry and ensure the teaching of chemical disciplines. During the first two years of its existence the department at the former Comenius Institute was located provisionally, and only in January 1952 it was relocated to the newly adapted facilities.

Prof. Bartík helped to built up the department from the very beginning. Later it was changed to the Department of Chemical Disciplines. He stayed with the department until the end of June 1990, i.e. for 40 years. He made an effort to obtain modern equipment and thanks to his organizational capabilities he was for the most part successful. He put considerable effort into setting up the radio-isotope laboratory that started its operation in 1962. He was the head of the Cathedral Department of Chemistry, Biochemistry and Toxicology from 1950 until the 15th January, 1971, when, for political reasons he was removed from his post. Subsequently, the department was "purposefully" abolished and a new Cathedral Department was formed together with the Department of Pathological Physiology. Prof. Bartík remained the head of the Department of Biochemistry and Toxicology till September 1972 when he was removed from that position for the same reasons as before.

After the discussion with Prof. Janeček from Brno, Prof. Bartík suggested the introduction of "Veterinary toxicology" in the curriculum of veterinary faculties, starting from the academic year 1952/53. He taught this subject until the end of the academic year 1970/1971. He also proposed to include biochemistry in the curriculum of veterinary faculties (in Brno and Košice) and this initiative was successful owing to his position as a vice-dean for educational activities. Biochemistry was taught for the first time in the academic year 1953/1954, after the approval by scientific councils of both faculties. He himself read lectures in veterinary chemistry (except for an enforced break from 1971 to 1980) until the end of the academic year 1988/1989. He worked his way up and became a recognized specialist and teacher in the field of veterinary biochemistry which was confirmed by an official invitation from the College of Veterinary Medicine in Brno in 1965 to succeed Prof. Janeček, the lecturer on veterinary biochemistry. Prof. Dr. Bartík resisted this lucrative proposal and decided to stay at the department which he helped to build up in Košice. He had not thought of the problems he would have to face several years later.

He was a vice-dean for educational activities (1953—1957), a member of the Scientific Council, consultant to postgraduate students in the scientific areas of biochemistry and toxicology, and the chairman of the Commission for the Defence of PhDs. These.

He was appointed Deputy Assistant Professor in 1955, Assistant Professor in 1957 and Deputy Professor for veterinary chemistry. He earned the scientific degree candidatus scientiarum (CSc.) at the Agricultural College in Brno by defending his thesis on the theme: *"Some quantitative relationships between metabolism of nitrates and nitrites in farm animals with regard to methaemoglobinaemia"*. The scientific degree DrSc. was awarded to Dr. Bartík by the Agricultural College in Nitra after the defence of his doctoral thesis *"The biochemistry of the intoxication of farm animals with nitrates and nitrites and the basics of their chemical diagnosis"*. He defended it at the College of Veterinary Medicine in Košice in 1968.

Prof. Dr. M. Bartík realized that the success of the department and the college is based first of all on the active scientific research and because of that he devoted considerable attention to scientific investigations from the very beginning. His department gradually became an important centre of biochemical and toxicological research. From 1969 to 1970 he was also the head of the biochemical laboratory of the Institute of Experimental Biology of SAS in Košice. The research activities of Prof. Bartík and his co-workers were oriented mainly on the following areas: 1. The metabolism and detoxication of nitrates and nitrites in farm animals involving the development of methodical procedures for chemical diagnosis of respective intoxications. 2. Enzymatic diagnosis and therapy in veterinary medicine — the kinetics of enzymes and its observation by means of radioactive and other substrates. 3. Polarographic analysis of some substances in biological materials. 4. Study of peptidic enzymes in animals and the development of analytical kitss for determining the activity of proteolytic enzymes. The radio-isotope laboratory of the department was focused on radio-immunochemical analyses and the use of radio-isotopes in determining enzymatic activities and enzymatic assays of substrates.

Prof. Bartík was also very active with regard to publishing his results. He himself or together with his co-workers published many specialized and scientific articles in domestic and foreign journals. He was the co-author of the following books: Bartík, M., Rosival, I.: *"Nitrates and Urea in Animal Nutrition",* 1971; Bartík, M., Piskáč, A. *et al.*: *"Veterinary Toxicology",* 1974, which was published also in English in 1981.

His successful 20 years of pedagogical and scientific activities were interrupted for political reasons in 1971, in the period of "normalization", because his two children did not return from Great Britain in 1968. He was recalled from the post of the head of the department, could not read lectures to full-and part-time students, and could not act as a consultant to postgraduate students. Upon the decision of the ruling party and the Ministry of Education the process of his appointment to university professor was stopped in 1970. Only 10 years later was he appointed Professor (starting from the 1st September, 1980) by the president of the Czechoslovak Republic.

It was both a family and personal tragedy as he became unwanted in his most productive years. He could not realize the plans he set out at the time of his entering the College which was detrimental to the department and school and also to

himself. Following the partial relief of the repressive measures after 1980 he could participate in the teaching process, but his research was still restricted.

He received an apology in the form of a rector's letter of the 2nd April, 1990, for the unjust, longhty punishment together with an appraisal of his civic and moral conduct and received his rehabilitation.

He was always known for the exceptional energy he put into his work, organizational spirit, and strong will. He was persevering, assiduous, and purposeful. He was not afraid of difficulties and obstacles He became exasperated with unfairness and tendentiousness. He was frank, direct and fearless when pointing out shortcomings and asked repeatedly for a revision of his case.

On the occasion of his 80th birthday, we wish to Prof. Bartík good health and many pleasant years of well-deserved rest.

Doc. MVDr. J. Jantošovič, PhD.
Prof. MVDr. Rudolf Cabadaj, PhD.
Doc. MVDr. et JUDr. Andrej Bugarský, PhD.
Doc. MVDr. Marián Kozák, PhD.

ADVANCED NOTE

This volume (No. 45) of *FOLIA VETERINARIA* will conclude with a supplementary issue based on a selection of the Proceedings of the International Scientific Conference devoted to the internal diseases of small animals, poultry, horses, and the history of veterinary medicine on the occasion of the 50th anniversary of the Clinic of Internal Diseases I of the U.V.M., Košice.

ERRATA

In Volume 45, Issue 2 of *FOLIA VETERINARIA,* p. 112 Professor Totolian is described as an Honorary member of the "Czech and Slovak Society of Microbiology". This should read the "Czechoslovak Society of Microbiology".

Doc. MVDr. Emil Pilipčinec, PhD.
Executive Editor