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FOLIA VETERINARIA, 46, 1, 3—7, 2002

A SCANNING ELECTRON MICROSCOPE STUDY OF THE LINING OF THE THIRD AND FOURTH CEREBRAL VENTRICLES OF EWES UNDER PHYSIOLOGICAL CONDITIONS AND HORMONAL STIMULATION

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ABSTRACT

The surface of the *recessus infundibuli* and the floor of the fourth cerebral ventricle were studied using the scanning-electron microscope in adult ewes during anoestrus, prooestrus and oestrus phases of the ovarian cycle and after the administration of Medroxyprogesteroni acetas (Veramix sponge sheep a.u.v., Pharmacia Upjohn, Belgium) and the subsequent application of the follicle-stimulating hormone (Folicotropin inj. sicc. a.u.v., Spofa, Prague, The Czech Republic). In control ewes in the period of oestrus, besides microvilli, a small number of supra-ependymal cells was also found, which, after hormonal stimulation, were missing. From the study it also follows that the lining of the floor of the fourth cerebral ventricle shows certain small differences between the control and experimental ewes. The study has confirmed in ewes a connection between the activity of sexual glands or the application of sexual hormones and the structures of the surface of the infundibular ependymal lining.

Key words: central nervous system; ependyma; hormonal stimulation; third and fourth cerebral ventricles; sheep

INTRODUCTION

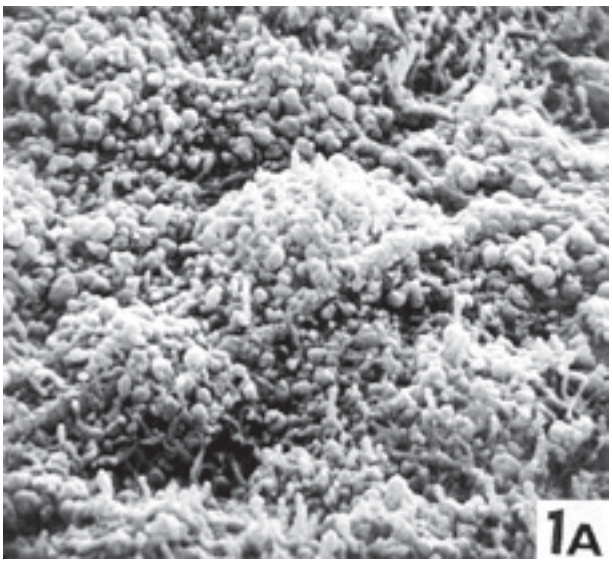
The ependyma, which lines the entire ventricular system of the brain, from the topographical point of view, does not belong to the sexual system. Functionally ependymal cells, especially tanycytes of the lower part of the third ventricle (in the *recessus infundibuli*), are connected with the activity of the sexual glands of females as well as males. The studies by several authors of lower vertebrates, laboratory rodents, and some domesticated mammals indicate a varied picture of the surface of the infundibular ependymal lining, which depends on the ovarian stage in females or the season in males (7, 8, 9, 2, 22, 4, 6, and others). Peculiarities in the structure of the ventricular wall in females suppose that ependymal cells, especially their highly specialised form — tanycytes — react in a characteristic way in the sexual cycle. The exact mechanism of this response has not been clearly explained yet.

The aim of this work is to record changes in the structure of the surface of apical membranes of the ependyma in the region of the infundibular recess of the third cerebral ventricle and on the floor of the fourth ventricle under physiological conditions, and after hormonal stimulation using the scanning-electron microscope.

MATERIAL AND METHODS

Ten adult healthy Merino sheep were used in our study. Six of them were used as controls which, according to the findings on their ovaries, were in anoestrus (March), prooestrus (June) and oestrus (October) cycles.

In the experimental group of sheep during June, there four other ewes were used. They were examined sonographically before the application of hormonal preparation Medroxyprogesteroni acetas 60 mg (Veramix sheep sponge a.u.v., Pharmacia Upjohn, Belgium) for the intactness of those sexual organs, and their previous reproductive cycles were also confirmed anamnestically to be without any problems. From day 12 of their administration we started to apply i.m. the follicle-stimulating hormone (Folicotropin inj. sicc. a.u.v., Spofa, Prague, The Czech Republic) twice a day (in the morning and in the evening) as follows: on day 12 — 4 mg × 2, on day 13 — 3 mg × 2 (in the evening the sponges were removed) and on day 14 — 2 mg × 2 (altogether 18 mg). During days 15 and 16 a detection of oestrus was carried out and on day 17 the experimental animals were killed under thiopental anaesthesia.



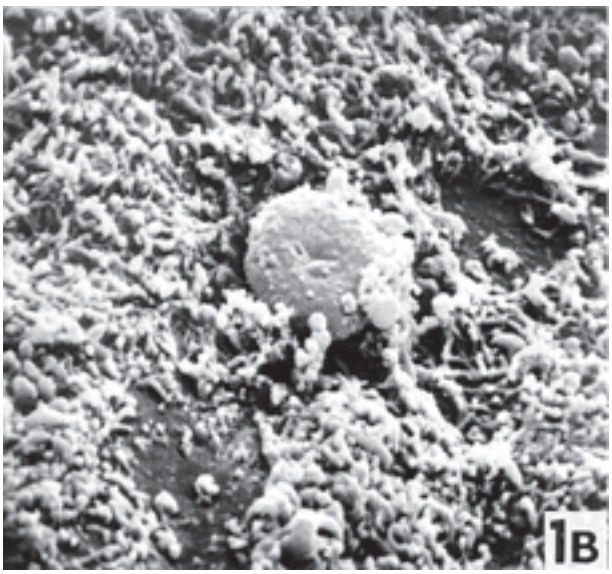


Fig. 1. A, B. Two details from the infundibular ependymal surface of an anoestrous ewe. A: dense microvilli-like structures, 5700 x. B: thin microvilli and scattered isolated small spherical protrusions. 6100 ×

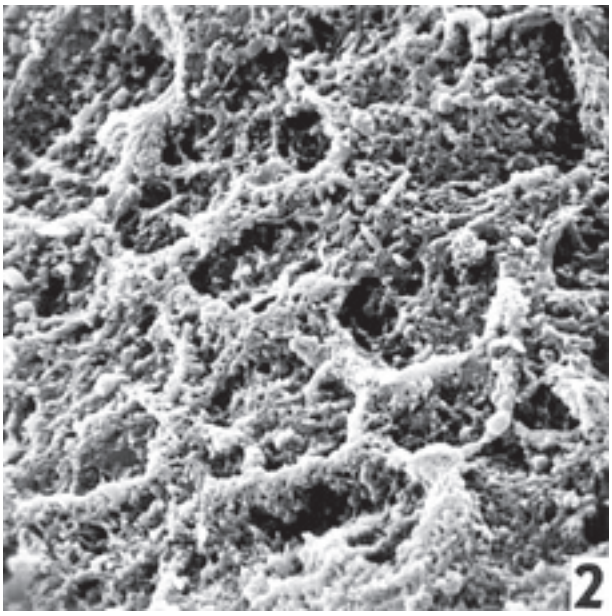


Fig. 2. The surface of the lining from the infundibular recess of a prooestrus ewe. Dense thick microvilli form a polygonal pattern. 5500 ×

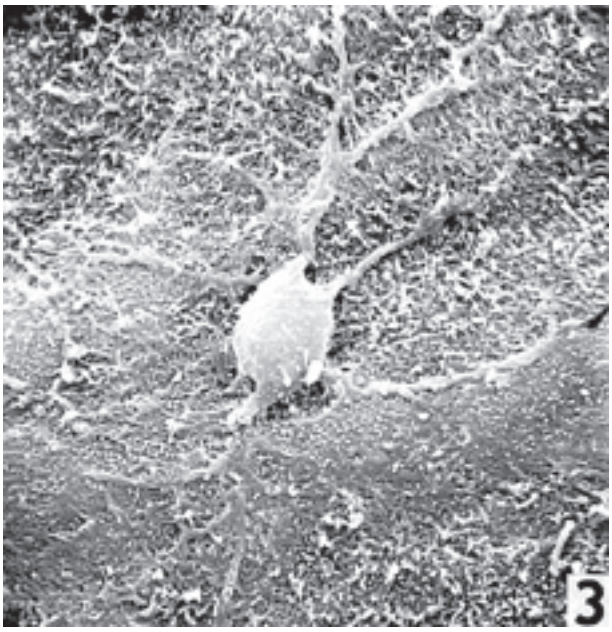
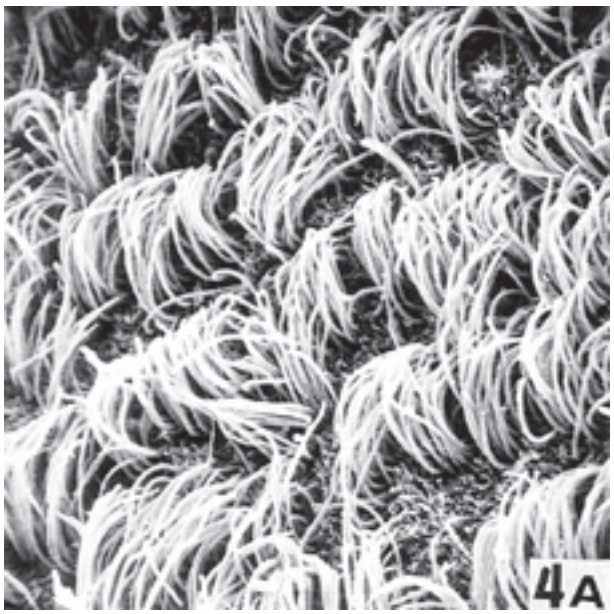


Fig. 3. Filiform microvilli and one of the supra-ependymal cells of an ewe in oestrus. 2500 ×

The blood vessels of the heads of all the ewes used immediately after bleeding were rinsed through the *a. carotis communis* by a phosphate buffer (0.2 mol.l^{-1}), then pre-fixed with Karnovsky solution, and the samples from the infundibular recess of the third, and the floor of the fourth, cerebral ventricles were post-fixed within 3 hours by 3 % solution of glutaraldehyde in a cacodyle buffer (0.2 mol.l^{-1}). The temperature of all solutions was 4 °C, and the pH 7.4. The samples were processed by the method of Murakami *et al.* (13). After dehydration in an increasing ethylalcohol order, they were dried using the method of the critical point, coated with gold in the vacuum, and studied in the scanning-electron microscope Stereoscan Cambridge 2A and Tesla BS 340 in connection with the picture analysis.

RESULTS

Control ewes. The surface of the *recessus infundibuli* lining of the third cerebral ventricle in the period of anoestrus is covered with short delicate microvilli (Fig. 1A). On the periphery of some apical membranes they are taller and denser. Based upon this, flat polyhedric cellules are formed. On the microvilli there are small spherical protrusions (Fig. 1A, 1B). The presence of cilia in this region was not recorded. The structure of the apical membrane surface of the *recessus infundibuli* during proestrus differs from that in the anoestrus stage of the ovarian cycle only by the presence of thicker



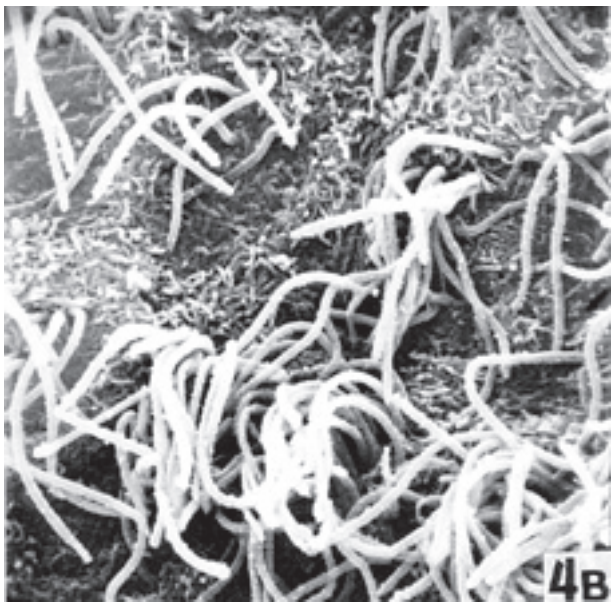
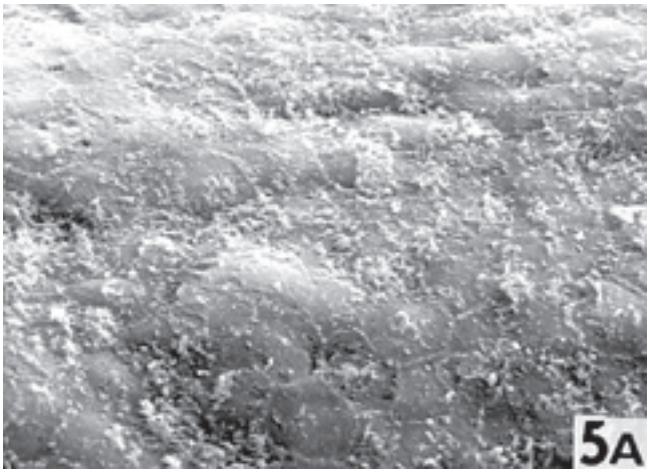


Fig. 4. A, B. The surface of ependymal lining from the floor of fourth brain ventricle of an oestrous ewe. Clusters of cilia are arranged in rows, A: 2400 ×. Under the cilia there are microvilli. B: 4500 ×



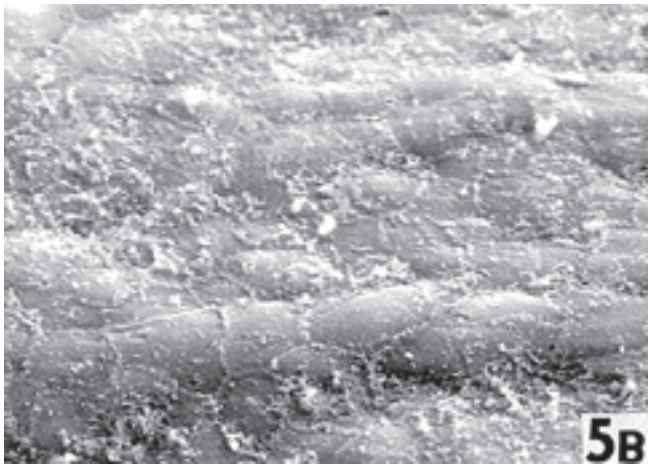
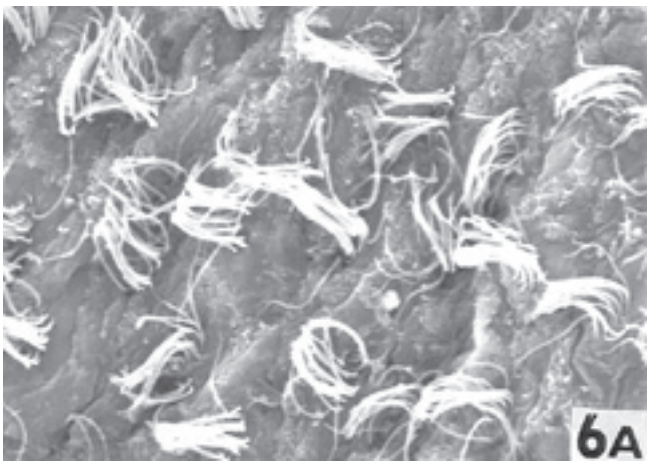


Fig. 5A, B. The irregular surface of the lining from the rostral (A) and middle parts (B) of the infundibular recess. The ewe after hormonal stimulation. Sparse, short, and thin microvilli form not very distinct polyhedric pattern. A, B: 2550 ×



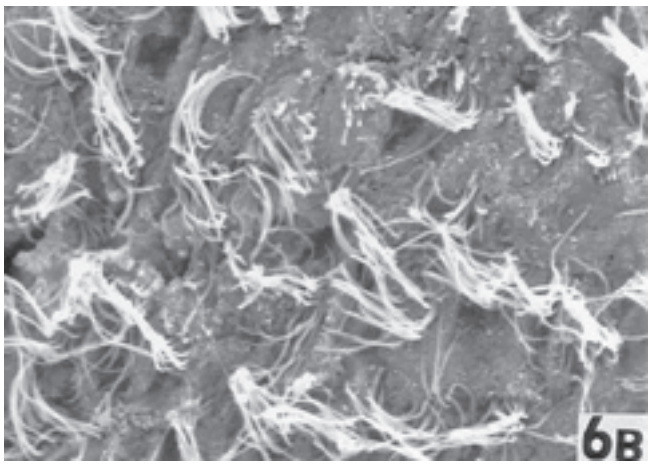


Fig. 6A, B. The irregular surface of the apical membranes of the ependyma from the floor of the fourth ventricle in a hormonally treated ewe. Ciliary clusters and microvilli are more sparse than in control ewes. A, B: 3100 ×

microvilli. On the floor, but also on the periphery of the polyhedric cellules (Fig. 2), the small spherical protrusions are dispersed. In the period of oestrus, the *recessus infundibuli* was covered with dense thick microvilli, which are arranged so they do not form a polyhedric pattern. On them there are supra-ependymal cells (Fig. 3) singly or in small groups which reach the transit zone. They have irregularly round-to-oval bodies with two or more processes, which form "ruffled membranes" only sporadically.

Independently of the study of the ovarian cycle, the surface of the apical membranes of the ependyma of the fourth cerebral ventricle floor was covered with short thin microvilli or microvillus-like thicker structures (Fig. 4B). Cilia typically protruded predominantly in clusters, arranged in irregular rows (Fig. 4A). In the region of the *sulcus medianus,* the cilia were denser than those along its sides. The occurrence of small spherical protrusions in the region of the floor of the fourth ventricle was sporadic. In the case of the fourth ventricle, no difference in the structure of the lining surface which corresponded with the stage of the ovarian cycle was recorded.

Experimental ewes. The findings on the surface of the ovaries after hormonal application: in one ewe there were altogether 6 freshly ovulated follicles, in three ewes on the ovary surface there were always more (up to 15) follicles prepared to ovulate.

After hormonal stimulation by the folliculo-stimulating hypophyseal hormone (Folicotropin) the ependymal surface in the *recessus infundibuli* is slightly undulated on some sites (Figs. 5A, 5B). Sporadically the apical membranes of the ependymal cells are weakly convex; they are covered with thin or thicker microvilli, which are not regularly distributed. In almost all the apical membranes on the periphery there is one row of slightly taller, more densely arranged microvilli. In this way, a slightly polyhedric appearance of the outer surface is formed (Figs. 5A, 5B). Neither spherical protrusions nor supra-ependymal cells were recorded.

In the region of the floor of the fourth cerebral ventricle (Figs. 6A, 6B), the apical membranes are convex, their surface is irregular, covered with only a small number of short delicate microvilli. Approximately in the centre of the apical membranes there are irregular depressions from which the clusters of several cilia protrude. Neither spherical protrusions nor supraependymal cells were observed.

DISCUSSION

In the literature it is known that the structure of the surface, especially of the lower part of the third cerebral ventricle *(recessus infundibuli)* changes depending on the sex of animal or the stage of the ovarian cycle (2). Literary data dealing with the relationship between the activity of sexual glands and the structure of ependymal lining of the ventricular system of the brain, apart from the sheep and goat, are not known. The first Kozlowski *et al.* (10) and Scott *et al.* (20) reported a description of the ependymal surface in the sheep using the scanning-electron microscope (SEM). Their recording is, however, rather general. Coates and Davis (4, 5) observed the surface of the *recessus infundibuli* using a SEM not only during the ovarian cycle of ewes, but also after hormonal stimulation with oestrogen — progesterone. Staníková *et al.* (21), Rajtová and Odehnal (15), Rajtová (16, 17, 18, 19), Pástorová *et al.* (14) referred to the study of the infundibular lining surface of the third cerebral ventricle by the SEM in ewes and goats in connection with the ovarian cycle, and the season of rams and bucks, but also after the application of hormones, which are used for cycle synchronization.

Coates and Davis (4, 5), dealing with ewes in oestrus and those treated with oestrogen — progesterone in the period of anoestrus, found on the surface of the infundibular recess in the SEM a similar picture: short, delicate microvilli, being thickened on the periphery of apical membranes, and therefore they formed a polyhedric pattern on which there were tiny, dispersed spherical protrusions. The presence of supraependymal cells was not reported by these authors, or by Kozlowski et al. (10). The same structure of the lining surface of the recessus infundibuli, as that described by Coates and Davis (4, 5) after hormonal stimulation, was also recorded in our experimental ewes.

These results differed from our controls during oestrus by the presence of sporadic or small groups of supraependymal cells. These cells occurred neither in anoestrous nor prooestrous ewes under our study. Their occurrence, however, was constant in female goats (Rajtová 17, 18, 19). During the ovarian cycle of goats, only their number was changed as it was e.g. in rats (2, 12). It is not known why the infundibular surface in the goat responds to the changes during the ovarian cycle and also after hormonal treatment in a way that differs from sheep. Probably it will be one of the interspecies differences.

The ependymal lining of the floor of the fourth cerebral ventricle in the control ewes has a much simpler structure and during the ovarian cycle, no changes on its surface were observed; there were neither supraependymal cells on it nor supraependymal neuronal elements as described by Mathew (11) in rats. After hormonal application, the structure of the fourth cerebral ventricle floor changed very little. Microvilli became thinner, apical membranes carried slight deepenings, from which clusters of cilia protruded.

Even if the ependymal lining in the *recessus infundibuli* as well as in the entire ventricular system does not form a direct part of the sexual system, substantially to a lesser or higher degree, all the processes connected with the ovarian cycle or hormonally directed reproduction are reflected in it.

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MORPHOLOGY AND DISTRIBUTION OF NITRERGIC NEURONS IN THE PHEASANT SMALL INTESTINE

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ABSTRACT

The aim of the present study was to examinate the morphology and distribution of nitrergic neurons in the pheasant ileum using the NADPH-diaphorase histochemistry.

Neuronal NADPH-d positivity in the pheasant ileum was demonstrated in the nerve cells and nerve fibres. The nerve cells were situated in the submucous and muscular layers and they were polygonal in shape and projected solitarily or arranged in groups. Nerve fibres entered into the ileum at its serosal surface frequently in the company of the ileal arterial branches. They were abundantly presented in the muscular and submucous layers of the ileum forming thicker nerves. Individual nerve fibres travelled through the submucosa into the *lamina propria mucosae* to create a plentiful nerve plexus. Some fine nerve fibres were distributed in the intestinal villi to achieve the epithelium covering crypts.

These findings support the fact that the pheasant ileum is characterized by dense distribution of NADPH-d positive nerve structures which may play significant roles in the small intestine functions of the pheasant.

Key words: NADPH-diaphorase; neurons; nitrergic; pheasant; small intestine

INTRODUCTION

NADPH-diaphorase is an oxidative enzyme which can be proved by a colour histochemical method. The presence of NADPH-d activity shows the specific sites where nitric oxide (NO) occurs (1). NO has been considered to be one of the most important neurotransmitters of nonadrenergic and noncholinergic inhibitory neurons of the central and peripheral nervous systems (11). NO has recently expanded its status from an environmental pollutant to a biological signaling molecule that mediates blood vessel relaxation and immune responses, kills pathogens, and serves as a neuromodulator in the central and peripheral nervous systems (15). NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), which has been identified in different parts of the central and peripheral nervous systems (5), endothelium and other tissues. Nerve fibres may be changed by many physiological and pathological conditions, e.g. ischaemia and reperfusion, stress, diabetes (7).

Evidence has been provided that neuronal NOS is identical to NADPH-d in the brain and peripheral nervous tissue (4). Thus the occurrence of NADPH-d positive nerve structures suggests the presence of neuronal NO synthase (NOS) which can participate in visceral functions. Snyder and Bredt (9) have reported that neuronal NOS appears in the nerve plexuses of the whole gastrointestinal tract (GIT) and there it mediates the physiological relaxation. The presence of NOS in the enteric nervous system was shown in various segments of the gut in a several of mammalian species (1, 3, 14). Innervation of the small intestine in lower vertebrates has been investigated only sporadically (2, 10, 6).

The present study was undertaken to investigate the occurrence, distribution and morphological features of nitrergic neurons in the pheasant ileum indirectly by NADPH-d histochemistry.

MATERIAL AND METHODS

We used twenty pheasants of both sexes aged of 1 to 5 weeks. The experimental animals were obtained from the animal house facility of the University of Veterinary Medicine where they were hatched and kept under routine specific-pathogen-free conditions. The birds were anaesthetised by intraperitoneal application of pentobarbital (50 $mg.kg^{-1}$) and perfused intracardially with Ringer's solution followed by a fixative solution containing 4 % paraformaldehyde in 0.1 % buffered glutaraldehyde with 0.1 mol sodium phosphate (pH 7.4). The fixatives were freshly prepared immediately prior to perfusion. The aboral segments of the ileum were carefully dissected out and stored in the same fixative for 2 hours; then stored overnight in 30 % sucrose in

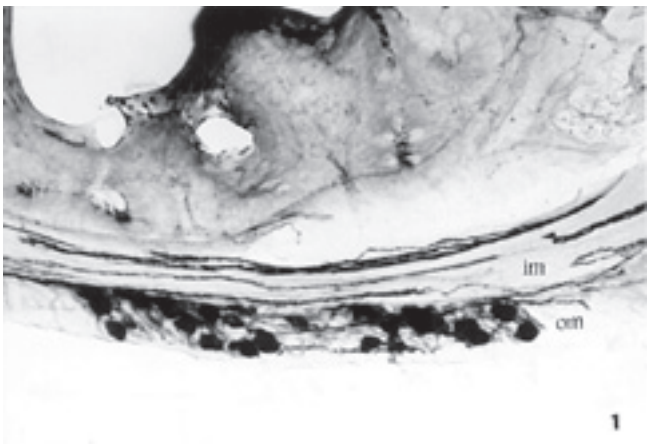

Fig. 1. The cross section through the wall of the pheasant ileum. Numerous NADPH-d positive nerve cells connecting with nerve fibres are located in the outer muscular layer (om). The inner muscular layer (im) consists of thicker nerves, circular in course. Magn. × 75

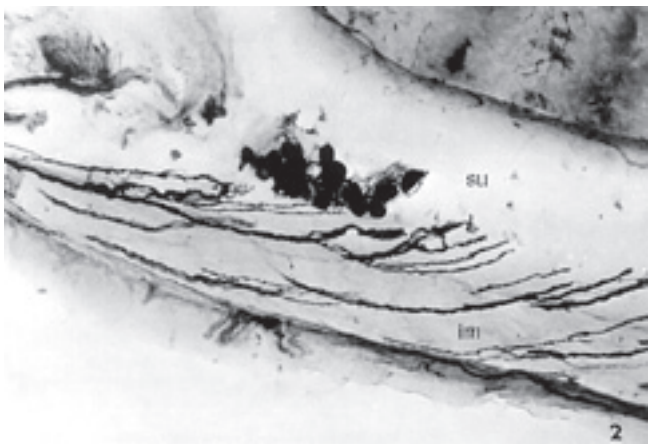

Fig. 2. The NADPH-d positive nerve fibres of the inner muscular layer (im) penetrate into the group of nerve cells situated in the *submucosa* (su). Magn. × 150

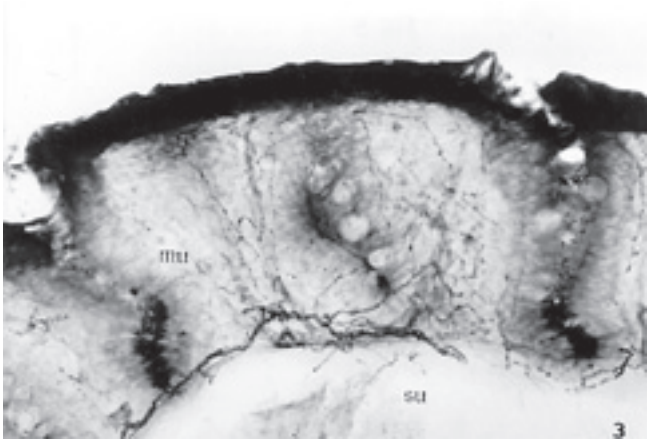

Fig. 3. Varicose NADPH-d positive nerve fibres travelling from the submucosa (su) form a rich plexus in the *mucosa* (mu). Magn. × 150

0.1 mol phosphate buffer (pH 7.4). Afterwardly they were cut in a freezing microtome at a thickness of 20 µm sections. The NADPH-diaphorase histochemical procedure according to Scherer-Singler *et al.* (8) were carried out on serial free-floating sections. The control sections were incubated in an identical solution, however, without NADPH.

After the incubation, the sections were washed in 0.1 mol phosphate buffer solution (pH 7.4), mounted on slides, air-dried overnight, and covered with Entellan.

RESULTS

NADPH-d activity in the wall of the pheasant ileum was detected in neuronal cell bodies as well as in the nerve fibres in several body regions. We observed plenty of the neuronal structures investigated in both layers of the *lamina muscularis externa*, in the *submucosa* and in the *mucosa.* Varicose nerve fibres expressing NADPH-d positivity penetrated in different places through the serosal surface of the ileum, the most frequently in the vicinity of the ileal arterial branches, to form thicker nerves. In their almost perpendicular course to the lumen of the ileum the nerve fibres infiltrated into the longitudinal (outer) layer of the *muscularis externa* and entered into the bodies of nerve cells situated there. The NADPH-d positive nerve cells polygonal in shape, were present in the *muscularis externa* scattered as solitary or in larger groups, forming the ganglia. Numerous thicker nerve fibres were demonstrated in a circular (inner) layer of the *muscularis externa* where they ran parallel with muscular fibres of this layer (Fig. 1). *Muscularis externa* NADPH-d positive nerve cells were not present in the circular layer of the *lamina muscularis externa*.

Nerve fibres expressing NADPH-d positivity penetrated from the circular layer of the *muscularis externa* into the submucosa often in the company of vessels supplying the ileum. Nerve fibres were connected with the bodies of the other nerve cells situated in the submucosa. The arrangement of the neurons expressing NADPH-d activity in the submucosa were similar to the neurons in the *muscularis externa*, they were present solitary or arranged in groups (Fig. 2).

Varicose nerve *muscularis mucosae* fibres travelled from nerve cells of the *tunica submucosae* through *lamina muscularis mucosae* into the *lamina propria mucosae* to form plentiful mucosal nerve plexus. Several nerve processes of the submucous nerve cells proceeded on to the luminal surface of the ileum in the intestinal villi and surounding crypts. They reached the epithelium covering the crypts and villi (Fig. 3).

The NADPH-d positive or negative nerve cells were not found in the mucosa of the ileum.

DISCUSSION

The neuronal NADPH-d positivity in the wall of the pheasant ileum was detected in two types of nerve structures: in the bodies of nerve cells and nerve fibres.

The bodies of nerve cells polygonal in shape were located in the myenteric plexus and the submucous plexus. Balaskas *et al.* (2) described the appearance and distribution of NADPH-diaphorase activity in myenteric neurons in different segments of the embryonic chicken gut as early as embryonic day 5.5 but the number of nerve cells increased with age. Van Ginneken *et al*. (3) detected that the number of NADPH-d expressing myenteric neurons in the pig duodenum doubled postnatally. In the chicken gut the formation of the myenteric plexus is followed by the formation of a circular muscle layer which is innervated by NADPH-d positive nerve fibres as early as embryonic 9.5 day, the longitudinal layer is formed latter (2).

Adeghate *et al.* (1) found that some neurons positive for NADPH-d were solitary, and some aggregated to form the ganglia within the muscular layer. It is in accordance with the presence of NADPH-d positive neurons in the *muscularis externay* which we described in the pheasant ileum. Nerve fibres and cells bodies expressing NADPH-d were observed in the myenteric plexus of all regions of the golden hamster small intestine. All neurons of the myenteric plexus that were seen to express NADPH-diaphorase were also found to be NOS-immunoreactive (12). We agree with statement of Wilhelm *et al.* (14) that the number and density of NADPH-d stained neurons in the myenteric plexus in the ileum is substantially species-specific different.

A high number of NADPH-d positive neurons and nerve fibres were found in the submucous layer of the pheasant ileum. Balaskas *et al.* (2) reported that in the chicken embryonic day 11.5 was also the first time at which submucous neurons expressing NADPH-d activity could be detected. Wang *et al.* (13) demonstrated an abundance of NADPH-d positive axon terminals in the submucous ganglia of the guinea-pig duodenum. Van Ginneken *et al.* (3) by applying NADPH-diaphorase on whole mount preparations demonstrated the myenteric and submucousal plexus in the pig duodenum. They discovered that the number of NADPH-d expressing myenteric and submucosal neurons was significantly higher in the oral duodenal segment compared with the aboral segment.

Varicose nerve fibres of the submucous ganglia of the pheasant ileum travelled through *lamina muscularis mucosae* into the *lamina propria mucosae* frequently in the perivascular course forming plentiful mucosal nerve plexus. This is also in accordance with the results of Van Ginneken *et al.* (3) who obtained them by investigating the pig duodenum.

These present results provided the morphological evidence for the presence and distribution of nitrergic neurons in the wall of the pheasant ileum. The topographical distribution of NADPH-diaphorase positive nerve structures may be an indication of several subpopulations of NO-synthetizing neurons which in the pheasant ileum may act either as motor neurons or as interneurons.

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THE INFLUENCE OF EXPOSING LAYERS TO NITRITES ON THE QUALITY OF THEIR PRODUCTS

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ABSTRACT

The 18 layers (hybrid Tetra) included in the experiment were divided into three groups. The first group served as a control and the layers in the second group were exposed to a nitrite load induced by *per os* administration of 5 mg $NaNO_2$ per head and day. In group three the nitrite dose was increased to 10 mg.

The administration of nitrites resulted in a load on the chickens, which was manifested by more than 7 % methaemoglobinaemia in group the three even by day 14 of administration; in the second group, which received 5 mg dose of $NaNO_2$, the methaemoglobinaemia also culminated on day 14 and then decreased gradually to the level observed in the control group. Methaemoglobinaemia in the first (control) group did not decrease below 5% throughout the experiment.

Residual levels of nitrites in the muscles and organs were lower at the end of the experiment than half way through. The highest accumulation of nitrites was observed in the kidneys and heart. The level of $NaNO_2$ in the egg vehiculum was considered negligible.

The administration of nitrites was reflected in the quality of meat after 14 days in terms of decreased water binding capacity.

We conclude that even in such animal species as poultry, the endogenous nitrites produced by a reduction of consumed nitrates in common feeding technologies induces a more important load on the body than exogenous nitrites.

Key words: layers; meat pH; nitrites; residua; water binding

INTRODUCTION

The increased yield of agricultural crops necessary for the optimum nutrition of farm animals depends on a constant application of nitrogen fertilizers. Their inadequate application either with regard to the time or the dose can have a negative influence on living bodies.

With regard to the health aspects an important impact was observed after the chronic supply of subtoxic quantities of nitrates, which resulted in a depression of metabolism (5). The load of exogenous substances on animals negatively affects their body and productivity and the residua in the products obtained are a subject of considerable concern.

Nitrates do not necessarilly pose a direct risk to farm animals if their doses are not high enough to cause typical toxicosis. Normally they are eliminated in urine (14, 16) by both animals and humans (15). Both the exogenous and endogenous reduction of nitrates to nitrites is conditional, as a rule, on the activity of some microorganisms. Various reactions linked up to the final products of enzymatic reactions of individual representatives of the microflora may play an important role with regard to the portion of reduced nitrates and the quantity of developed nitrites. This dependence determines the role of the transformed nitrites and their acute or chronic action in respective pathological reactions. This concerns firstly the production of methaemoglobin and secondly the potential production of nitrosamines.

Because our previous experiments showed that the endogenous reduction of nitrates to nitrites in layers is negligible we directed our attention to the influence of nitrite loads of exogenous origin on poultry and its products.

MATERIAL AND METHODS

The experimental groups were formed from 18 layers 20 months of age (hybrid Tetra). They were placed individually in cages and fed with a complete mixed feed for productive layers HYD-10 NV NORM TYP. The feed and drinking water were supplied *ad libitum.*

The layers were divided into three groups, with six hens in each. During the first two days intended for acclimatization (6) blood samples were taken from the layers to determine the initial values of methaemoglobin (MtHB) and the basic parameters of the blood picture. The first group served as a control; the layers in the second group were supplied sodium nitrite *per os* at a dose of 5 mg per head and day in drinking water; the layers from the third group received 10 mg per head and day. The experiment lasted 28 days.

Every 7 days an analysis of the blood picture was performed and the eggs were examined for nitrites. After 14 days three hens from each group were killed and the nitrite residues were determined in the meat and internal organs. The basic parameters of meat quality (pH and water binding capacity) were determined at the same time. The same determinations were carried out after 28 days.

The level of nitrites was determined spectrophotometrically on a copper-coated cadmium reduction column using the ISO method for the determination in meat and meat products. The values of pH were registered after 20 min and 24 h (pH_1 and pH_{24}) using a pH meter DIGI-88. The analysis of the blood picture and the determination of MtHB were carried out according to the Veterinary Examination Methods (1983).

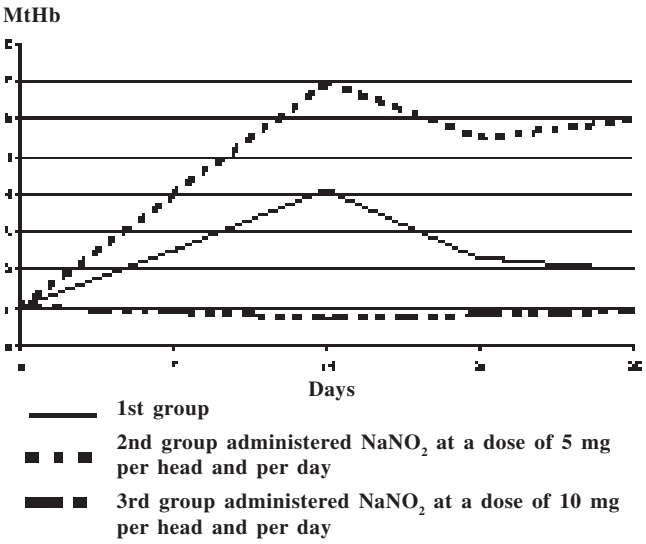

Fig. 1. The values of methaemoglobin in individual groups of layers exposed to $NaNO_2$ load

Table 1. The values of nitrites in the blood serum, muscles, organs and eggs of layers according to groups on days 7, 14, 21, and 28 of the experiment

Organ	Groups	n	Day 7	Day 14	n	Day 21	Day 28
			$NaNO_2$ mg.kg^{-1}				
Blood serum	1st	6	0.01	0.01	3	0.02	0.00
	2nd	6	0.14	0.17	3	0.12	0.09
	3rd	6	0.32	0.30	3	0.34	0.14
Kidney	1st	3	_	0.01	3	_	1.09
	2nd	3	_	1.24	3	_	1.69
	3rd	3	_	1.92	3	_	2.74
Heart	1st	3	_	0.72	3	_	1.02
	2nd	3	_	1.29	3	_	0.92
	3rd	3	_	1.52	3	_	0.72
Muscle	1st	3	_	0.01	3	_	0.08
	2nd	3	_	0.57	3	_	0.61
	3rd	3	_	1.09	3	_	0.44
Liver	1st	3	_	0.00	3	_	0.01
	2nd	3	_	0.65	3	_	0.38
	3rd	3	_	0.78	3	_	0.60
Egg white	1st	6	0.01	0.00	3	0.00	0.00
	2nd	6	0.02	0.02	3	0.01	0.01
	3rd	6	0.12	1.22	3	0.02	0.01
Yolk	1st	6	0.01	0.00	3	0.00	0.00
	2nd	6	0.14	0.13	3	0.01	0.02
	3rd	6	0.19	0.08	3	0.02	0.01

Table 2. Values of pH and water binding capacity on days 1 and 28 of the experiment

Group	n		Day 14 of the experiment			n	Day 28 of the experiment		
			pH_1	pH_{24}	Water (%)		pH_1	pH_{24}	Water (%)
1st	3	$\bar{x}$	6.38	6.09	2.14	3	6.20	6.02	2.44
		SD	0.15	0.36	0.30		0.32	0.15	0.40
		cv	2.63	2.18	7.16		3.10	2.34	5.19
2nd	3	$\bar{x}$	6.33	5.79	6.04	3	6.19	6.03	3.28
		SD	0.94	0.49	5.52		0.40	0.72	0.40
		cv	9.12	6.52	28.22		7.82	5.12	5.82
3rd	3	$\bar{x}$	5.84	5.76	7.19	3	6.55	6.18	9.24
		SD	0.80	0.72	3.00		3.14	2.45	4.18
		cv	10.20	12.14	32.28		27.95	31.48	22.92

Legends: 1st group – control group; 2nd group – group administered $NaNO_2$ at a dose of 5 mg per head and per day; 3rd group – group administered $NaNO_2$ at a dose of 10 mg per head and per day
$\bar{x}$ — Mean; SD — Standard deviation; cv — Coefficient of variance

RESULTS AND DISCUSSION

As with the studies of the majority of authors (5, 1, 4) the response to the oral administration of nitrate to layers investigated in our study was evaluated on the basis of methaemoglobin level dynamics (MtHB) in their blood. The opinions about the physiological limits of methaemoglobin differ. While Piskáč and Kačmár (13) stated that physiological values in the respective animal species should not exceed 2.5% MtHB, the tolerable level of MtHB according Lebeda and Přikrylová (12) may reach up to 5% of total haemoglobin.

Our experiment showed (Fig. 1) that the highest values of MtHB were registered in layers from the third group on day 14 of the administration of nitrites (10 mg per head and day). Its values (7.1%) were very low in comparison with 46% MtHB stated by Baranová *et al.* (3) in hens supplied with nitrates. On the other hand, they correlate with the results presented by Ďureč- ko *et al.* (8). These differences may be explained only by the presence of aggressive microflora responsible for a massive reduction of the nitrates supplied.

Nitrite resides found in blood serum, meat, organs, and eggs of layers are presented in Table 1. While the highest values of nitrites were observed in the blood serum of layers from the third group, the differences in nitrite levels between the second and third groups in other organs were minimal. The only exception was the kidneys which showed significant ($P<0.01$) differences in this parameter. The residua in the heart resembled those in the kidneys, however, as with other organs, nitrites culminated on day 14 and decreased almost by 50% by the end of the experiment.

Similar results were published by Baranová and Maľa (4). With regard to the penetration of nitrites into eggs our results agree with those of Baranová and Maľa (4), not with the values presented by Koré- neková *et al.* (10). Although both sets of authors mentioned registered residues twice as high in the yolk compared to the white, our observations showed no differences between the two egg components.

Experiments with the administration of nitrites to layers initiated papers about their potential supply in feed (11) and drinking water (7, 2). The transfer of nitrates into animal bodies, particularly through vegetable feed, is many times higher and more frequent in comparison with nitrites. While the nitrates put a load on the body only after their reduction, nitrites immediately bind to haemoglobin and other tissues and, because of that, the participation of nitrates in the load is judged according to the result of their reduction. We refer to the endogenous conversion of nitrates which essentially depends on the activity of nitroreductases of the microflora of individual animal species including humans (18). Because of that the quantity of endogenously developed nitrite can only be assessed in comparison with the value measured after the exogenously applied nitrite.

The administration of nitrites to layers caused no marked qualitative changes in their meat. Table 2 failed to register fluctuations in the values of pH_1 and pH_{24} in the experimental groups. It should be mentioned that these values are not so decisive in poultry as, for example, in pigs (14, 9). Higher significance has been ascribed to water binding capacity (15). The results obtained proved that the administration of $NaNO_2$ failed to affect this parameter in both the second and third groups.

The analysis of blood pictures showed no significant changes.

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A COMPARISON OF TWO CONTRAST AGENTS, OMNIPAQUE AND HEXABRIX, FOR POSITIVE-CONTRAST ARTHROGRAPHY OF THE SCAPULO-HUMERAL AND KNEE JOINTS IN DOGS

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ABSTRACT

Two contrast materials Omnipaque 240 and Hexabrix 320 for the detailed X-ray diagnosis of the diseases of the scapulo-humeral and knee joints in dogs were used.

The punction of both joints and the application of contrast media were provided. Lactate dehydrogenase activity and total protein concentrations in collected synovial fluid samples as well as the radio-opacity caused by X-raying of the above-mentioned two agents were tested.

The quality of all obtained arthrograms was high.

From the biochemical point of view Omnipaque contrast medium induced only a mild inflammatory process as well as it was degraded more quickly. The other contrast medium, Hexabrix 320, persists in examined joints longer. These parameters were proved by decline in the total protein level and the lactate dehydrogenase activity. From this point of view we conclude that Omnipaque 240 is a more suitable medium for positive contrast arthrography in dogs.

Key words: arthrography; contrast media; dog

INTRODUCTION

Radiological evaluation is probably the commonest special method, used in surgical, neurosurgical, and orthopaedic practice, and not only in human but also in veterinary medicine.

Šourek (11) states that apart from detailed general, neurological and orthopaedic examinations, the importance of radiological and biochemical methods lies in determining an accurate diagnosis of certain joint diseases and also in indicating their surgical treatment. Radiological methods have brought unusually valuable information about the extent, localization, and sometimes the character of the pathological process.

Movement disorders (limping), which can be detected by using simple and contrast radiograms involve degenerative diseases, congenital anomalies, luxations, fractures, infective and neoplastic diseases (7).

The history of contrast media is comprehensive and connected with the development of the whole of medicine and especially radiology.

The development of contrast media is far from being a closed chapter. On the contrary, we can wait for the future development of new contrast media. Because of the more frequent use of contrast media throughout the whole world, today there is an evident need not only for their extensive production, but also for their more thorough and quicker evaluation from different points of view.

Contrast media are used in the urogenital system, myelography, epidurography, portal venography and arthrography. In arthrography, the injection of positive or negative contrast agents into a joint cavity demonstrates the articular surfaces and outlines of the joint capsule.

The aim of this study was to test and clarify the duration of the activity of two positive contrast media, Omnipaque and Hexabrix, and to compare their quality.

MATERIAL AND METHODS

Five adult (from 2 to 5 year-old) mixed-breed dogs, weighing approximately 14.6 kg (range 9 to 23 kg) were selected for the study. All dogs were kept at the Clinic of Surgery, Orthopaedics and Radiology of University of Veterinary Medicine, Košice. They were fed daily by standard way. Water was given *ad libitum.* Dogs were in good condition and exhibited no clinical signs of any disease.

Two contrast materials, Omnipaque 240 and Hexabrix 320, for the detailed X-ray diagnosis were used. **Omnipaque** (f.

Nycomed, Norway) is an X-ray contrast medium designed for arthrography and arteriography. It is a tri-iodinated, non-ionic water-soluble contrast medium with a molecular weight of 821 (iodine content 46.4 %). **Hexabrix** (f. BykGulden, Germany) is an X-ray contrast medium for arteriography and arthrography. It is a 59 % aqueous solution, which contains Meglumin and Natriumioxalate.

Injection procedure

For anaesthesia atropine (f. Spofa, The Slovak Republic) (0.05 mg.kg^{-1} of body weight) and a combination of xylazine (f. Spofa, The Czech Republic) (2 mg.kg^{-1}) and ketamine (f. Spofa, Slovak Republic) (10 mg.kg^{-1}) were used. Both the right shoulder and stifle joint were aseptically (6, 8) injected with 1—1.5 ml Omnipaque (240 mg.ml^{-1}) and the contralateral joints with 1—1.5 ml Hexabrix (320 mg.ml^{-1}). The injection time was recorded as time 0. One, thirty and sixty minutes after injecting the contrast media the diagnostic quality of the radiographs was evaluated.

Selected biochemical determination

Total protein concentration and LDH activity were determined in synovial fluid. Synovial fluid (0.2 to 0.3 ml) was collected for baseline laboratory analysis from each joint immediately before the contrast agent was injected. Samples were obtained while the dogs were anaesthetized days 0, 1, 3, 7, and 14 after arthrography and were determined for lactate dehydrogenase activity and total protein concentration.

Lactate dehydrogenase was measured kinetically. The principle of the method (4) is that LDH (E.C. 1.1.2.7. L-lactate: NAD oxidoreductase) catalyses the change of lactate to pyruvate with a simultaneous reduction of NAD to NADH, which reduces iodonitrotetrazolium violet to red formazan in the presence of N-methylphenazonium methylsulphate.

Total protein concentration was determined according Pruzanski *et al.* (10). There is a principle that protein and peptides react with the biuret reagent to form a violet complex suitable for photometric determination.

RESULTS

Assessment of radiographic quality

The comparison between Hexabrix and Omnipaque revealed no 1-minute differences after their injection. The miscibility of both contrast media with the synovial fluid was good and intra-articular structures were sharply delineated. However, thirty minutes after injecting Hexabrix 320, the arthrograms obtained better radio-opacity than those with Omnipaque. There was a higher contrast of them. After 60 minutes, joint structures were hardly visible with either media (Figs. 3—12).

Analysis of synovial fluid

The results of synovial fluid analysis in scapulo-humeral and knee joints indicate a higher total protein concentration and LDH activity in synovial fluid after Hexabrix injection (Figs. 1 and 2) as well as the quicker degradation of Omnipaque contrast agent from examined joints (Table 1).

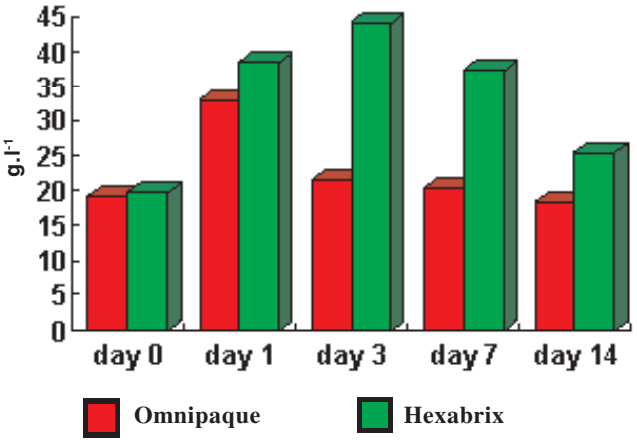


Fig. 1. Total protein concentration in synovial fluid

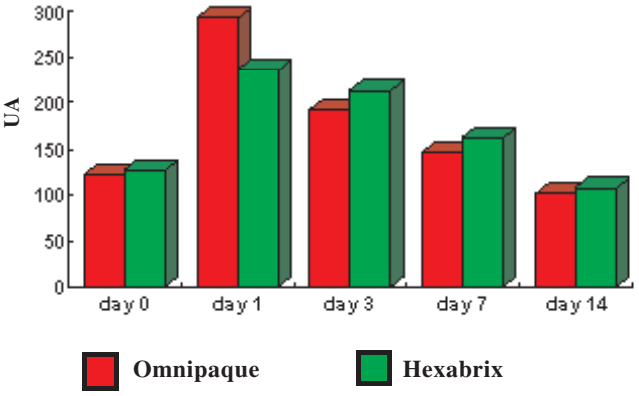
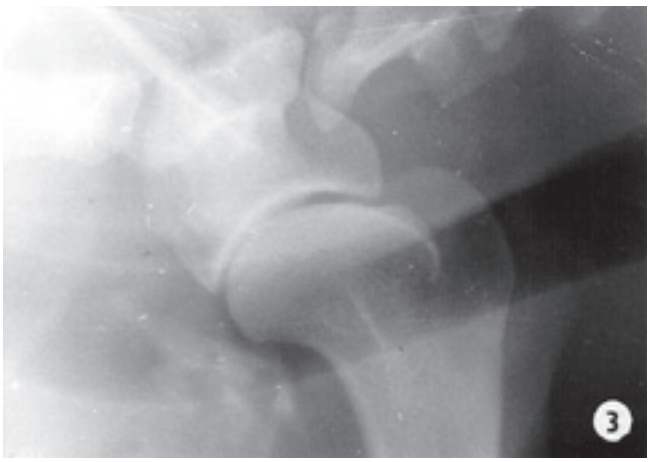
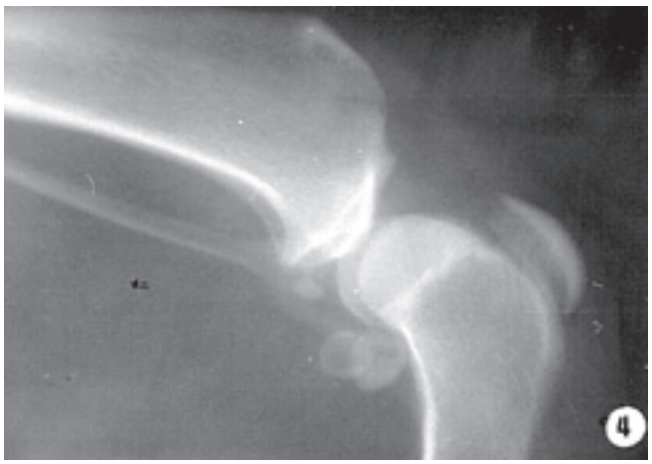


Fig. 2. Lactate dehydrogenase activity in synovial fluid

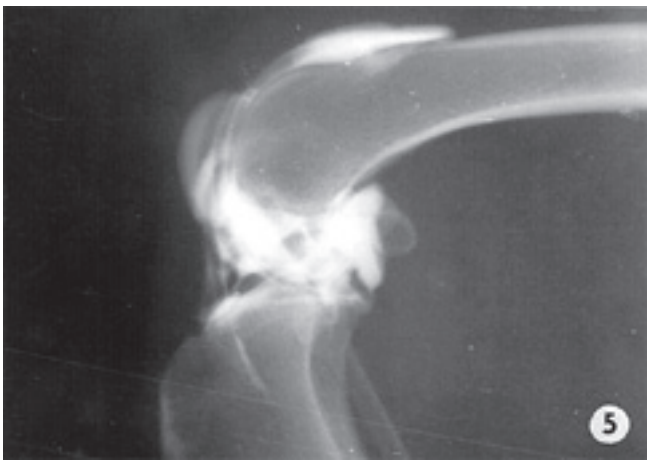
Table 1. The results of synovial fluid analysis after the injection of Omnipaque or Hexabrix in scapulo-humeral and knee joints

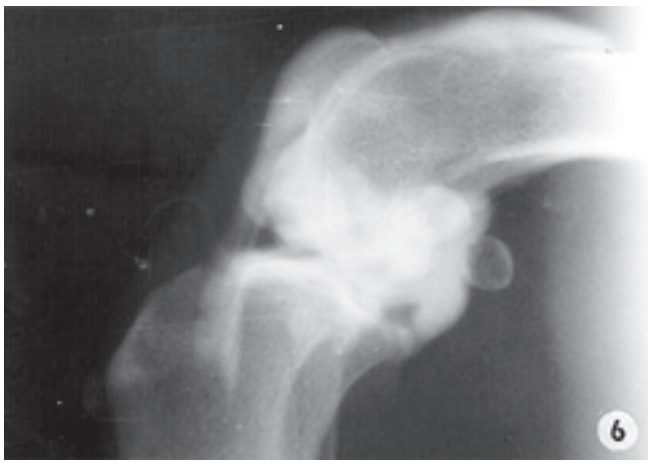
	Time after the injection (d)				
	0	**1**	**3**	**7**	**14**
OMNIPAQUE					
Total protein conc. (g.l^{-1})	19.183 ± 6.999	33.104 ± 15.689	21.472 ± 7.423	20.236 ± 6.761	18.220 ± 5.829
Lactate dehydrogenase (U.l^{-1})	122.657 ± 32.921	292.579 ± 105.495	192.217 ± 99.949	146.011 ± 62.989	101.707 ± 37.57
HEXABRIX					
Total protein conc. (g.l^{-1})	19.72 ± 1.818	38.531 ± 5.845	44.142 ± 11.720	37.339 ± 10.863	25.496 ± 8.995
Lactate dehydrogenase (U.l^{-1})	126.738 ± 37.385	236.033 ± 45.601	213.527 ± 66.664	161.367 ± 42.969	107.367 ± 33.157



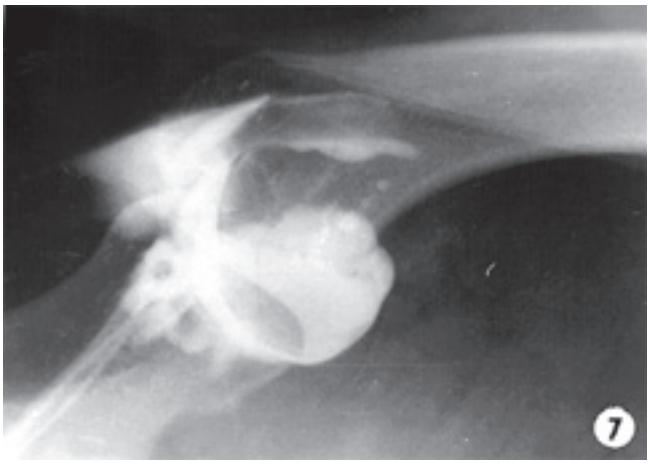


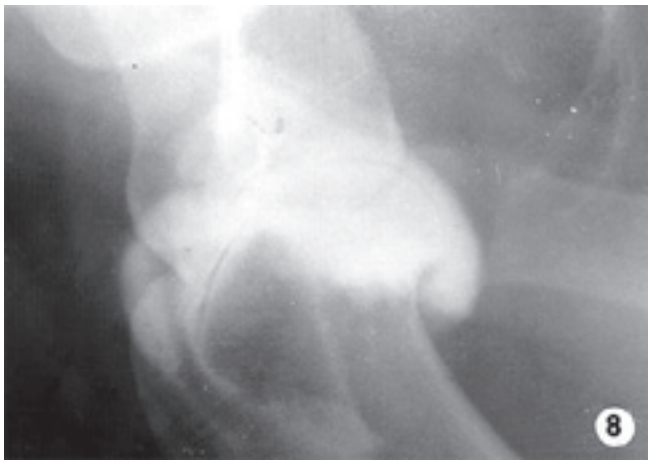
Figs. 3, 4. Simple mediolateral radiograms of the scapulo-humeral and stifle joint of dogs before injection with contrast media



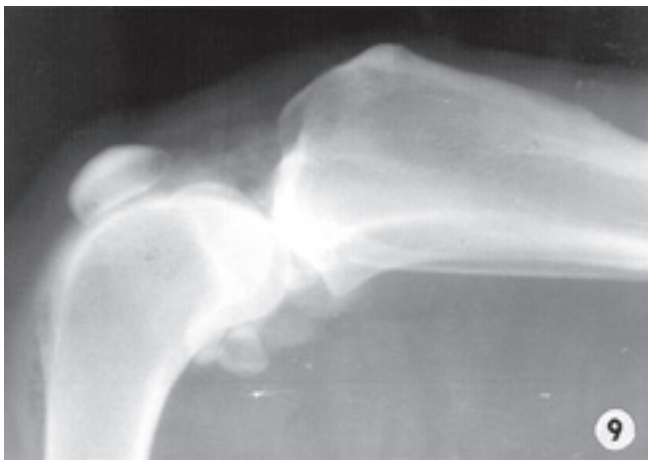


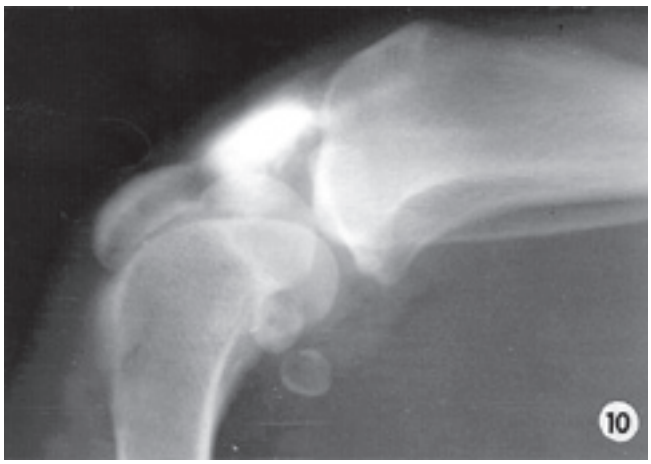
Figs. 5, 6. Mediolateral radiograms of the stifle joints of dogs, comparing Omnipaque with Hexabrix 1 minute after injection. The radiographic quality is similar between the two contrast agents



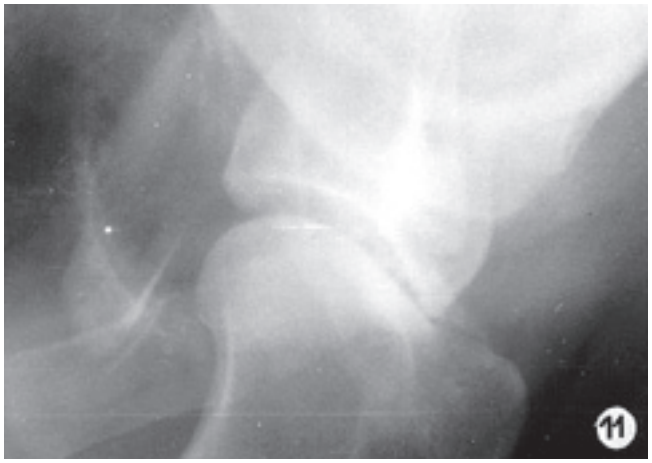


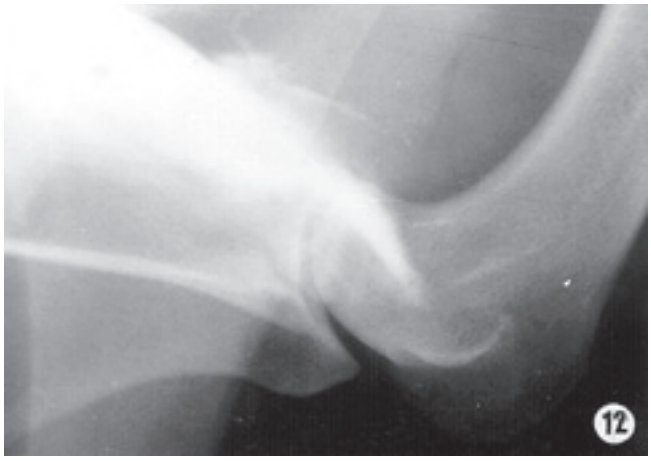
Figs. 7, 8. Mediolateral radiograms of the scapulohumeral joint of dogs, comparing Omnipaque with Hexabrix 1 minute after injection. The radiographic quality is similar between the two contrast agents





Figs. 9, 10. Mediolateral radiographs of the stifle joints of dog, comparing Omnipaque with Hexabrix 30 minutes after injection. The radiographic quality is superior in Hexabrix





Figs. 11, 12. Mediolateral radiograms of the scapulohumeral joint of dogs, comparing Omnipaque with Hexabrix 60 minutes after injection. Omnipaque is completely resorbed and Hexabrix is still visible but of no diagnostic importance

DISCUSSION

The present study revealed the high diagnostic effects of two radiographic contrast media — Omnipaque and Hexabrix. They are very useful in the diagnostic work for the visualisation of any changes, which are undetected under native radiograms. However, these materials have also had the adverse effects (painful application, degenerative lesions) to the examined organs (10). From the biochemical point of view almost all contrast media used in X-ray diagnosis can induce mild inflammatory processes. All adverse effects are caused by the combination of chemotoxic and osmotic effects, and by the ionic composition of the solutions (1, 3).

There is a high affinity of contrast agents to erythrocytes, enzymes as well as to serum proteins. That is why these reactions are used for the determination of contrast media toxicity in the body (4). To obtain information about examined joint reactions to contrast agents LDH activity as well as protein concentration in synovial fluid was provided.

LDH is frequently used for the diagnosis of acute inflammation processes, because this enzyme is a cytoplasmic one (4). Normal synovial fluid generally has a low protein concentration ($< 2.5\,g.dl^{-1}$). Concentration of protein will increase with an inflammatory process.

In our experiment an increase in mean total protein concentration and LDH activity was observed after the injection of Hexabrix. The increase in these values indicates local inflammation, which could be caused by the mechanical irritation resulting from injection, the type of contrast medium, or both (9, 5). The increase in LDH activity may correlate with cell mortality (2).

To obtain summary information about usefulness of chosen contrast agents in practice their radio-opacity were also evaluated. The quality of all obtained arthrograms in the present study was very high. There were no differences between Hexabrix and Omnipaque in this field, although, thirty minutes after the injection with Hexabrix, the arthrograms had better radiographic quality, than those with Omnipaque. It indicates to longer irritation of joint because of persistency of Hexabrix in

it. This fact gives the possibility to evaluate the arthrograms longer. It does not have any influence for routine diagnostic work because appraising of obtained X-rays is done immediately after processing.

Our study clarified the utility both contrast agents — Omnipaque 234 and Hexabrix 320 in routine diagnostic work.

Omnipaque is a more suitable medium. There is its quicker degradation related to the quicker decline in total protein level and lactate dehydrogenase activity to initial levels. The examined joints are exposed to consequence of contrast agent presence for a shorter time.

Finally the using of contrast agent Omnipaque 240 result in obtaining of high quality arthrograms without any severe inflammatory changes of examined joints.

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THE EFFECT OF PROTECTED FAT AND PROTEIN SUPPLEMENTS ON RUMEN METABOLISM, ON SOME PARAMETERS OF INTERMEDIARY METABOLISM, AND ON THE QUALITY AND PRODUCTION OF MILK IN DAIRY COWS

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ABSTRACT

Thirty dairy cows were given calcium soaps of fatty acids (CSFA) 390 g per head daily (CSFA — 0.5 kg Megalac® per head and day) or 180 g per head protected protein (rape seed meal) with 230 g CSFA per head daily (CSFA + P — 0.9 kg Megapro® per head and day) as part of their daily ration composed of maize silage, alfalfa haylage, hay and concentrate.

pH, NH3 and VFA (volatile falty acids) in the rumen and urea in the blood serum decreased in the CSFA group. A more marked decrease in pH and the highest values of NH3 and VFA, as well as increased urea concentration were observed in the CSFA + P group. The serum concentration of total lipids, cholesterol and triglycerides increased significantly and glucose slightly in both experimental groups (CSFA and CSFA + P). The values of acetoacetic acid, BHB acid, and NEFA were not influenced by CSFA and CSFA + P supplements. Added CSFA and CSFA + P increased milk production and slightly reduced the concentration, but not the yield of milk fat and protein.

Key words: Ca soaps of fatty acids (CSFA); intermediary metabolism; lactating cows; milk composition; milk production; rumen fermentation

INTRODUCTION

Energy is often a limiting factor during the early alctation period. Fat added to the daily ration of ruminants have adverse effects on fibre fermentation in the rumen. Protected fats (saponification of oils by calcium) increase the energy value of the daily ration without an adverse effect on fibre content and digestion, thus increasing the effect of energy utilization. Calcium soaps of fatty acids are not soluble at the normal pH of rumen content, they are resistant to rumen fermentation without an adverse effect on the digestibility of the daily ration (29).

Increase in the protected fat level increases acetate concentration in the rumen (15) followed by an increase in milk production. This is accompanied by a slight decrease in protein concentration in milk (6, 19). Casper and Shingoethe (4) suppose that the decrease in protein concentration in milk is due to the lower release of growth hormone and the indirect reduction in amino acid utilization by the mammary gland. Cant *et al.* (3) suggest that fat added to the daily ration decreases blood flow in the mammary gland and decreases the nutrient supply in the mammary gland. The inclusion of combined protected fat and protein supplements (13) or high levels of non-degradable proteins in the rumen (22), in order to reduce the adverse effect of added fat on milk protein concentration, was only partially successful.

The aim of our work was to find out the effect of protected fat supplement and combined protected fat and protein supplements on nutrient supply, ruminal and intermediary metabolism and milk quality and production in dairy cows.

MATERIALS AND METHODS

The experiment was carried out on 30 healthy dairy cows (Black Pied breed; ten animals in third and fourth lactation per group) in the first phase of lactation (upto day 70 in milk) fed basic ration containing corn silage, alfalfa haylage, hay and concentrate mixture (control group). The last mentioned was supplemented with 390 g of protected fat (calcium soaps of palm oil fatty acids — in 0.5 kg of Megalac®, AGRO-BEST, Běstovice, CzR) in the CSFA group or with 180 g of protected protein (rape seed meal) and 230 g of protected fat (in 0.9 kg

of Megapro®, AGRO-BEST, Běstovice, CzR) in the CSFA + P group, with the aim of increasing the energy and protein content of the basic ration in first phase of lactation (from calving upto day 70 in milk). The formulation of daily rations and the content of ingested nutrients and energy in individual groups is shown in Tab. 1.

Rumen content and blood were taken 16 and 36 days after the beginning of the feeding of CSFA or CSFA + P supplements. The pH of the rumen content was determined by potentiometer, NH_3 titrimetrically, volatile fatty acids (VFA) isotachophoretically. The values of total proteins, urea, glucose, total lipids, cholesterol, triglycerides, alkaline phosphatase (ALP), alanine aminotrasferase (ALT), aspartate aminotrasferase (AST), Ca, P, Mg in the blood serum were determined by Biola tests (La Chema Brno, CzR), whereas non-esterified fatty acids (NEFA) were determined by colorimeter according to Duncombe (8), and the values of fatty acids by isotachophoresis. The energy efficiency of VFA production was calculated according to Orskov *et al.* (21). Nutrient content in feedstuffs was determined directly by analytic methods according to the *Decree of the Ministry of Agriculture of the Slovak Republic* No. 1497/4/1997-100 (5). Data about the production and quality of milk were gathered during monthly checks of herd yield.

RESULTS AND DISCUSSION

The evaluation of the daily ration — The basic daily ration was deficient in energy and protein and provided PDI (Protein digested in the small intestine) supply of 74.2 % and an energy supply of 89.3 % when compared with the standardized requirements of nutrients for the specific animal category. The CSFA supplement increased the supply of energy (98 % of the requirement), and the combined CSFA supplement with protein improved the total supply of protein to 82.2 % and energy to 98 % of the requirements. The fibre content in the daily ration in all groups corresponded to the required standard specified for the animal category. Added CSFA and CSFA + P reduced the deficit, particularly that of energy, but did not provide an adequate protein supply in the daily ration.

Table 1. The formulation of daily rations and the content of ingested nutrients and energy in the individual groups of dairy cows

Feed (kg)	Groups CSFA + P	CSFA	Control
Corn and alfalfa silage	16	16	16
Meadow hay	3.0	3.0	3.0
Concentrate mixture	9.0	9.0	9.0
Megapro®	0.9	–	–
Megalac®	–	0.5	–
Dry matter (kg)	17.2	16.8	16.4
PDI (g)	1514.7	1367.1	1367.1
% of requirements met	82.2	74.2	74.2
NEL (MJ)	122.3	122.1	111.5
% of requirements met	98	98	89.3
Crude fibre (kg)	2.5	2.4	2.4

PDI — protein digested in the small intestine

The effect of protected fat or protected fat with proteins on rumen metabolism. The parameters of the rumen metabolism in dairy cows from individual groups are shown in Tab. 2.

pH values of rumen content detected after 16 days of feeding CSFA and CSFA + P supplements were in all the groups observed, including the control, higher than the upper limit of the reference range (30) (Fig. 1). After 36 days all values were in the reference range for optimal ruminal fermentation. In both periods observed the highest pH of the rumen content was detected in the control group, lower in the group with added CSFA and the lowest in the group with added CSFA + P. Differences among the groups were not statistically significant. The pH decrease in rumen content in sheep fed CSFA supplement was detected also by Yildiz (35), Drochner and Yildiz (7) and Ammann (1) when fed CSFA supplement with proteins. Nevertheless, Ohajuruka *et al.* (20) and Savoini *et al.* (25) did not record changes in the pH of the rumen content after including CSFA supplement in the daily rations of dairy cows, Heller (11) even recorded an increase in pH.

Ammonia concentration in the rumen (Fig. 2) after 16 as well as after 36 days of the experiment was lower in all groups than the lower limit of the reference range for the optimal support of rumen microorganism multiplication (15—25 mg % of ammonia) (30) resulting from the protein deficiency in the basic daily ration. When comparing each group, we detected the lowest ammonia values in the group with CSFA supplement, after 16 days of observation, and highest in the group with combined CSFA + P supplement. Similar results were detected by Brinkman and Abel (2), Kim *et al.* (16) and Yildiz (32) when observing the effect of CSFA supplement in the daily rations. After 36 days of the experiment the lowest ammonia concentration was detected in the control group, the values in both experimental groups were slightly lower when compared with the values of the same groups after 16 days of the experiment.

The total amount of VFA (Fig. 3) after 16 days of the experiment was lower in the group with CSFA supplement than the lower limit of the reference range. In the control group and in the group with CSFA + P the values were at the lower limit of the reference range. According to Rodriguez *et al.* (23) the ammonia concentration in rumen may be reduced due to the limited VFA production. After 36 days of the experiment higher VFA levels were detected in both experimental groups. Again, there is an evident negative correlation between VFA production and ammonia content in the rumen. The lower concentration of total VFA in the case of feeding higher fat doses indicates possible interference with rumen microorganism activity or with lowered availability of fermentable carbohydrates in the rumen (16).

After 16, as well as after 36, days of the experi-

ment, we recorded a lower production of propionic acid when the amount of different VFA was observed and this resulted in a higher ratio between C2 and C3 in all groups. The energy efficiency of VFA production was not influenced by the CSFA, or CSFA + P supplements.

The effect of protected fat supplement, or protected fat and proteins on some parameters of intermediary metabolism. The parameters of intermediary metabolism in the blood serum of dairy cows are shown in Table 3.

Values of total protein after 16 days of the experiment were higher in all groups than the upper limit of the reference range (28). After 36 days these were in the upper limit. The differences among the groups were not significant. Similar results were detected by Moallem *et al*. (18) and Salimei *et al.* (24) when observing the effect of CSFA supplement.

When observing urea values in blood serum, we recorded lower levels in the group with the CSFA supplement and higher in the group with CSFA + P supplement, after both days 16 and 36 of the experiment, when compared with the control group (Fig. 4). A decrease in urea concentration was also detected by Salimei *et al.* (24) and Moallem *et al.* (18) when fed CSFA, and an increase in urea concentration is recorded by Rodriguez *et al*. (23) when fed a combined CSFA supplement and proteins. Lower ammonia concentrations in the rumen and urea in blood serum when fed a CSFA supplement, according to Kim *et al.* (16), may result from the more effective utilization of fermentable carbohydrates in the rumen for microbial protein synthesis or the inhibition of microorganism proteolytic activity.

The lowest glucose values in the blood serum of dairy cows were detected in the control group in both samplings, which indicates the above-mentioned energy deficiency. Both the CSFA supplement and the combined CSFA+P resulted in a glucose concentration increase, more obvious after 36 days of the experiment (Fig. 5). All the values detected after 16 days of the experiment were under the lower limit of the reference range. A significantly low glucose level correlates considerably with a low amount of propionic acid in the rumen content, being the main precursor of gluconeogenesis. After 36 days the glucose levels in both experimental groups were above the lower limit of the reference range. The increase in glucose levels correlates with an increase in propionic acid level in the rumen as well as with the protective effect of an energy supply like fat for the energy balance in animals. Despite this, in the control group, a low glucose level persists. Sklan (27), Salimei *et al.* (24) and Moallem *et al*. (18) did not prove the effect of the CSFA supplement on glucose concentration in a nutritionally balanced daily ration.

When evaluating lipid metabolism, after both 16 and 36 days of the experiment, we detected an increase in total lipid, cholesterol and triglyceride levels in the experimental groups, more obviously in the group with CSFA+P supplement (Fig. 6). The values of total lipids the in the group with the CSFA supplement after 36 days, and in the group with the CSFA+P supplement after both 16 and 36 days of feeding were higher than the upper limit of the reference range. Cholesterol values after 16 days of feeding both preparations ranged

Table 2. Parameters of rumen metabolism in dairy cows after 16 and 36 days of the CSFA and CSFA + P supplementation

Parameter/Group Sampling	CSFA+P	CSFA 16th day	Control	CSFA+P	CSFA 36th day	Control
pH	6.85 ±0.35	6.96 ±0.39	6.99 ±0.12	6.48 ±0.44	6.53 ±0.14	6.66 ±0.26
NH_3 mg %	12.1 ±4.9	9.7 ±1.3	11.4 ±2.8	10.7 d ±3.6	8.4 ±2.5	5.4a ±1.0
Acetic acid mmol.l^{-1}	69.1a ±5.7	54.0 d ±6.2	58.9 ±5.6	67.3 ±15.6	72.5 ±4.9	54.4 ±3.4
Propionic acid mmol.l^{-1}	14.7 ±1.4	12.1 ±2.4	12.5 ±2.0	17.5 ±4.7	17.0 ±0.8	14.3 ±3.5
Butyric acid mmol.l^{-1}	9.4 ±2.3	9.0 ±2.4	10.2 ±2.5	17.6 ±12.2	11.2 ±3.4	6.9 ±1.4
Sum of VFA mmol.l^{-1}	93.2a	75.1 d	81.6	102.3	100.7	75.6
Energy efficiency of VFA production %	71.8	72.3	72.1	73.7	72.5	73.0
C2:C3	4.7:1	4.6:1	4.8:1	3.9:1	4.3:1	4.0:1

ad — $P<0.05$; C2:C3 — acetic acid : propionic acid

within the reference values. However, after 36 days, the values measured in both experimental groups were higher than the upper limit of the reference range. The values of triglycerides detected in the experimental groups were higher when compared with the control group, but all the values were within the reference range. Similar results were obtained by Hightshoe *et al.* (12), Horton *et al.* (14), Sklan (36), Hagens *et al.* (10), Savoini *et al.* (26), Salimei *et al.* (24), Espinoza *et al.* (9) and Moallem *et al.* (18) when observing the effect of a CSFA supplement in the daily ration of dairy cows.

On our observing the enzyme profile, the ALP values detected in the group with CSFA+P supplement on day 16, and in the control group in both samplings (on day 16 and 36) were higher than the upper limit of the reference range. The lowest average ALP values were detected in the group with the CSFA supplement, which corresponds with the results given by Moallem *et al.* (18).

The activity of liver transaminases (ATP, AST) was markedly higher in the first sampling (early lactation phase). Increased activity indicating that the liver load was of approved AST values in the control group and in the group with the CSFA supplement. In the group of animals with the CSFA+P supplement, the lowest activities of both enzymes were on average detected, with a marked protective effect of the increased protected protein supply. Whilst Hagens (10) describes a decrease in AST value after including CSFA supplement in the daily ration, Moallem *et al.* (18) recorded an increase in values up to day 30 after the delivery of dairy cows.

On our observing the mineral profile, the lowest values of serum calcium were detected in the control group in both samplings. The CSFA supplement increased the calcium concentration in the blood serum, and in the group with CSFA+P supplement, this was more noticeable. Calcium values in the control group were on average level of the reference range in both samplings, in the group with the CSFA supplement these were at the lower limit of the reference range after 16 days.

The values of serum phosphorus in the control group and in the group with CSFA+P supplement in both samplings were, on average, at the lower limit of the reference range. In the group with the CSFA supplement these were above the limit of this range. Both in

Table 3. Parameters of intermediary metabolism in blood serum of dairy cows after 16 and 36 days of CSFA and CSFA+P supplementation

Parameter/Group Sampling	CSFA+P	CSFA 16th day	Control	CSFA+P	CSFA 36th day	Control
Total protein g.l^{-1}	86.5	98.7	86.7	76.8	79.6	82.5
	± 7.1	± 9.9	± 6.1	± 7.5	± 11.5	± 2.5
Urea mmol.l^{-1}	6.28a	4.55 d	5.17	3.79b	2.18	2.25a
	± 0.56	± 0.98	± 0.89	± 0.68	± 0.41	± 0.41
Glucose mmol.l^{-1}	1.02	0.78	0.73	2.99c	2.99c	1.53a
	± 0.26	± 0.28	± 0.29	± 0.18	± 0.19	± 0.13
Total lipids g.l^{-1}	5.37b	4.63d	2.83a	6.25c	5.70d	3.76a
	± 0.64	± 0.69	± 0.83	± 0.76	± 1,0	± 0.95
Total cholesterol mmol.1^{-1}	4.54 d	4.26	2.96a	6.75b	5.98c	3.48a
	± 0.62	± 0.73	± 1.0	± 0.77	± 1.1	± 0.52
Triglycerides mmol.l^{-1}	0.67d	0.60	0.30a	0.62	0.54	0.41
	± 0.18	± 0.23	± 0.02	± 0.06	± 0.17	± 0.1
ALP mkat.l^{-1}	1.04a	0.70d	0.95	0.79	0.68	0.95
	± 0.18	± 0.09	± 0.22	± 0.13	± 0.21	± 0.23
ALT mkat.l^{-1}	0.32	0.33	0.37	0.22	0.18	0.20
	± 0.043	± 0.065	± 0.046	± 0.032	± 0.048	± 0.036
AST mkat.l^{-1}	0.48	0.53	0.61	0.50	0.47	0.44
	± 0.027	± 0.066	± 0.107	± 0.045	± 0.072	± 0.078
Ca mmol.l^{-1}	2.54	2.23	2.18	2.47	2.31	2.18
	± 0.106	± 0.138	± 0.371	± 0.210	± 0.336	± 0.136
P mmol.l^{-1}	1.59	1.69d	1.30a	1.41	1.62	1.59
	± 0.159	± 0.195	± 0.165	± 0.171	± 0.341	± 0.487
Mg mmol.l^{-1}	0.77	0.74	0.68	1.05	1.10	0.98
	± 0.050	± 0.059	± 0.029	± 0.070	± 0.057	± 0.181
NEFA mmol.l^{-1}	0.25	0.25	0.26	0.25	0.26	0.26
	± 0.009	± 0.015	± 0.011	± 0.021	± 0.022	± 0.011
Acetoacetic acid mmol.l^{-1}	1.7	1.8d	1.4a	1.6	1.5	1.7
	± 0.19	± 0.13	± 0.28	± 0.14	± 0.78	± 0.53
BHB acid mmol.l^{-1}	0.7	1.0	0.7	0.8	0.7	1.4
	± 0.21	± 0.49	± 0.42	± 0.44	± 0.38	± 0.12

ab — $P<0.001$; ac — $P<0.01$; ad — $P<0.05$; BHB — betahydroxybutyric acid
ALP — alkaline phosphatase; ALT — alanine aminotransferase; AST — aspartate aminotransferase

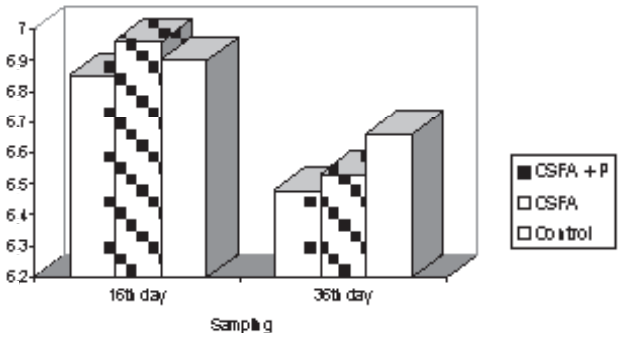

Fig. 1. pH of the rumen content of the dairy cows fed the CSFA and CSFA+P supplements

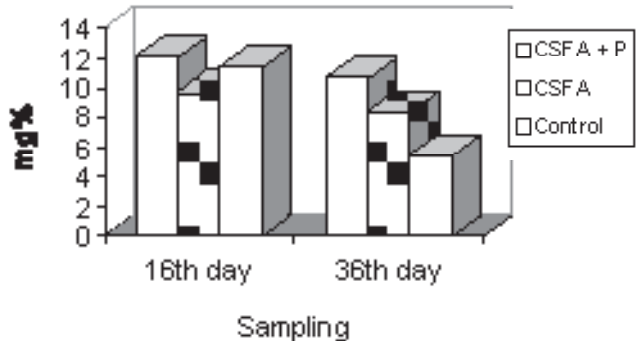

Fig. 2. Ammonia content in the rumen of the dairy cows fed the CSFA and CSFA + P supplements

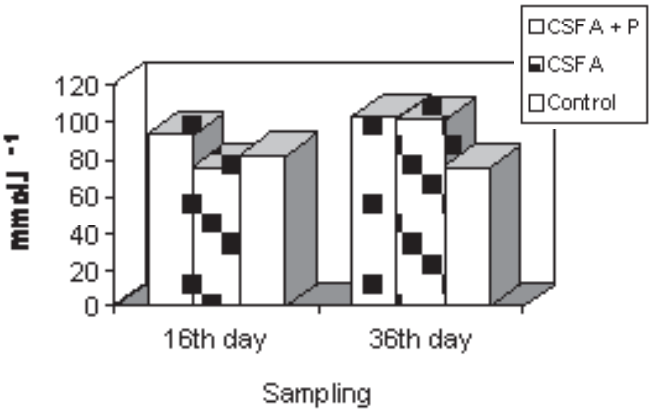

Fig. 3. VFA content in the rumen of the dairy cows fed the CSFA and CSFA+P supplements

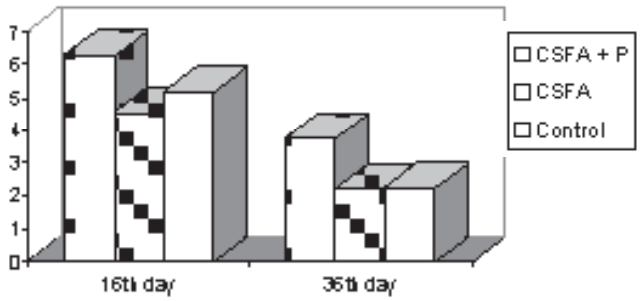

Fig. 4. Urea in the blood serum of the dairy cows fed the CSFA and CSFA+P supplements

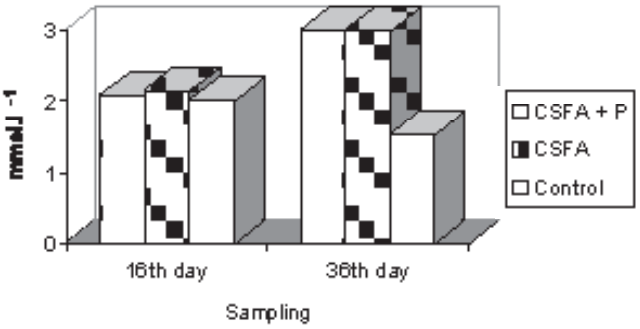

Fig. 5. Glucose in the blood serum of the dairy cows fed the CSFA and CSFA + P supplements

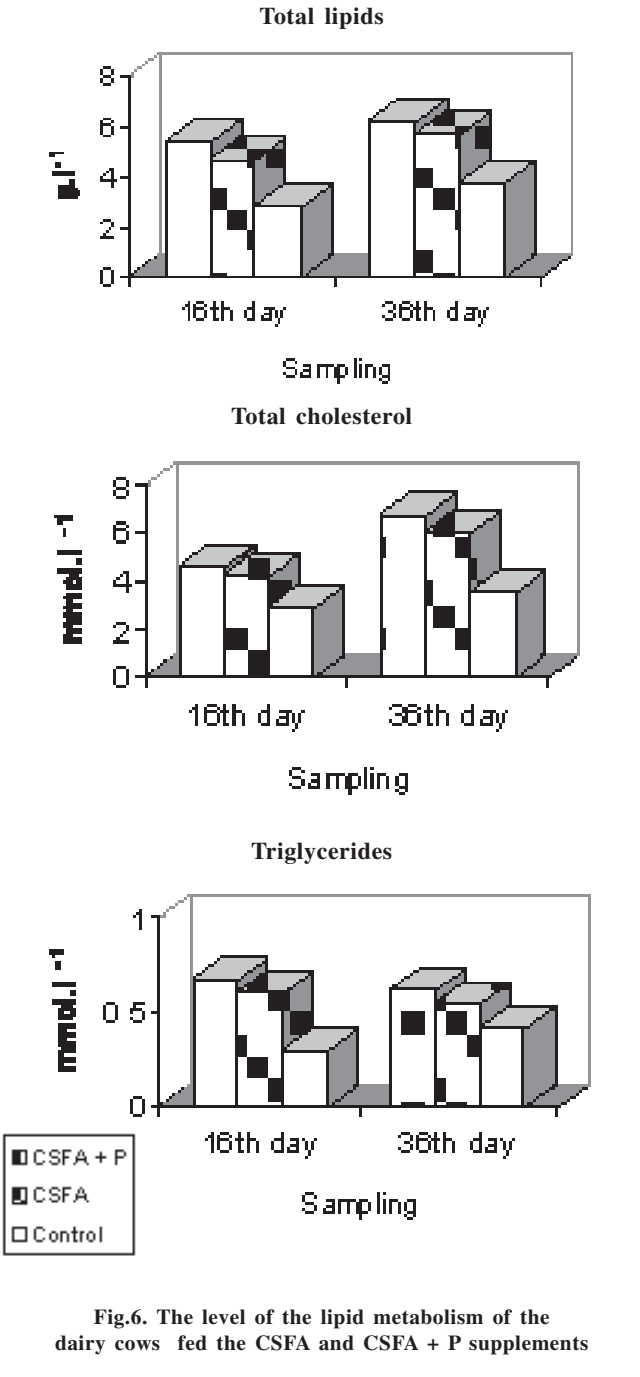

Fig.6. The level of the lipid metabolism of the dairy cows fed the CSFA and CSFA + P supplements

individual and in average values, the decrease in blood serum inorganic phosphorus level correlates with the increase in alkaline phosphatase reflecting the metabolic activity of bone tissue.

After 16 days the magnesium concentration in the blood serum of dairy cows was detected at the lower limit of the reference range with the lowest average value in the control group. The values of serum magnesium detected after 36 days showed an increase to the medial level of the reference range in all groups.

The values of NEFA as well as acetoacetic and betahydroxybutyric (BHB) acids varied slightly between the groups within the reference range levels, and were not influenced by the CSFA, or CSFA+P supplements. Similar results were obtained by Horton *et al.* (14) and Moallem *et al.* (18). The parameters of lipolytic processes in the organism were in close correlation with the body condition of the animals without excessive fat reserves and with a limited process of lipolysis.

The effect of protected fat supplement, or protected fat and protein supplement on milk production and its quality. Data about milk, milk fat and protein production, as well as milk fat and milk protein concentrations in individual groups are shown in Table 4.

The CSFA, or CSFA+P supplements in the daily ration of dairy cows in the first phase of lactation caused a slight decrease in fat and proteins in milk. The increase in milk production and relative decrease in fat and protein concentrations in milk of dairy cows were also observed by Rodriguez *et al.* (23) and Kowal- ski (17). In our observation, the increase in energy concentration (by 8.7 %) in the daily ration resulted in an increase in daily production of milk, fat, and proteins by 10—14 % in comparison with the control group. An equal increase in energy and protein supplement (150 g PDI) (CSFA+P) increased the production of milk, fat, and proteins by one third when compared with the control group. The combined protected fat and protein supplement did not prevent a decrease in the protein concentration in milk.

CONCLUSION

The effects of protected fat and protein supplements were observed in dairy cows in the first phase of lactation. The daily ration in the control group did not provide sufficient energy and protein supply and this markedly influenced the animal's condition and the levels of energy and protein metabolism.

Limited protein supply in the daily ration influenced the level of rumen metabolism by a significantly decreasing the ammonia level in the rumen content, particularly in the control group and in the group with the protected fat supplement (CSFA) which correlates with the low urea level with the exception of the group with the protected fat and protein supplement (CSFA+P) where the protein supplement reduced the protein imbalance. The protected fat supplement (CSFA) stabilized the level of energy metabolism (glucose, triglycerides, cholesterol, total lipids) without a negative effect on rumen fermentation. Optimal levels of energy and protein metabolism indices were detected when feeding the combined protected fat and protein supplement (CSFA+P). Protected fat, or protected fat and protein supplements increased milk production with a subsequent slight reduction of milk fat and protein concentrations.

Table 4. Milk production and composition in dairy cows up to 60 days after calving

Parameter/Group	CSFA + P	CSFA	Control
Milk production (l.day^{-1})	33.9	27.9	24.5
Milk fat production (kg.day^{-1})	1.38	1.14	1.03
Protein production (kg.day^{-1})	0.97	0.80	0.72
Milk fat (%)	4.08	4.10	4.20
Milk protein (%)	2.85	2.87	2.92

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AN EVALUATION OF THE MICROBIOLOGICAL DIFFUSION METHODS AS A TOOL FOR SCREENING MONENSIN RESIDUES IN THE TISSUES OF BROILER CHICKENS

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ABSTRACT

In Slovakia, the official primary control of residues of antibacterial substances in foods of animal origin has been carried out by using the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953 and by using the four-plate test (FPT) with *Bacillus subtilis* BGA and *Micrococcus luteus* ATCC 9341 (2, 5). These methods are based on inhibiting the growth of a test micro-organism in agar medium with the subsequent production of an inhibition zone. The aim of the present study was to evaluate both microbiological methods mentioned above for the detection of monensin residues in the tissues and blood serum of broiler chickens, and to determine the minimum inhibiting concentrations (MICs) and the most sensitive test micro-organism for the monensin tested.

According to our results, obtained by the screening of the tissues and blood sera of broiler chickens, we detected that the FPT showed a greater sensitivity to the monensin residues than the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953. The most sensitive test micro-organism of the FPT was *Micrococcus luteus* ATCC 9341. However, according to the results obtained by the screening of standard solutions of monensin, the MIC of monensin for *Micrococcus luteus* ATCC 9341 was only 1 µg.ml^{-1}. The more sensitive test micro-organism was *Bacillus stearothermophilus* var. *calidolactis* C953 with the MIC of 0.05 µg.ml^{-1}.

The MIC of 0.05 µg.ml^{-1} represents the detection limit of monensin, and with respect to a tolerance of 0.05 mg.kg^{-1} is used as a safe residue level; in our judgement, the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953 is more suitable for the screening of monensin residues at the levels of concern.

Key words: broiler chickens; microbiological methods; monensin; residues

INTRODUCTION

Monensin, a monocarboxylic polyether ionophoric antibiotic, is a natural product derived from the fermentation of *Streptomyces cinnamonensis* and was approved as the first of the group of ionophores in the 1970's for the control of coccidiosis (24). Coccidiosis is the most important disease affecting the economics of the broiler chicken industry. Current coccidiosis-control strategies depend heavily upon the prophylactic feeding of anticoccidial drugs (13). Monensin, involved in shuttle and rotational programmes, is given continuously in the feed as a prophylactic measure to avoid potentially large losses (4). The recommended level of its use for broiler chicken is 100 mg. kg^{-1} of complete feed.

Monensin is also being used in other animal species as a growth stimulant and to improve the efficiency of feed utilization. Typical fortification levels of the feeds range from 20 to 110 mg.kg^{-1}, depending on the species concerned. Monensin is known to be readily absorbed, metabolized and excreted, but a mandatory three-day withdrawal period must be observed before the chickens are slaughtered for human consumption (8).

It is believed that the widespread used of this polyether ionophore in the broiler chicken industry represents a potential hazard to humans, as residues of this drug may persist in edible tissues. There are no reports describing ionophore toxicity in humans following the consumption of meat containing residual concentrations of this drug. Nevertheless, the cardiovascular properties of all the ionophores mean that there is the potential for an adverse effect on human health. These properties have been completely characterized for monensin (14, 15, 21).

Although withdrawal periods from medicated feeds are required prior to slaughter, the possibility that monensin (or other polyether ionophores) in foods could exacerbate the condition of individuals affected with cardiovascular problems remains. The need to identify and quantify monensin in various matrices such as feed, poultry tissues, eggs, etc., is, therefore, obvious (12).

Monitoring the residues of anticoccidial drugs in foods presents a major problem to regulatory authorities (12, 22). Despite the fact that it is now mandatory (6, 11) to include the monitoring of residues of anticoccidial drugs in the national residue-testing programmes, a serious weakness exists due to a lack of suitable tests. The inclusion of anticoccidials in national residue-testing programmes necessitates the establishment of both screening and confirmatory methods suitable for routine monitoring purposes (9, 10) and the establishment of internationally accepted maximum residue limits (MRLs) for those anticoccidials, including monensin, for which no definitive MRL has been established (7).

In Slovakia, according to the veterinary-hygiene legislation, food for human consumption should contain either no residues of veterinary drugs, or levels that are well below those set by the regulatory authorities. For monensin residues in animal tissues, a tolerance of 0.05 $mg.kg^{-1}$ is used as a safe residue level (23). In order to monitor monensin residue levels in poultry tissues, simple, reliable, fast, and sensitive screening methods are required. The purpose of a screening test is to give a quick result whether the analyte is either not present in the sample or is at or below the level of concern (e.g. the safe levels or MRL) (19). Screening methods are designed 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 (1, 12).

Microbiological agar diffusion methods form the basis of screening methods for monitoring the presence of drug residues, which possess antibiotic or antibacterial activity in foods of animal origin. These methods are based on inhibiting the growth of a test micro-organism in an agar medium with the subsequent production of an inhibition zone. Their sensitivity to the residual concentrations of any antibacterial substance depends on the test micro-organism and on the composition and the properties of the medium used. There is a whole gamut of such methods with the various test micro-organisms used worldwide. In Slovakia, the official primary testing for residues of antibacterial substances in foods of animal origin has been carried out by using the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953 (at pH 8.0) as a test micro-organism and by using the four-plate test (FPT) with *Bacillus subtilis* BGA (at pH 6.0, 7.2, and 8.0) and *Micrococcus luteus* ATCC 9341 (at pH 8.0) as the test micro-organisms (2, 5).

In the present study, both microbiological methods mentioned above were used for the detection of monensin residues in the tissues and blood serum of broiler chickens. The diameters of inhibition zones around the samples or discs were measured for the detection of positivity and compared with the inhibition zones produced by monensin standard solutions. The MICs of standard solutions of monensin for the test micro-organisms and the most sensitive test micro-organism were determined.

MATERIAL AND METHODS

Animal treatment and processing of the samples. Twenty 17-day old broiler chickens (hybrid Ross) weighing 0.510 ± 0.012 kg were used in the experiment. The experimental chickens were randomly divided into four groups of five each and they were placed in individual animal-care approved cages with the free access to feed and water. Two types of feeds, medicated feed BR2 and un-medicated feed BR3 (MON-free) were used.

The chickens in the first group (I) were fed with BR3, and monensin (MON) (Elancoban-100 medicated premix, Eli Lilly and Co., USA) was given to the chickens orally by intracrop administration. The dose for the individual chickens was based on their weight and the average daily consumption of feed. Each chicken was weighed every day and 80 $mg.kg^{-1}$ of medicated premix (containing 8 mg of Na-monensin sodium) dissolved in 5 ml of drinking water was administered orally. This dose was estimated to be equivalent to 100 mg of monensin sodium per kg of feed.

The chickens in the second group (II) were fed with BR3 and a medicated premix of monensin was mixed into the feed at the dose of 100 $mg.kg^{-1}$ of feed.

The chickens in the third group (III) were fed with the commercially-produced feed BR2 containing 100 $mg.kg^{-1}$ of monensin in complete feed.

The chickens in the fourth group (IV) were fed with BR3 and served as an untreated control group.

The chickens were treated in these ways until they were 38 days old. On day 38, which represented day 0 of the withdrawal period, one chicken from each experimental group was killed. Blood and tissues (left and right breast muscle, left and right upper and lower thigh muscle, heart, liver, kidney, gizzard, spleen, lung, fat and skin) were removed and frozen. The blood samples were centrifuged at 3000 *g* for 10 min, then the blood serum was separated and stored at –20 °C until analysis.

After day 38, the remaining chickens from all the experimental groups were fed with BR3, un-medicated MON-free feed, until they were 42 days old. During these days, which represented days 1, 2, 3, 4 post withdrawal, the chickens were killed and processed as described above. Control tissues were obtained from the fourth group fed with MON-free feed.

Standard solutions

Monensin sodium salt (M 5273), as a standard, was obtained from Sigma Chemical CO. (St. Louis, MO, USA). A stock solution (500 $mg.ml^{-1}$) was prepared by dissolving an appropriate amount of monensin standard in methanol of p.a.quality (Merck, Darmstadt, Germany). Working solutions of monensin were prepared by serial dilutions with methanol. Monensin was tested using concentrations of 50, 10, 5, 1, 0.5, and 0.05 $\mu g.ml^{-1}$. The stock and working solutions were stored in the refrigerator at +4 °C.

Test micro-organisms and test media

B. stearothermophilus method: *Bacillus stearothermophilus* var. *calidolactis* C953 (Merck 11499), Test agar acc. to Kundrat (Merck 10662).

The four-plate test: *Bacillus subtilis* BGA (Merck 10649), *Micrococcus luteus* ATCC 9341 (CCM 559), Test agar pH 6.0 (Merck 10663), Test agar pH 7.2 (Merck 15787), and Test agar pH 8.0 (Merck 10664).

Principle

Diffusion of any antibacterial substance from a piece of tissue or from a paper disc impregnated with tissue fluid, liquid sample, or standard solution into an agar layer previously seeded with susceptible test micro-organisms present results in the formation of a zone around a sample or disc, in which the growth of the test micro-organism is inhibited. The size of the zone is directly proportional to the concentration of the antibacterial substance within a concentration range, specific for each substance.

Testing of tissue samples

B. stearothermophilus method: Tissue samples (left and right breast muscle, left and right upper and lower thigh muscle, heart, liver, kidney, gizzard, spleen, lung, fat and skin) and blood serum collected from the experimental and control broiler chickens were removed from a deep freezer. Cube-shaped samples with the minimum dimensions of 7 × 7 × 7 mm were prepared with a lancet from the tissues, and two replicate cube-shaped samples from each tissue sample were placed on the surface of the agar media in Petri plates.

The four-plate test: Tissue samples (as in the *B. stearothermophilus* method) and blood serum collected from the experimental and control broiler chickens were removed from a deep freezer. A cylindrical core 8 mm in diameter was obtained from each frozen tissue sample with a cork borer. Two millimetre slices were cut with a lancet from each end of each core, and were discarded. Eight 2 mm slices were then cut from the remaining tissues. Two replicate slices from each tissue sample were placed on the surface of each agar medium in the Petri plates.

The testing of blood serum and monensin standards

Filter paper discs (Whatman 1; 12 mm in diameter) were moistened with 0.1 ml of the blood sera and placed in parallel on the surface of each agar medium in the Petri plates. Monensin standards were dissolved in methanol to prepare the concentrations mentioned above. Filter paper discs were moistened with 0.1 ml of the tested concentration of monensin and placed on the surface of the test plates. At the same time, filter paper discs were moistened with the solvent used, and served as a control. The commercially-produced antibiotic paper discs of penicillin, sulphadimidine and streptomycin were used to check the quality of each prepared agar medium.

The plates were incubated as follows: the plates seeded with *Bacillus stearothermophilus* var. *calidolactis* C953 at 63—65 °C for 3—5 h, the plates seeded with *Bacillus subtilis* BGA at 30 °C for 18—24 h, and the plates seeded with *Micrococcus luteus ATCC 9341 at 37 °C for 18—24 h. After incubation, the plates were evaluated and the diameters of the clear inhibition zones surrounding the tissue samples and paper discs moistened with blood sera and standard solutions were measured in millimetres.*

RESULTS

A positive result was defined as the complete inhibition of the growth of a test micro-organism around both tested samples on one or more plates, in annular zones: 1) in the case of *Bacillus stearothermophilus* var. calidolactis C953 method not less than 3 mm wide for

Table 1. The mean diameters of the inhibition zones (mm) observed in the tissues of broiler chickens after intracrop administration of monensin (group I)

Target tissue	Test micro-organism: *Bac. st.* pH 8				*Bac. st.* pH 6				*Bac. subt.* pH 7.2				*Micr. lut.* pH 8				*Micr. lut.* pH 8			
	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*
LBM	0	0	0	0	1	0.5	0	0	0.5	0	0	0	0	0	0	0	**2**	**2**	1	1
RBM	0	0	0	0	1	0.5	0	0	0.5	0	0	0	0	0	0	0	**2**	**2**	1	1
LUTM	0	0	0	0	0.5	0	0	0	0.5	0	0	0	0	0	0	0	1.5	1	0	0
RUTM	0	0	0	0	0.5	0	0	0	0.5	0	0	0	0	0	0	0	1.5	1	0	0
LLTM	0	0	0	0	1	0.5	0	0	0.5	0	0	0	0	0	0	0	**2**	1.5	1	0
RLTM	0	0	0	0	1	0.5	0	0	0.5	0	0	0	0	0	0	0	1.5	1	0	0
Heart	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	**2**	1	0	0
Liver	2	2	2	1.5	**2**	**2**	**2**	1	**2**	**2**	1	1	**2**	**2**	1	1	**3**	**2.5**	**2**	1.5
Kidneys	2	2	1	1	1.5	1	0	0	1	0	0	0	1	0.5	0	0	**2**	**2**	1	1
Gizzard	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	**2**	1	1	0.5
Spleen	2	2	1.5	1	**2**	1	0	0	?	?	?	?	?	?	?	?	**2**	**2**	1.5	1.5
Lungs	2	2	2	1	**2**	1	1	1	**2**	1	1	1	**2**	1	1	1	**3**	**3**	**2**	1.5
Fat/Skin	**3**	**3**	2	2	**2.5**	**2**	1.5	1	1.5	1	1	1	1	1.5	1	0	**3**	**2**	**2**	**1.5**
Serum	**2**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1.5**	**1**	**1**	**1**

Legends: *Bac. st. — Bacillus stearothermophilus* var. *calidolactis* C953; *Bac. subt. — Bacillus subtilis* BGA; *Micr. lut. — Micrococcus luteus* ATCC 9341; * — days of withdrawal period; LBM — left breast muscle; RBM — right breast muscle; LUTM — left upper thigh muscle; RUTM — right upper thigh muscle; LLTM — left lower thigh muscle; RLTM — right lower thigh muscle; ? — not examined (because of spleen's small size); bold numerals represent the positive results

Table 2. The mean diameters of the inhibition zones (mm) observed in the tissues of broiler chickens after the administration of monensin in feed (group II)

Target tissue	Test micro-organism: *Bac. st.* pH 8				*Bac. st.* pH 6				*Bac. subt.* pH 7.2				*Bac. subt.* pH 8				*Micr. lut.* pH 8			
	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*
LBM	2	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	1.5	1.5	0.5	0.5
RBM	2	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	1.5	1	0.5	0.5
LUTM	0.5	0.5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0.5	0.5	0	0
RUTM	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.5	0	0
LLTM	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0.5	0	0
RLTM	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.5	0	0
Heart	2	1	1	0	**2.5**	1.5	0	0	0	0	0	0	0	0	0	0	1.5	1	0	0
Liver	2	1.5	1.5	1	**2**	**2**	1	0.5	1	0.5	0.5	0	**2**	1	0.5	0.5	**4**	**3**	**2.5**	1.5
Kidneys	2	1.5	1	0	1.5	1	0	0	1	0	0	0	1	0.5	0	0	**2**	**2**	1	0.5
Gizzard	2	2	1.5	0	**2**	1	0	0	0	0	0	0	0.5	0	0	0	**2**	1.5	0	0
Spleen	2	2	1.5	1	**2**	1	0	0	?	?	?	?	?	?	?	?	**2**	**2**	1.5	1.5
Lungs	2	2	1	1	**2**	**2**	1	1	1.5	1	0.5	0	1	1	1	0	**4**	**3**	**2.5**	1.5
Fat/Skin	2	2	1.5	1.5	**2**	**2**	**2**	1	1.5	1	1	1	**2**	1	1	1	**2**	**2**	**2**	**1.5**
Serum	**1.5**	**0.5**	**0.5**	**0.5**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**

L e g e n d s: see Table 1

Table 3. The mean diameters of the inhibition zones (mm) observed in the tissues of broiler chickens after the administration of monensin in feed (group III)

Target tissue	Test micro-organism: *Bac. st.* pH 8				*Bac. st.* pH 6				*Bac. subt.* pH 7.2				*Bac. subt.* pH 8				*Micr. lut.* pH 8			
	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*	0*	1*	2*	3*
LBM	2	1	1	0	1.5	1	1	0.5	1	1	0	0	1	1	0.5	0	**3**	**2**	**2**	1
RBM	2	1	1	0	**2**	1	0.5	0	1	0	0	0	1.5	1	0	0	**3**	**3**	**2.5**	**2**
LUTM	0.5	0.5	0	0	1	1	0.5	0	1	0	0	0	1	1	0	0	**2**	1.5	1.5	1
RUTM	0.5	0.5	0	0	1	1	0	0	0.5	0	0	0	1	1	0	0	**3**	**2**	**2**	1.5
LLTM	1	0.5	0.5	0	1	1	0.5	0	1	0.5	0	0	1	1	1	0	**2**	**2**	1	1
RLTM	0.5	0.5	0	0	1	1	0.5	0	1	1	0	0	1	1	0	0	**3**	**2**	**2**	1
Heart	3	3	2	2	**2**	**2**	1	1	**2**	1	0	0	2	2	1	0	**3**	**2**	**2**	1
Liver	**5**	4	3	3	**4**	**4**	**3**	**2**	**3**	**2**	**2**	1	**3**	**2**	**2**	**2**	**6**	**5**	**4**	**3**
Kidneys	3	3	2	2	**4**	**3**	**3**	**2**	**2**	**2**	1	1	**3**	**3**	**2**	1	**4**	**3**	**3**	**2**
Gizzard	2	1	1	1	**2**	1	1	0	1	0	0	0	1	1	0	0	**2.5**	**2**	**2**	1
Spleen	3	3	2	2	**3**	**2**	**2**	1	?	?	?	?	?	?	?	?	**3**	**3**	**2**	**2**
Lungs	4	3	2	2	**5**	**4**	**3**	**3**	**3**	**2**	**2**	1	**3**	**3**	**2**	**2**	**5**	**4**	**4**	**3**
Fat/Skin	**6**	**5**	**4**	**3**	**3**	**3**	**2**	**2**	**2**	1	1	1	**3**	**2**	1	1	5	**4**	**3**	**3**
Serum	**2**	**2**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**2**	**2**	**2**

L e g e n d s: see Table 1

meat and 5 mm for internal organs; and 2) in the case of the four-plate test method not less than 2 mm for both, meat and internal organs. The minimum acceptable annular zone diameters for the commercially-produced antibiotic paper discs were 6 mm for penicillin, sulphadimidine and streptomycin. The lowest concentration of the standard solution of monensin which completely inhibited the growth of the sensitive test micro-organism was recorded for monensin tested as the minimum inhibiting concentration (MIC).

All the results are summarized in Tables 1, 2, 3, and 4. As Table 3 shows, the largest inhibition zones were observed in the third group fed with the com-

Table 4. The mean diameters of inhibition zones (mm) produced by standard solutions of monensin

Concentration ($\mu g.ml^{-1}$)	Test micro-organisms: *Bac. st.* pH 8	*Bac. st.* pH 6	*Bac. subt.* pH 7.2	*Bac. subt.* pH 8	*Micr. lut.* pH 8
50	8	6	3	4	7
10	6	3	**1**	2	4
5	4	1	0	**1**	2
1	3	**0.5**	0	0	**0.5**
0.5	**2**	**0**	**0**	**0**	**0**
0.05	**0.5**	0	0	0	0

L e g e n d s: see Table 1

mercially-produced feed BR2 containing 100 mg.kg^{-1} of monensin in the complete feed. In this group, using the microbiological method with Bacillus stearothermophilus var. calidolactis C953, we detected two positive target tissues only, the liver on day zero of the withdrawal period, and the fat and skin still on the third day of the withdrawal period. On the fourth day, no positive results were detected.

Using the FPT, more positive results were observed. In the case of Bacillus subtilis BGA at pH 6.0, the right breast muscle and the gizzard were positive up to day zero of the withdrawal period, the heart up to the first day of the withdrawal period, the spleen up to the second day of the withdrawal period, and on the third day of the withdrawal period the liver, the kidneys, the lungs, and the fat and skin were still positive.

In the case of Bacillus subtilis BGA at pH 7.2, the fat and skin were positive up to day zero of the withdrawal period, the kidneys up to the first day of the withdrawal period, the liver and the lungs up to the second day of the withdrawal period, and on the third day of the withdrawal period, no inhibition zones other than the permitted levels set were found in any target tissues.

In the case of Bacillus subtilis BGA at pH 8.0, the fat and skin were positive up to the first day of the withdrawal period, the kidneys up to the second day of the withdrawal period, and on the third day of the withdrawal period the liver and the lungs were still positive.

In the case of Micrococcus luteus ATCC 9341, all the target tissues were positive on day zero of the withdrawal period, the left lower thigh muscle up to the first day of the withdrawal period, the left breast muscle, the right upper thigh muscle, the right lower thigh muscle, the heart, the gizzard up to the second day of the withdrawal period, and on the third day of the withdrawal period the right breast muscle, the liver, the kidneys, the spleen, the lungs, the fat and skin, and also the blood serum were still positive. On the fourth day of the withdrawal period, the liver, the lungs, and the fat and skin were still positive, and the diameters of the inhibition zones reached 2 mm. On the fifth day of the withdrawal period, the inhibition zones in all the above-mentioned tissues were less than 2 mm.

In group I (Table 1) and in group II (Table 2), fewer positive results were detected and the diameters of the inhibition zones of the target tissues were very similar. In group I, using the microbiological method with Bacillus stearothermophilus var. calidolactis C953, the fat and skin were only positive on the first day of the withdrawal period. Using the FPT, the left breast muscle, the right breast muscle, the left lower thigh muscle, the heart, the liver, the kidneys, the gizzard, the spleen, the lungs, and the fat and skin were positive on day zero of the withdrawal period. However, on the third day of the withdrawal period the inhibition zones in all the above-mentioned tissues were less than 2 mm.

In group II, using the microbiological method with *Bacillus stearothermophilus* var. *calidolactis C*953, no positive results were found. Using the FPT, the heart, the liver, the kidneys, the gizzard, the spleen, the lungs, and the fat and skin were positive on day zero of the withdrawal period, and, as in group I, on the third day of the withdrawal period, the inhibition zones in all the above-mentioned tissues were less than 2 mm.

As Table 4 shows, monensin inhibited the growth of all three test micro-organisms. However, the sensitivity of individual test micro-organisms to the standard solutions of the monensin evaluated according to the production of inhibition zones was different.

The MIC of monensin for *Bacillus stearothermophilus* var. *calidolactis* C953 was 0.05 µg.ml^{-1}, the MIC of monensin for *Bacillus subtilis* BGA at pH 6.0 was 1 µg.ml^{-1}, the MIC of monensin for *Bacillus subtilis* BGA at pH 7.2 was 10 µg.ml^{-1}, the MIC of monensin for *Bacillus subtilis* BGA at pH 8.0 was 5 µg.ml^{-1}, and the MIC of monensin for *Micrococcus luteus* ATCC 9341 was 1µg.ml^{-1}.

According to our results, and in spite of the fact that a tolerance of 0.05 mg.kg^{-1} is used as a safe residue level, the most sensitive test micro-organism for the detection of monensin residue was *Bacillus stearothermophilus* var. *calidolactis* C953.

The diameters of inhibition zones caused by commercially-produced antibiotic paper discs on plates were 8 mm for penicillin, 9 mm for sulphadimidine, and 10 mm for streptomycin. The control paper discs moistened with the solvent used did not produce the zones of microbial inhibition.

DISCUSSION

Consumer confidence in the safety of food has become a priority issue for all those involved in the food supply chain. Manufacturers of veterinary medicinal products, livestock producers, veterinary practitioners, and regulatory authorities all have a responsibility to ensure that human health is not placed at risk by the presence of veterinary drug residues in food. Residue detection can be used to monitor the usage of approved substances, to ensure that recommendations concerning dosages and withdrawal periods are being followed and to provide information on the occurrence of residue violations in animal-derived food products destined for human consumption (3).

In the present study, the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953 and the FPT were used for the detection of monensin residues in the tissues and blood serum of broiler chickens. In general, an inhibition test is useful for the detection of an antibacterial substance or a group of antibacterial substances, if the detection limits of these substances are at or below the safe levels or MRL. The detection limit of a test plate was considered as the lowest monensin concentration or MIC, which revealed an inhibition zone on a test plate. The MICs of monensin towards the test micro-organisms were based on standard solutions of monensin.

According to our results, obtained by the screening of the tissues and blood sera of broiler chickens, we detected that the FPT showed a greater sensitivity to the monensin residues than the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953, and that the most sensitive test micro-organism was *Micrococcus luteus* ATCC 9341. Similar results were recorded by Nagy *et al.* (17, 18) who determined the salinomycin residues in the tissues of broiler chickens by using the same microbiological diffusion methods.

For monensin, a three-day withdrawal period is recommended. However, in group III, on the fourth day of the withdrawal period, the liver, the lungs, and the fat and skin were still positive, and the diameters of the inhibition zones reached 2 mm. The chickens in the third group were fed with the commercially-produced feed BR2, and in comparison with the group I and the group II, the higher diameters of the inhibition zones were probably caused by the higher monensin concentration in this feed.

According to Okerman *et al. (*20), the microbiological tests do not require an extraction or clean-up procedure and the tissues are laid directly on agar media. Therefore, it is possible that some tissue components can change the composition of the medium and influence the inhibitory zone produce by an antibiotic residue present in the sample.

According to our results, obtained by the screening of tested concentrations of monensin standard solutions, we judge that in spite of the fact that *Micrococcus luteus* ATCC 9341 had been detected as the most sensitive test micro-organism for the screening of the tissues and blood sera of broiler chickens, its MIC was only 1 $\mu g.ml^{-1}$. The more sensitive test micro-organism for the detection of monensin residue was *Bacillus stearothermophilus* var. *calidolactis* C953 and its MIC was 0.05 $\mu g.ml^{-1}$. The highest sensitivity of *Bacillus stearothermophilus* var. *calidolactis* C953 to the monensin residues was also reported in our previous study (16). The MIC of 0.05 $\mu g.ml^{-1}$ represents the detection limit of monensin, and with respect to a tolerance of 0.05 $mg.kg^{-1}$ is used as a safe residue level, we evaluate that the microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C953 is more suitable for the screening of monensin residues at the levels of concern. Testing the standard solutions of monensin in conjunction with the screening of the monensin residues in the animal tissues serves for the approximate quantification of monensin residues in animal tissues.

The monitoring of monensin residues in foods of animal origin, especially in poultry meat, should be a critical point in the protection of the food chain against the penetration of residues of this drug from the viewpoint of hygiene and public health. The microbiological agar diffusion methods are highly valuable in routine residue testing and can also be used to obtain the primary proof of the presence of monensin residues in the tissues of broiler chickens. However, the postscreening verification of monensin in suspected samples must be performed by an integrated test system utilizing confirmatory techniques (6, 10).

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MORPHOLOGICAL AND IMMUNOLOGICAL CHANGES IN THE SMALL INTESTINE OF PIGS DURING THE FIRST 42 DAYS OF LIFE

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ABSTRACT

Selected morphological and immunological parameters were observed in piglets during the first 42 days of life. The lowest activity of the immune system was found in piglets slaughtered two hours after birth. On day 2, significantly higher values ($p < 0.05$) of the phagocytic activity of neutrophils (PANe) were recorded. The PANe decreased ($p < 0.01$) most markedly after weaning.

The most marked growth of *villi* length was recorded in the duodenum between day 2 and day 7 ($p < 0.001$) and a significant decrease ($p < 0.05$) was found one week after weaning. The length of the *villi* in the jejunum reached its maximum on day 14 — 762.14 mm. A significant decrease ($p < 0.001$) in the length of the *villi* was recorded in the jejunum on day 21. In the ileum, the length of the *villi* generally decreased, except for day 28. A significant difference ($p < 0.001$) was observed when day 7 was compared with day 2.

$CD3^+$ T-lymphocytes were observed for the first time in the duodenum on day 2 and their number significantly increased ($p < 0.001$) again on day 21. Two weeks after weaning, decreased number of $CD3^+$ T-cells ($p < 0.05$) was recorded. In the jejunum significant differences ($p < 0.001$) were recorded on days 2, 21, and 28, resp. A significant decrease ($p < 0.001$) in the target $CD3^+$ T-cell population in the jejunum was recorded one week after weaning. A significant growth ($p < 0.001$) ($p < 0.05$) ($p < 0.01$) ($p < 0.001$) of the number of $CD3^+$ T-cells according to age was found in *villi* of ileum on days 2, 7, 21, and 42, respective.

Key words: lymphocytes; intestine;; piglets *villus*; weaning

INTRODUCTION

The gastrointestinal tract and immune system of piglets undergo changes after birth that are reflected in the development and health status of the body. During the first days after birth, the weight of the small intestine and its absorption ability markedly increases (16).

All compartments of the porcine small intestine contain lymphoid cells at birth. During the first few days of life, maternal antibodies and leukocytes are taken up by the intestinal epithelium (7). The optimal activity of the immune system at a local level and as a whole is crucial for preserving the health status of young piglets. One of the factors that could negatively influence the gut and the immunity is weaning. According to Cera *et al*. (3) occurring stress and dietary changes have a negative effect on small tissue development.

Activated helper T-lymphocytes in the *lamina propria* probably help in local responses (1). T-suppressor lymphocytes enter the epithelium to become intra-epithelial lymphocytes (IEL) and regulate responses by suppressor and contrasuppressor activities. They are also cytoxic for luminal pathogens (1). According to Croitoru and Ernst (4) IEL can play an important role in the induction and regulation of mucous membrane immunity and IgA antibody production.

The aim of this study was to evaluate the development and changes in the morphological and immunological parameters in the small intestine during the first 42 days of piglets' lives bred in normal conditions and weaned on day 28.

MATERIAL AND METHODS

The design of the experiment

In the experiment, we included 16 crossbreed Slovak white x landrace piglets, reared by the sow and weaned on day 28.

From day 5, the piglets were fed with commercial feed mixtures for early-weaning piglets ČOS1 and ČOS2 (Poľnonákup Spiš, Slovakia). Samples of biological material were taken from two animals two hours after birth and then on days 2, 7, 14, 21, 28, 35, and 42.

Sample collection and parameters determined

Blood was collected from the *sinus venosus infraorbitalis*. In the blood we determined the total number of leukocytes (Le), the differential blood picture (DBP), the phagocytic activity and the index of the phagocytic activity of neutrophils (PANe, IPANe, resp.) For immunohistochemical analysis, samples from particular parts of the gut were taken immediately after killing. The tissue samples from the duodenum were taken 5 cm distal from the influx of the *ductus pancreaticus*. Samples from the jejunum and ileum were taken from their medial parts. The total number of leukocytes was measured by a routine method using a Burker chamber. The DBP was established on blood smears stained according to Pappenheim.

Phagocytic activity (PA) and the index of phagocytic activity (IPA)

The parameters were determined by means of microspheric synthetic hydrophilic particles (MSHP — Artim Prague, The Czech Republic) according to Větvička *et al*. (15). Within one hour of sampling, a 0.1 ml aliquot of heparinized blood was incubated with 0.05 ml a solution of microspheric particles, prepared according to the manufacturer' s instructions. The incubation lasted for one hour at 37 °C in a plastic test tube with regular mixing. After the incubation, the blood smear was prepared, dried for 24 h and stained by panoptic staining according to Pappenheim. Then the PANe and the IPANe were determined for 200 cells from each sample using objective magnification 100×. Each of the potentially phagocytizing cells (monocytes, neutrophil granulocytes, eosinophils, basophils) which contained 3 or more phagocytized particles was considered to be a phagocytizing cell.

Immunohistochemistry

Samples of the small intestine tissue were fixed in 10 % neutral formalin and embedded in paraffin wax. Tissue sections (5 to 6 µm thick) were placed on coated slides and deparaffinized in xylene, 96 % benzyl alcohol, and 70 % ethyl alcohol. After inhibiting the endogenous peroxidase in 3 % H_2O_2, the sections were washed in distilled water and digested in 0.4 % pepsin in 0.01 N HCl. Sections were incubated for 16 hours at 4 °C with polyclonal rabbit anti-human T cell CD3 antibody (Dakopatts, Glostrup, Denmark) as the primary antibody in a dilution of 1:300. The secondary antiserum of anti-rabbit origin (Biogenex Laboratories, San Ramon, CA, USA) and the peroxidase-antiperoxidase complex (PAP) were incubated at room temperature for 30 min. The biotin from the secondary antibody reacts with avidin from the peroxidase-antiperoxidase complex. The reaction was visualized by diaminobenzidine (DAB).

The evaluation of villi length and the presence of $CD3^+$ T-cells

The length of the *villi* and the number of lymphocytes in the small intestine were counted using the Meopta micrometer system (Meopta, Czech Republic), which includes ocular and objective micrometers. The coefficient calculated by magnifying the objective 40× was 2.5. The length of the seven *villi* was measured in each part of the small intestine by using the Jenamed 2 microscope (Carl Zeiss Jena, Germany). Positive $CD3^+$ T cells were counted in seven *villi* in a 250 µm long segment from the top of the *villus* by magnifying the objective 40×.

One-way ANOVA with Tukey's multiple comparison test was used to analyse the results.

RESULTS

The total number of leukocytes varied during the experiment between 12.00 $G.l^{-1}$ and 28.75 $G.l^{-1}$. The lowest number of leukocytes was established in the piglets slaughtered two hours after birth. In the following days we observed an increased number of leukocytes, and on day 14 we recorded the first maximum 26.5 $G.l^{-1}$. Up to day 28 the number of white blood cells decreased and a second maximum was reached on day 35—28.75 $G.l^{-1}$. The percentage of lymphocytes in the DBP increased during the first 14 days of life from 33.50 % to 82.00 %. In the next 14 days, this number decreased and on day 28 was 49.00 %. One week after weaning on day 35, the percentage of lymphocytes increased again to 64.67 % and one week later on day 42 it reached 69.00 %. The ercentage of neutrophil granulocytes showed a correlation opposite to the population of lymphocytes. Immediately after birth the percentage of neutrophils reached the value of 66.00 %, which during the first 14 days decreased to 17.50 %. A consecutive increase culminated on day 28—47.00 %. In the next two samplings, the percentage of neutrophils in the whole white blood cell population decreased and two weeks after weaning it reached 27.50 % (Tab. 1).

The phagocytic activity of neutrophil granulocytes was at its lowest level in piglets two hours after birth. On day 2, a definite boost was recorded and significantly higher values were observed in the phagocytic activity of neutrophils (PANe) ($p < 0.05$). The increase lasted until day 14 when the index of the phagocytic activity of neutrophils (IPANe) reached 9.70, and the PANe 73.21 %, which was significantly higher ($p < 0.05$) compared to day 7. A temporary but significant decrease of PANe ($p < 0.05$) on day 21 was relieved by the repeated significant ($p < 0.05$) enhancement. The next reduction ($p < 0.01$) of neutrophils phagocytic activity was recorded on day 35, one week after weaning. IPANe recorded similar changes without significance also, except for day 42 (Tab. 1).

The length of the gut *villi* in particular parts of the small intestine changed according to age. In the duodenum a consecutive increase was recorded in the *villi* length from 335.71 µm to 574.29 µm on day 21 of life. A significant increase ($p < 0.001$) was recorded on day 7 and the next significant increase ($p < 0.05$) on day 21. On day 35, one week after weaning, the average length of the

Table 1. Non-specific immune parameters during the first 42 days of life.
Significant differences at level: a, b, c, d — $p < 0.05$; e — $p < 0.01$

Day	Le ($G.l^{-1}$)	Ly (%)	Ne (%)	PANe	IPANe
0	12.00 ± 3.50	33.50 ± 2.50	66.00 ± 3.00	20.61 ± 0.60[a]	1.57 ± 0.13
2	16.25 ± 1.75	52.50 ± 0.50	46.00 ± 1.00	44.28 ± 0.73[a]	3.23 ± 0.20
7	18.25 ± 6.75	62.50 ± 0.50	36.00 ± 0.00	52.10 ± 5.04[b]	9.32 ± 2.68
14	26.50 ± 1.00	82.00 ± 8.00	17.50 ± 8.50	73.21 ± 1.79[b/c]	9.70 ± 2.55
21	21.50 ± 0.00	65.00 ± 4.00	33.00 ± 5.00	48.35 ± 5.49[c/d]	6.35 ± 3.35
28	22.00 ± 0.82	49.00 ± 4.00	47.00 ± 7.00	73.69 ± 2.02[d/e]	15.64 ± 3.22
35	28.75 ± 1.75	64.67 ± 2.05	33.00 ± 1.00	44.65 ± 3.54[e]	8.09 ± 0.35
42	28.00 ± 1.00	69.00 ± 5.00	27.50 ± 4.50	52.57 ± 4.57	5.72 ± 2.48

Legends: Le ($G.l^{-1}$) — the total number of leukocytes; Ly (%) — the percentage of lymphocytes; Ne (%) — the percentage of neutrophils; PANe — the phagocytic activity of neutrophils; IPANe — the index of phagocytic activity of neutrophils. Values are presented as mean ± SD

Table 2. The length of the villi and the number of $CD3^+$ T-lymphocytes in the *villus* of the small intestine.
Significant differences at level: a, d, e, f, i, l, m, p, q, r, s, t, z — $p < 0.001$; g, j, k, v — $p < 0.01$; b, c, h, n, u — $p < 0.05$

Day	The average length of the *villus* (μm)			The number of $CD3^+$ T-lymphocytes in *villus*		
	duodenum	*jejunum*	*ileum*	*duodenum*	*jejunum*	*ileum*
0	335.71 ± 16.99	591.43 ± 10.59	526.43 ± 17.87[h]	0[l]	0.21 ± 0.25[p]	0[t]
2	359.29 ± 24.85[a]	607.14 ± 36.34	475.00 ± 29.28[h/i]	4.00 ± 0.85[l]	7.29 ± 1.06[p]	4.00 ± 0.76[t/u]
7	491.43 ± 28.75[a]	658.57 ± 39.89[e]	405.71 ± 38.21[i]	5.36 ± 0.64	6.57 ± 0.86	6.29 ± 1.58[u]
14	521.43 ± 49.40[b]	762.14 ± 44.71[e/f]	432.86 ± 34.21[j]	7.36 ± 0.87[m]	7.29 ± 1.83[q]	5.21 ± 0.65[v]
21	574.29 ± 22.59[b]	614.29 ± 27.18[f]	375.00 ± 19.27[j/k]	11.64 ± 1.64[m]	11.64 ± 1.22[q/r]	7.79 ± 1.53[v]
28	548.57 ± 14.32[c]	604.29 ± 33.85	442.14 ± 25.05[k]	10.36 ± 1.16	15.93 ± 2.54[r/s]	8.21 ± 1.06
35	495.71 ± 16.57[c/d]	552.86 ± 43.41[g]	440.00 ± 18.32	10.50 ± 1.65[n]	11.64 ± 1.79[s]	7.50 ± 1.00[z]
42	412.14 ± 20.33[d]	646.43 ± 58.35[g]	405.00 ± 14.88	7.93 ± 1.59[n]	11.71 ± 1.77	10.64 ± 1.60[z]

Legend: values are presented as mean ± SD

villi in the duodenum significantly ($p < 0.05$) decreased to the value 495.71 μm. One week later, a significant ($p < 0.001$) decrease in the *villi* was again observed. The most marked growth in *villi length in the duodenum was recorded between day 2 and day 7 (Tab. 2).*

The length of the villi in the jejunum increased between day 0 and day 14 when it culminated at 762.14 μm. A significant difference ($p < 0.001$) was recorded on day 14. This was followed by a significant decrease ($p < 0.001$) to 614.29 μm. Consistent decrease in this value continued for one week after weaning when the average length of the *villi* in the jejunum was 552.86 mm. On day 42, a repeated increase ($p < 0.05$) of the *villi* in jejunum was recorded. The most marked increases in the length of the jejunum's *villi* were noted between days 7 and 14, and between days 35 and 42 resp. (Tab. 2).

The first recorded average height value of the *villi* in the ileum was 526.43 μm. During the first three weeks of life this decreased to 375.00 μm with significant differences ($p < 0.05$) on day 2, ($p < 0.001$) on day 7, and ($p < 0.01$) on day 21. On day 28, a gentle increase ($p < 0.01$) in the *villi* in the ileum to the value 442.14 mm was observed. It was subsequently offset by a decrease, but the average length of the *villi* in the ileum did not fall under 400.00 μm. (Tab. 2).

The first $CD3^+$ T-lymphocytes were observed in the duodenum on day 2 ($p < 0.001$) compared to day 0 and their average number was four to a *villus*. In the following days their number increased and the maximum during the entire experiment reached the value of 11.64 positive cells on the *villus* on day 21 ($p < 0.001$) compared to the previous week. After day 21, we recorded only a decrease in the number of these immunocompetent cells, and on day 42 it was 7.93 ($p < 0.05$) *vs* day 35 (Tab. 2).

In the jejunum, the first $CD3^+$ T-lymphocytes were already observed two hours after birth, but their numbers were negligible and the average value was 0.21. On day 2 the number of these cells increased ($p < 0.001$) to 7.29 in one *villus*. During the first two weeks of the experiment, the number of this immunocompetent cell population was of the level of 7.00 cells to one *villus*. A more marked increase ($p < 0.001$) was observed on day 21 — 11.64, and the maximum of $CD3^+$ T cells in the *villi* of the jejunum was reached on day 28 — 15.93 ($p < 0.001$). After weaning (day 35), the number of the

T-cell population observed fell to 11.64 ($p < 0.001$) and similar numbers were recorded on day 42 (Tab. 2).

In the *villi* of the ileum an increase in $CD3^+$ T-lymphocytes was observed between days 0 and 2 ($p < 0.001$), and between days 2 and 7 ($p < 0.05$) to 6.29 positive cells to one *villus*. After a temporary decrease on day 14, their numbers started to grow again. On day 21, the length of the *villi* was significantly higher ($p < 0.05$). One week before weaning the number was 8.21. One week after weaning, this had dropped to the value of 7.50, but on day 42, it finished at the level of 10.64, ($p < 0.001$ *vs* day 35) (Tab. 2).

DISCUSSION

The ability to recognize and to respond to antigenic structures is mediated by two cell populations — T-and B-lymphocytes. T-lymphocytes recognize the antigen as accurately as B-lymphocytes do. T-cells recognize foreign antigenic peptides caused by complex enzymatic processing with the innate MHC complex on the antigen-presenting cells (APC). Specific T-cell receptors (TCR) are the heterodimerous glycoproteins present on the cell in connection with CD3 molecules. The molecular complex of CD3 is non-covalently associated with TCR and serves to transmit of the activating signals into the cell (8).

Free $CD3^+$ T lymphocytes were not present in the *villi* of the gut two hours after birth. Only in the jejunum were a negligible number of these cells found — 0.2 cell to the *villus*. On day 2 the number of $CD3^+$ T-cells started to grow and in 250 mm long segment from the top of the *villus* we observed the highest number in the jejunum. According to Rothkötter *et al.* (10) the postnatal increase of IEL largely depends on the breeding conditions: in germ-free animals there was a constant level, while in conventionally bred pigs the numbers increased. The lymphatic tissue of the gut responds to daily contact with massive numbers of antigens by rapid development, which occurs mainly in the early postnatal period of life.

The lymphatic tissue of the gut consists of organized tissue and free diffused lymphocytes under an epithelial layer in the *lamina propria*. The other lymphocytes — intraepithelial lymphocytes are present directly in the epithelial segment of the mucous layer. T-cells mature in the thymus, but one of the extra-thymic places where their maturation can pass is the gut wall. By this maturation T-cells achieve CD3 sign and TCR type (α, β or γ, δ) and in the murine gut they constitute 50—60 % of lymphocytes (14). Apart from the ileum, in other parts of the small intestine, a greater age correlated in our experiment with an increase in the $CD3^+$ T-cell number in the intestinal *villi*. In the duodenum this reached its maximum on day 21 and in the jejunum on day 28. These changes correlate with Rothkötter *et al.* (9) who isolated 26.8×10^6 intraepithelial lymphocytes from the jejunal wall of normal adult pigs compared with 5 day old pigs with a 10-fold lower total lymphocyte yield.

In the first week of life, the maternal antibodies from colostrum and milk play a protective role and partly suppress the development of the innate immunity of the offspring as was shown by Nagahata *et al.* (6) in the first 3 weeks of the lives of calves. A similar effect has been seen in the time scheme of our experiment when the marked increase in $CD3^+$ T-cells was observed after day 14 of life in all parts of the small intestine.

The weaning period mainly influences the ecology of the gut, when the dietary changes can lead to disturbances in the composition of the microflora. Stress that is combined with weaning negatively influences the activity of the immune system also. According to Sola-no-Aguilar *et al.* (12) weaning had a significant negative effect on the numbers of $CD4^+$, $CD8^+$ cells in blood and on the numbers of lymphocytes isolated from mesenteric lymph nodes and ileal sites. In our experiment, one week after weaning, the most pronounced decrease in the $CD3^+$ T-lymphocyte number in the jejunum was observed ($p < 0.001$). In the duodenum and ileum the numbers of the immunocompetent cells decreased only slightly.

The changes observed in non-specific immune parameters, especially increased the number of leukocytes and mostly decreased the percentage of neutrophils in the blood, are in correlation with observations of Sell-wod *et al.* (11). They concluded that neutrophil emigration into the intestinal lumen of piglets could occur in response to $K88^+$ *E. coli*. The non-specific immunity evaluated by the phagocytic activity of neutrophil granulocytes decreased largely ($p < 0.01$) after weaning.

The negative influence of the stress caused by weaning was observed at the local level in the gut and also in the depression some of the non-specific defense mechanisms. It is in a consequence of dietary and microflora changes, when beneficially acting bacteria are replaced by other species without these effects (2).

The length of the intestinal *villi* and its changes differ according to the particular part of the intestine. Tang *et al.* (13) found shorter *villi* in small intestine in piglets at 3 days post weaning (age 15 days) and they observed longer *villi* in the jejunum and ileum on day 34. We found after birth increase in the *villi* length in duodenum and jejunum. By contrast, in the ileum, the *villi* were shorter during the first few weeks. Weaning mainly affected the duodenum and jejunum. In the case of the ileum, the influence of weaning on the length of the *villi* was negligible.

Cera *et al.* (3) observed the shortening of the *villi* between days 21 and 35 in piglets according to the gut environment changes connected with food and microflora. For young growing pigs, the great surface of the gut lumen is important for ensuring optimal digestion and absorption. The short period after weaning is characterized by a lower food intake and generally by an energy deficient state (5).

We found that one week after weaning the *villi* length in the jejunum had decreased. Renewed growth of the

villi was recorded two weeks after weaning. These sudden changes in the small intestine of the pigs during weaning, especially during the 14 days after weaning, negatively influence the total utilization of nutriments. The reduced *villi* surface could be a factor in decreasing the absorption ability, dehydration, and intestinal infections.

CONCLUSIONS

According to the results presented, we can conclude that the influence of the weaning on *villi* length differs in particular parts of the small intestine. The most pronounced negative changes were recorded in the duodenum and jejunum. We can also conclude that the $CD3^+$ T-cell distribution in the *villi* was visible two days after birth in all parts of the small intestine and grew in correlation to the age of the animals. The most negative effect of the weaning on this cell population was recorded in the *jejunum.*

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ANENCEPHALY AND ASSOCIATED MALFORMATIONS IN A OVINE FOETUS (A CASE REPORT)

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ABSTRACT

An unusual case of anencephaly and associated malformations recorded in a bovine foetus is reported. The gross morphological features were acrania, anophthalmia, arrhinia, anotia, duplication of clearly ridged hard palate, absence of soft palate, malformed tongue, rudimentary neck, gastroschisis, polydactyle limbs, hypoplastic left lung and liver and aplasia of spleen, pancreas and urinary bladder.

Radiographic examination revealed acrania, scoliosis, malformed rib cage, improperly developed and malpositioned scapula, radius and ulna, pelvic bones, femur and tarsals. Though there was no evidence of carpals but the metacarpals and phalanges were in duplicate.

Key words: anencephaly; associated malformations; bovine foetus

INTRODUCTION

Congenital malformations of the central nervous system (CNS) are more common than those of any other organ system in both laboratory and farm animals (8). This may be because the development of the CNS begins at an earlier stage of embryogenesis compared with other organ systems. Disturbances with the normal development of the CNS may also affect the development of organ systems including the skeleton. Anencephaly, which means the absence of the brain, occurs due to a primary defect in the anterior neural tube closure during embryonic development leading to defective development of the central nervous system (5).

In this communication, we report a case of anencephaly and associated malformations in a dead bovine foetus.

MATERIALS AND METHODS

Case History: The foetus, was prematurely delivered (with obstetric intervention as the animal went into lateral recumbency after showing severe straining) to a three year-old stall-fed Jersey crossbred after 5 months of gestation. The foetus was weighed and the gestational age was determined by the crown-rump length (7). The gross morphological changes in the foetus were recorded, and radiographic studies of the different regions were carried out by standard procedures.

RESULTS

The foetus weighed 4.3 kg and had a length of 53 cm. The gestational age was approximately to150 days. The gross morphological features were an improperly developed head region lacking normal facial features, gastroschisis, polydactyle limbs, and a fully developed tail (Fig. l). The head region revealed acrania with three fluid filled pouches, anophthalmia, arrhinia, anotia, an improperly developed mandible, a duplication of the clearly ridged hard palate, an absence of the soft palate and malformed tongue (Fig. 2). The neck region was rudimentary, whereas the thorax and abdomen were partially developed. The thoracic cavity revealed a well-developed heart and right lung. The left lung was hypoplastic. The skin and muscles of the ventral abdominal region were not fused at the midline, leading to the evisceration of some abdominal organs (Fig. 3). In the abdomen, the forestomachs were fully developed, the liver was rudimentary, and the spleen and pancreas were absent. The intestines, kidneys and ureters were moderately developed. The urinary bladder was absent and a rudimentary penile urethra was observed. testes were present in the abdominal cavity; however,

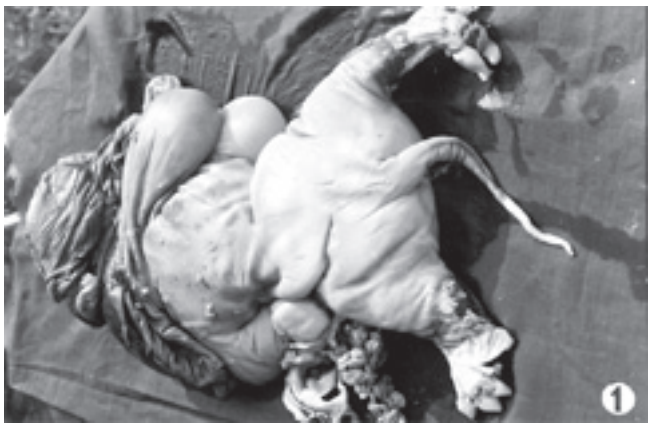

Fig. 1. Dorsal view of the foetus showing acrania with improperly developed head region lacking normal facial features, three fluid filled pouches, gastroschisis, polydactyl hind limbs and a well-developed tail

Fig. 2. Close-up view of the head region showing anophthalmia, anotia, arrhinia, duplication of clearly ridged hard palate and multiple tongue buds

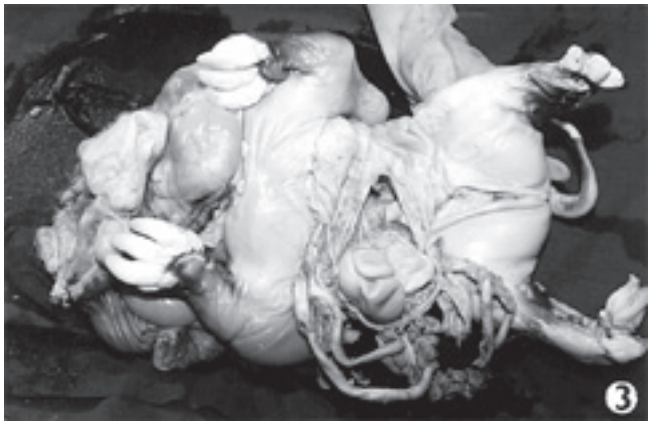

Fig. 3. Ventral view of the foetus showing rudimentary neck region, polydactyl forelimbs, improper fusion of skin and abdominal muscles and evisceration of stomach and intestine

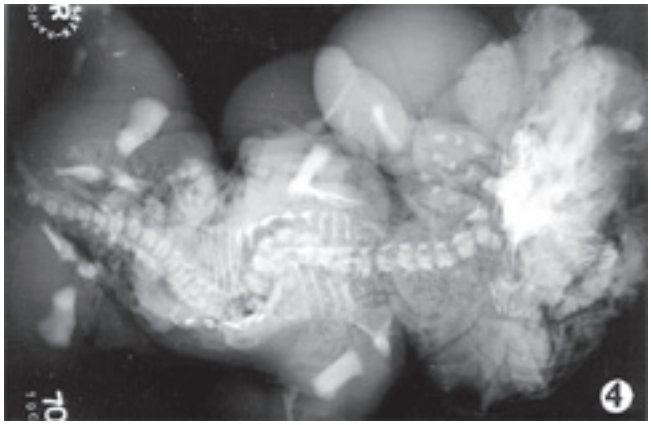

Fig. 4. Radiograph (dorsal view) showing scoliosis, malformed rib cage, malpositioned scapula, moderately developed humerus, radius and ulna, absence of carpals, duplication of metacarpals and phalanges

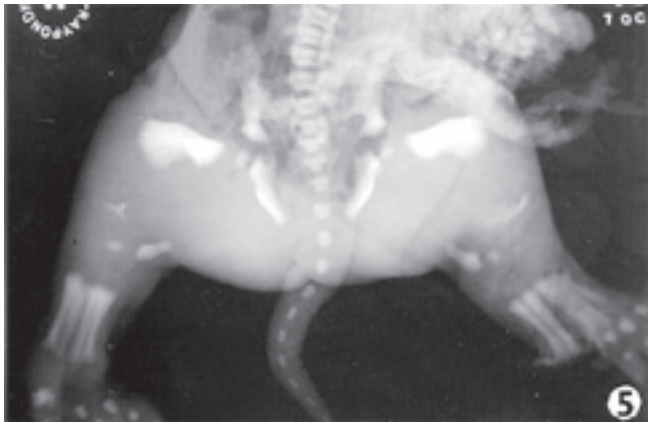

Fig. 5. Radiograph showing poorly developed pelvis and femur, rudimentary tibia and fibula, improperly developed tarsals and duplication of metatarsals and digits

the scrotal sac was mal-positioned on the medial aspect of the left thigh region.

Radiographic examination of the foetus revealed the absence of a cranium, two partially developed upper jaws, and multiple mandibles with dentition. The vertebral column revealed severe scoliosis (Fig. 4). The rib cage was malformed. In the forelimbs, there was evidence of a malpositioned scapula, a moderately developed humerus, an improperly developed and malpositioned radius and ulna. There was no evidence of carpals, but the metacarpals and phalanges were each duplicated. The pelvic bones and femur were poorly developed and the tibia-fibula was rudimentary. The tarsals were improperly developed; the metatarsals and digits were each duplicated (Fig. 5).

DISCUSSION

Malformations of the foetus in mammals are not uncommon, but the atypical congenital anomaly presented here is a rarity. Based on the morphological features, this case is categorized as one of ariencephaly and associated malformations. Neural tube defects result from a combination of genetic and environmental factors and therefore are classified as having a multifactorial aetiology.

An autosomal recessive mode of inheritance has been attributed to the increased occurrence of anencephaly in some laboratory animals and in a Czech breed of swine (8). There is relatively little information on the spontaneous incidence of anencephaly in farm animals. Incidences have been reported in calves, lambs and piglets (3, 6, 8).

Anencephaly has been induced experimentally in animals with the use of physical agents (radiation, excess carbon dioxide with anoxia, hyperthermia), and with chemicals and drugs (actinomycin D, bromodeoxy uridine, cadmium, cyclophosphamide, cytochalasins, diethylene glycol, dimethylether, diphenylhydantoin, ethylene thiourea, meth-amphetamine, methyl mercury, mycotoxins, salicylates, sodium arsenate, sulphonamides, thalidomide, triethanolmelamine, trypan blue, vitamin A toxicity (1, 8). It has been associated in some animals with the consumption of certain plants, infections of the dam with Akabane, and Cache valley fever viruses, and also due to stress following overcrowding (4, 8, 2).

In human medicine, despite the high prevalence of neural tube defects and the significant rate of illness associated with them, much remains to be learnt about their complex multi-factorial aetiology. Evidence suggests that these defects a are aetiologically heterogeneous and may follow foetal insults such as maternal diabetes, hyperthermia, folic acid deficiency, and anticonvulsant (valproate) therapy (5).

Anencephaly may be associated with defects of the face, palate, limbs, and internal organs (8). Experimentally, anencephaly with the complete absence of the brain, calvarium, and face accompanied by gastroschisis was induced in mice following the subcutaneous administration of colchicine (1 $mg.kg^{-1}$ body weight (9)). Anophthalmia and gastroschisis have also been reported to occur secondary to anencephaly in laboratory animals (8). The features described above were observed in the present case.

According to Jones (5), a single localized anomaly in the early stages of morphogenesis can lead to secondary anomalies which may further result in multiple anomalies in later stages. The abnormal development of the foetus is the result of a malformation sequence in which there has been a single localized poor formation of the tissue that initiates the chain of subsequent defects. Accessory tissue such as polydactyly may be presumed to have been initiated at approximately the same time as the normal tissue. In the present case, the anterior neural pole defect might have resulted in CNS developmental defects further leading to secondary malformations. Since, the history of the case did not suggest the involvement of any extraneous factors, it is plausible that polygenetic factors might have contributed to the dysmorphogenesis.

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PRACTICAL EXPERIENCE WITH TIMING THE MATING OF BITCHES ON THE BASIS OF PROGESTERONE LEVEL IN THE PERIPHERAL BLOOD

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This paper was originally presented at an international scientific conference organized on the occasion of the 50th anniversary of the Clinical Department No. 1, the University of Veterinary Medicine Košice, 22—23 June 2001

ABSTRACT

The concentration of serum progesterone was determined in 179 bitches in heat and the time for mating (or subsequent examination) was determined using the following scheme: progesterone below 2 ng.ml^{-1} — repeated examination after 3—5 days; 2—3 ng.ml^{-1} — repeated examination or mating after 3—4 days; 3.1—4.5 ng.ml^{-1} — mating after 2—3 days; 4.6—8 ng.ml^{-1} — mating after 1—2 days; 8.1—16 ng.ml^{-1} — immediate mating. The pregnancy was diagnosed by ultrasonography within 25—30 days of mating. One hundred and thirty nine (77.7 %) out of 179 bitches that mated according to this scheme were pregnant and 40 (22.3 %) were unfertilized.

Key words: bitch; mating; progesterone

INTRODUCTION

The determination of the optimum time for mating raises problems in many cases due to the considerable physiological variability in the length of individual sexual cycle phases and uncommon clinical symptoms. In bitches, prooestrus lasts 3 to 27 days and oestrus 3 to 21 days (7). The total length of heat in 75 % of them ranges between 10 and 21 days on average. A number of breeding approaches and examination methods with varying reliability have been used to determine the suitable time for mating, eg. mating on the basis of discharge changes or compliance to mate, according to the length of heat determined by vaginal cytology or depending on the determination of hormonal profile. The level of blood serum progesterone (P_4) enables us to determine rather accurately the phase of the sexual cycle in the bitch and, in addition, to define with greater precision the optimum time for mating the bitch in heat. The aim of the paper is to assess the level of conception in bitches mated according to the scheme devised.

MATERIALS AND METHOD

In the period from 1998 to 2000, we carried out, at our clinic, single or repeated determinations of blood serum progesterone levels in 179 bitches in heat between, 1.5 and 6 years old. The progesterone levels were used as a basis for determining a suitable time for mating. The blood intended for determining the progesterone was sampled from the *v. cephalica antebrachii* into plastic Eppendorf tubes and sent for examination immediately. The progesterone was determined by the chemiluminescence method in a faculty laboratory.

On the basis of the results obtained, the bitches were scheduled for a repeated examination. Their optimum time of mating was recommended according to the following scheme: at progesterone levels below 2 ng.ml^{-1} repeated examination was recommended after 3—5 days; progesterone equal to 2—3 ng.ml^{-1} resulted in repeated examination or mating after 3—4 days; bitches with progesterone reaching 3.1—4.5 ng. ml^{-1} were recommended for mating after 2—3 days, and those with 4.6—8 ng.ml^{-1} level for mating after 1—2 days; in case of 8.1—16 ng.ml^{-1} progesterone levels immediate mating was recommended (Tab. 1).

In those bitches which could be mated repeatedly, the first mating was timed to the days close to the ovulation (around 5 ng.ml^{-1}) and the second 48 h later. The pregnancy was diagnosed by ultrasonography within 25—30 days of mating by the apparatus Aloca, model SSD 500, equipped with a 5 MHz linear probe.

RESULTS AND DISCUSSION

The ultrasonographic examination conducted 25—30 days following the mating revealed that of the total number of 179 bitches covered, according to the progesterone level, 137 were gravid and 40 unfertilized. It has been stated in general, that the success of mating or

Table 1. Interpretation of findings and determination of the term for mating of bitches in relation to progesterone concentration

Progesterone	Phase of the sexual cycle	Recommended term of mating
Below 2 ng.ml^{-1}	Prooestrus	Re-examination necessary after 3—5 days
2—3 ng.ml^{-1}	Day of the LH peak	Re-examination or mating after 3—4 days
3.1—4.5 ng.ml^{-1}	Period after the LH peak	Mating after 2—3 days
4.6—8 ng.ml^{-1}	Post-ovulation period	Mating after 1—2 days
8.1—16 ng.ml^{-1}	End of the fertile period	Mating as soon as possible

insemination based on the level of plasma progesterone may reach 80 %. Moreover, it has been assumed that a more accurate assessment of ovulation may increase the size of the litter (1).

The scheme devised was based on the following data. During the late proestrus of bitches the production of progesterone in the follicular cells of ovaries is induced by the luteinizing hormone (LH) (7). The pre-ovulation peak in the level of luteinizing hormone lasts for one to two days (3). The ovulation ensues approx. 2—3 days following this LH peak (4). During the ovulation oocytes of the 1st order are gradually released and the total time for the ovulation of all the follicles is highly variable and may last 12—72 hours (3). During the first three days, the oocytes released into the oviduct undergo the first stage of meiotic division and mature to the oocytes of the second order (6). Only after this period are they capable of fertilization in the course of the following 1—2 days (4). Unconserved dog's sperms, after seven hours capacitation, retain the ability to fertilize in the reproductive tract of bitches for 4 to 6 days (3).

During the anoestrus and almost all prooestrus the concentrations of progesterone fluctuate below the basal level of 0.5 ng.ml^{-1}. At the end of prooestrus and the beginning of the oestrous phase of the sexual cycle the level of progesterone increases above the critical value of 1 ng.ml^{-1} (4). In the subsequent period, due to continuing luteinization of follicular cells, the level of progesterone gradually increases and, at the LH peak, reaches the level of approximately 2.6 ± 0.3 ng.ml^{-1} in the majority of bitches 3). During ovulation the progesterone concentration ranges from 4.8 to 6 ng.ml^{-1} (3).

The rate of increase in progesterone concentrations depends on the total number and functional activity of yellow corpuscles which are affected particularly by the size and breed of the bitch. That is why the end of the fertile period cannot be assessed accurately. In general, more ovulations and subsequently also higher numbers of yellow corpuscles occur in medium and large breeds in comparison with small ones, therefore the progesterone concentration in these breeds rises more rapidly immediately before and after the ovulation. It can be assumed that oocytes of breeds with smaller litters lose fertility and vanish at progesterone concentrations of about 11 ng. ml^{-1} and in large breeds at approximately 17 ng.ml^{-1} and, because of that, mating at these concentrations is in the majority of cases unsuccessful. The data presented indicate that the concentrations of progesterone ranging from 7 to 14 ng.ml^{-1}, depending on the breed, appear the optimum for mating.

Our observations revealed some conditions in the unfertilized bitches that could explain their inability to conceive (infertility of the mating dog, forced mating, hypoluteinism, anovulation cycle).

CONCLUSION

On the basis of the highly successful conception of the bitches (even the problematic ones) that were scheduled for mating depending on blood serum concentrations presented in our scheme, this method may be recommended for widespread use in veterinary practice. This method is generally available as progesterone levels can be determined in both veterinary and human medical institutions.

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SPERMATIC GRANULOMAS IN BONNET MONKEYS* (AN ULTRASONOGRAPHIC STUDY)

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ABSTRACT

The ultrasonographic appearance of the testis, epididymis and *ductus deferens* with regard to the localization of spermatic granulomas were studied in long-term vasectomized monkeys. Scrotal sonography is considered to be highly sensitive in detecting intrascrotal abnormalities and also very useful in differetiating testicular from paratesticular lesions. However, from our study, we concluded that although ultrasonographic evaluation may give valuable information in the diagnosis of sperm granulomas in monkeys, the diagnosis of microscopic granulomas could not be detected. The ultrasonographic detection of a spermatic granulomas was possible only in the later stages of its development.

Key words: bonnet monkey *(Macaca radiata); ductus deferens;* epididymis; spermatic granuloma; testis; ultrasonography; vasectomy

INTRODUCTION

A spermatic granuloma is a chronic inflammatory lesion, which appears when spermatozoa extravasate from the reproductive duct system into the surrounding connective tissue (12). These extravasated spermatozoa are surrounded by macrophages and lymphocytes and it is the site where spermatozoal phagocytosis takes place (11). The extravasated spermatids or spermatozoa were treated as foreign antigens as they appear after puberty, when immune tolerance has already been established (7).

The objective of the paper is to evaluate the effectiveness of ultrasonography in diagnosing spermatic granulomas in testis, epididymis, and *ductus deferens* after a long period (2 years) of vasectomy in bonnet monkeys. This paper is a part of our multidisciplinary study on the patho-physiology following vasectomy in bonnet monkeys.

MATERIAL AND METHODS

Animal used: Six bonnet monkeys were used as non-human primate model in this study. The animals required for the research were procured from the Guindy Wild Life Park, Chennai, India after the obtaining necessary permissions. Healthy adult male monkeys of body weight ranging from 5 kg to 7 kg were selected for the study. The fertility of the animals was confirmed by semen analysis during the quarantine period. Quarantine and animal maintenance were carried out according to the guidelines formulated by international (Canadian Council for Experimental Animal Care) and local bodies (Laboratory Animal Science Association of India). The ethical committee of Dr. A. L. M. Pgibms, University of Madras approved the protocol of the work. Details of animal maintenance are described elsewhere (14).

Surgical procedure and postoperative care: The animals were anaesthetized using thiopental sodium (Pentothal® Abbott Laboratories, India) 30 $mg.kg^{-1}$ of body i. p. Under aseptic conditions, a vertical incision for 1—2 cm was made over the scrotum just lateral to the root of the penis. The spermatic cord was exposed and its layers were incised exposing the *ductus deferens.* Care was taken to avoid any sort of vascular injury. The *ductus deferens* was gently squeezed to remove the sperm from the site of incision. After making sure that the *ductus deferens w*as devoid of sperm at the surgery site, two

* Animal experiments were approved by the ethical committee of our institute, and animal maintenance was according to the recommendations of the Canadian Council for Experimental Animal Care and the Laboratory Animal Science Association of India.

ligatures were made one at either end using 5-0 silk suture threads, without including any blood vessels along the *ductus deferens* which were carefully spared. The intermittent segment of about 1 cm of the *ductus deferens* was incised and removed. The wound was closed in layers. No antibiotic was administrated postoperatively. Analgesics were administered for two days postoperatively after which no medication *ductus deferens* administered. The ultrasonographic observations on two years of post-operative period and sham operated control animals were discussed.

Ultrasound imaging: The scrotum and its contents were imaged ultrasonographically using Colour Doppler HDI 1500 (ATL-India Ltd [Philips Company]). A high frequency probe 5—12 MHz was used. Before scanning, the animals were anaesthetized using thiopentone sodium. General anaesthesia was preferred to facilitate the approach without much constraint of the animal. The animals were placed in a supine position with their hind limb stretched apart with slightly bent knees. The hairs of the scrotum were clipped and the ultrasound transmission gel was applied. Transverse and saggital images of the testis and epididymis were made.

In accordance with the original protocol, the animals were sacrificed at the end of intended experiments by an overdose of anaesthesia. Immediately after respiration ceased, the animals were fixed by trans-cardial perfusion with formal saline after flushing the blood with normal saline. Post-mortem and histopathology examination were carried out.

RESULTS

The results reported here are on the ultrasonographic and histological observation made four long-term vasectomized and two sham-operated control animals. In the control animals, the mediastinum and visceral tunica were highly echogenic, the testis and epididymis were of medium echogenic and the *ductus deferens* was hypoechoic (Fig. 1a; 1b and 3a; 3b). In the experimental animals, the spermatic granulomas were seen close to the upper pole of the testis (where the *ductus deferens* was severed surgically) and were difficult to locate. They were seen as mixed echoic structures. In the other two animals, spermatic granulomas could not seen. In all the vasectomized animals, the enlargement of the mediastinum and epididymis were seen; howewer, epidymis, testis, and mediastinum appeared similar to that of control animals in echogenicity (Figs. 2a, 2b, 4a, 4b, 5a, 5b).

Post-mortem analysis confirmed the presence of the granulomas seen in the ultrasound studies. Without such post-mortem observation, it would have been difficult to identify granulomas from the ultrasound images alone. As the granulomas had created impressions on the surrounding structures (Fig. 6), there was a possibility of misinterpreting these as a pathological condition of the surrounding tissues rather than granulomas of the *ductus deferens* itself.

The enlargement of the *ductus deferens* and epididymis was due to the accumulation of sperm. However, unlike the ultrasound observations, histological studies revealed several pathological changes in the experimental animals when compared with control animals. The changes seen in the experimental animals were: a) Hypertrophy of myoid layer. b) Distorted seminiferous tubules. c) Decreased spermatogenesis. d) An increase in the connective tissue proportion of the testis. e) Microscopic spermatic granulomatous reactions in the epididymis and infiltrations at many places in the proximal segment of the *ductus deferens* (such infiltrations include lymphocytes and plasma cells). f) Hyaline degeneration of cells of the duct system. It is noteworthy that there was no indication of these changes in the ultrasonographic observations.

DISCUSSION

Spermatogenesis continues even after 2 years in vasectomized monkeys. Obstruction of the *ductus deferens* leads to a distension of the efferent ducts of the testis, epididymis tubules, and proximal end of the *ductus deferens*, due to the accumulation of spermatids and spermatozoa, which were evident from the distended appearance in sonography and post mortem. The pressure exerted on the duct system cause tubular rupture or small leakage. Extravasated cells in the connective tissue induce the immune response of the host, resulting in infiltration and followed by formation of spermatic granulomas (10, 3).

In the present study, the spermatic granulomas resulting from vasectomy were more common in the epididymis and *ductus deferens* than in the testis. The testis seems to show a resistance or inhibition to granuloma formation. Observations by Itoh *et al.* (6) agree with our results.

Spermatic granulomas especially those which are smaller in diameter, could not be diagnosed by ultrasonography, until the lesion became encapsulated and indurations of surrounding tissues were palpable (8). The exact location of large granulomas became difficult to interpret sonographically, as these granulomas appeared to push into the adjoining structures, which may mimic as lesions of the same tissues (9). Therefore, extra care is necessary in locating granulomas sonographically.

Histopathological study, revealed degenerative changes in the testis and epididymis, with microscopic multiple granulomas, which appeared normal or hypoechoic. It seems to be very difficult to diagnose lesions of this nature sonographically without histological support (5). Larger granulomas near the proximal *ductus deferens* were anechoic or of mixed echogenecity with or without a hyperechoic capsule surrounding the lesion. Our observation suggests that the formation of granulomas differs from animal to animal.

Though we have not tried to correlate the lesion age with ultrasonographic appearance in this study, periodical scanning may be needed to confirm the lesion after the onset of pain after vasectomy. Further, the ultrasound appearance of the lesion and its alteration (1) with the

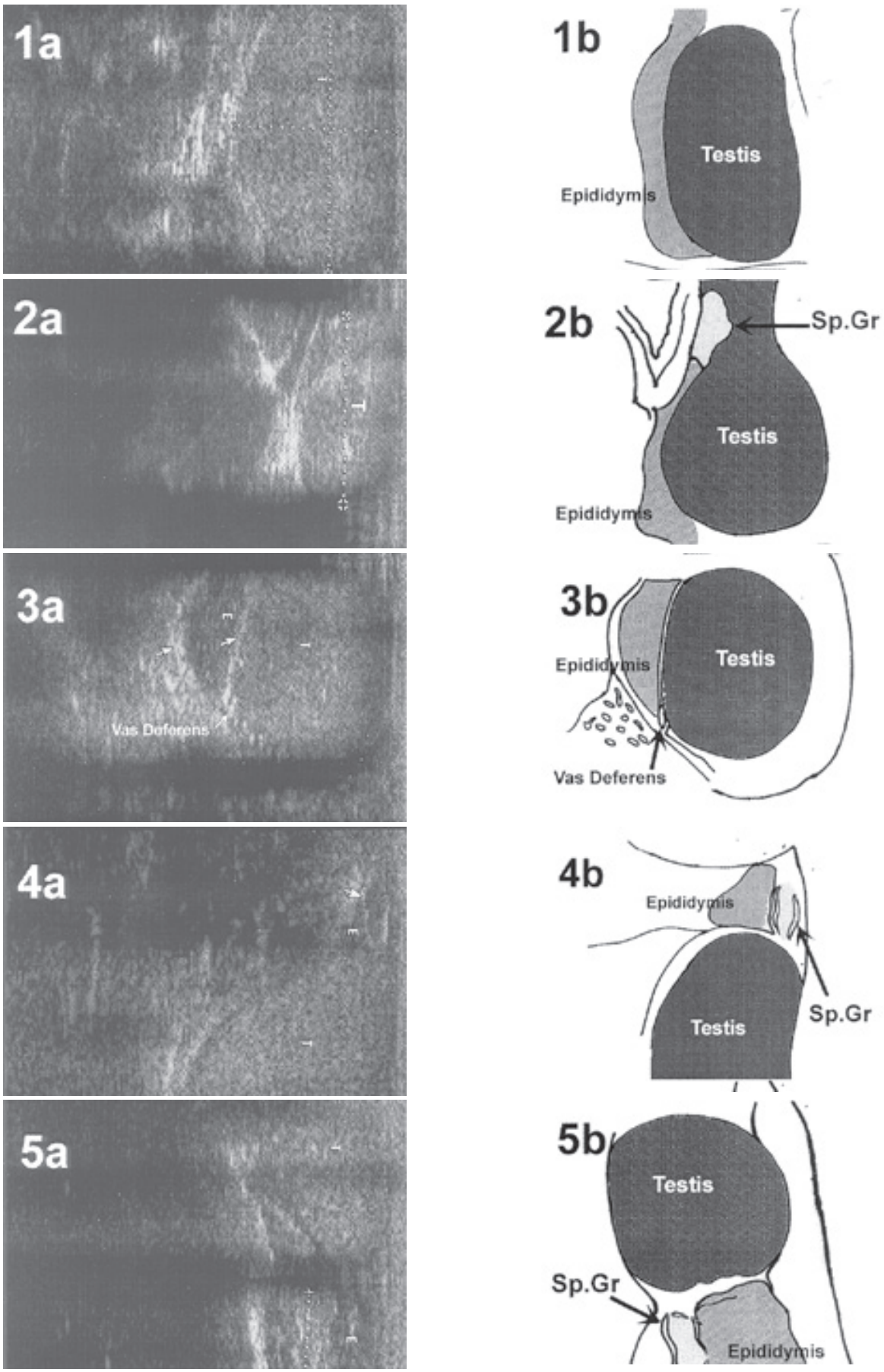

Plate 1. Ultrasound pictures along with schematic representations of the same. In the plate; Fig. 1a & 1b; 3a & 3b are pictures of normal animal, and Fig. 2a & 2b; 4a & 4b; 5a & 5b are pictures of experimental animals. (Sp. Gr. — spermatic granuloma)

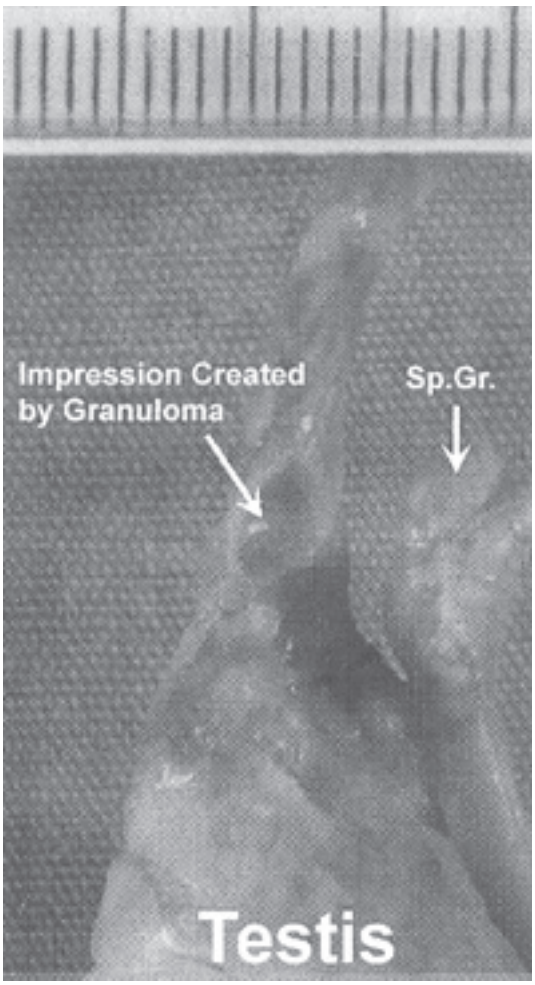

Fig. 6. A photograph showing a large granuloma (Sp. Gr.) in the proximal *ductus deferens* segment and its impression on the surrounding structures

time lapsed between consecutive scanning can be useful in diagnosis. It may elucidate orchialgia, whether it is due to increased epididymal pressure or due to the appearance of a sperm granuloma at the vasectomy site (16).

A large granuloma near the surgery site can impede the blood supply as a result of its compression, which may cause testicular infarction (2) or epididymal inflammation (13). During ultrasound examination of the scrotal contents, the presence of such granulomatous compression on the blood vessel or other surrounding structures could be useful.

In conclusion, our observation showed that **e**pididymal granulomas multiple and microscopic in nature could not be detected conclusively using ultrasound. Similarly, testis showed degeneration histologically, but it appeared as more or less normal echoically. Only large granulomas could be detected in scanning. Ultrasound imaging has its limitation in visualizing various pathological changes in the testis, epididymis, and *ductus deferens* in relation to spermatic granulomas. There is no uniformity in the appearances of granulomas after vasectomy ultrasonographically.

Ultrasound was widely used in human and veterinary care, especially in screening for infertility among animals and removing the affected animals from the colony, where, granulomas were induced by infectious origin (15). The seminal characteristics of the affected animals may not constant enough to diagnose granulomas, as the sperm quality varies according the location of granuloma and is based on uni or bilateral lesions (8). In diagnosing the testicular and para-testicular lesions, a good correlation between pathological and ultrasonic findings (5) is need for a more varied type of therapeutic approach.

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FELINE TUMOUR SUPPRESSOR GENE p53 — MUTATION IN A CASE OF BRONCHIAL GLAND CARCINOMA (A Preliminary Report)

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ABSTRACT

Tumours localized in the lung of cats were subjected to molecular genetic studies. Highly conserved regions of the tumour suppressor gene p53, including typical tumour hot spots were analysed. A 2 bp GT deletion at the splice donor site of intron 7 was present in a bronchial gland carcinoma.

Key words: carcinoma; cat; lung, mutation; p53; splice site

INTRODUCTION

The majority of the lung tumours in cats are malign and are of epithelial origin (17). They often represent bronchial carcinomas, in rarer cases adenocarcinomas and squamous carcinomas.

The tumour suppressor gene p53 is mutated in 60 % of human tumours (3, 16). A majority of the mutations are localized between codons 120 and 290 of this highly conserved gene. In human lung tumours, p53 mutations are common and range from 33 % in adenocarcinomas to 70 % in small cell lung cancers. In the domestic cat, missense mutations have been reported in cases of fibrosarcoma (5, 7, 9, 11), osteosarcoma (8), lymphosarcoma and lymphoma (4, 12), and mammary carcinoma (6, 9).

The aim of the work was to screen feline lung tumours for p53 mutations and to assess the type of the mutations. We now report a p53 mutation in a further feline tumour type.

MATERIAL AND METHODS

DNA was extracted from blood and tumour samples from 8 feline patients by standard techniques (10). Histopathologically, the investigated lung tumours were classified as 4 bronchial gland carcinomas, 2 bronchial squamous cell carcinomas and 2 bronchioloalveolar carcinomas according to the World Health Organization (2). No extrapulmonary metastases were detected in any of the tumours.

A segment of the feline p53 gene corresponding to exon 5 (codons 126—186), intron 5 (82 bp), exon 6 (codons 187—225), intron 6 (231 bp), exon 7 (codons 226—260), intron 7 (267 bp) and the 5´ part of exon 8 (codons 261—298) of the p53 gene (human numbering; 15) was amplified using PCR and the products were sequenced as described earlier (9). Three sets of primers were used to span the region of p53 that contains exons 5 to 8. The first primer pair, 17 bp and 20 bp respectively, was sense 5´-TACTCCCCTCCCCTCAA-3´/antisense 5´-TCGGGCG GCTCGTAGGGCAC-3´. The second primer pair, 21 bp each, was sense 5´-AGCATCTCATCC-GAGTGGAAG-3´/antisense 5´ ATGCAGGAACTGTTACACA-TG-3´and the third primer pair, 20 bp and 24 bp respectively, was sense 5´-GTCGGCTCTGACTGT ACCAC-3´/antisense 5´-CTCAGGGCAAGGCTCCCC CCTCTT-3´.

The polymerase chain reaction (PCR) buffer was composed of 50 mM NaCl, 10 mM Tris-HCl, pH 8.0; 1.5 mmol $MgCl_2$. The enzyme Ampli-Taq-DNA polymerase (Perkin-Elmer-Cetus, Cypress, CA, USA) was used and 35 amplification cycles were performed. Each temperature cycle consisted of template denaturation for 3 minutes at 97 °C, primer annealing for 1 minute at 53 °C and extension for 1 minute at 73 °C.

The PCR products, 385 bp spanning codons 126 to 226, 325 bp from codons 192 to 243 and 489 bp from codons 225 to 298 were analysed by 4 per cent NuSieve/agarose gel electrophoresis.

Amplification resulted in a single discrete band and no nonspecific bands were observed. The products were eluted from the 4 per cent NuSieve/agarose gels using the Gene cleanII Kit (Bio 101 Inc., La Jolla, CA, USA). The PCR-products were sequenced directly to avoid potential sampling errors that could

have arisen if the PCR-products had been cloned and sequenced from one or only a few clones/cat. Sequencing was performed on an ABI 373 A, using a Taq Dye Deoxy Terminator Cycle Sequencing Kit, according to the manufacturers instructions (Applied Biosystems, Foster City, CA, USA).

All sequences were obtained for both strands. The DNA was amplified and sequenced three times to exclude PCR artefacts.

RESULTS

The 8 cats investigated showed normal sequences with one exception. This tumour was a bronchial gland carcinoma (Fig. 1) localized in the lung close to the bifurcation of an eighteen years old grey male Persian cat.

The alteration was an 2 bp guanine and thymine deletion at the boundary exon 7/intron 7 within the splice donor site of intron 7. The sequencing of the 2 bp deletion read as …TCCAAgtagg… → …TCCAAagg…. The triplet TCC and the two bases AA represent codon 260 and the two first positions of codon 261, respectively. For comparison of the feline nucleotide sequence, see Mayr *et al.* (7). This deletion was restricted to the tumour and remained undetected in control peripheral blood leucocytes, thus implying its somatic nature.

DISCUSSION

A broad panel of tumour suppressor p53 mutations including splice site mutations is now known in human lung cancer (13, 1, 14, 16). However, no reports are available about p53 mutations in feline lung cancer so far.

In our present study we detected a 2 bp p53 intron 7 splice donor site deletion. To date, the only feline splice site mutations in cats known so far were found in a pleomorphic sarcoma and a fibrosarcoma, respectively. (7). They were 23 bp and 6 bp deletions including the intron 5 splice acceptor site and intron 7 splice donor site, respectively.

The deletion of the intron 7 splice donor site in our present study could be predicted to directly affect the nature of the encoded p53 protein by making impossible correct splicing of the p53 transcript at the exon 7/8 boundary. Possibly the deleted intron is not spliced out during the mRNA maturation process. As a result larger sized p53 mRNA could be expressed and no or an altered p53 specific protein could be formed. Moreover, the existence of multiple aberrant splice products of high diversity in different frequencies leading eventually to chaotic p53 expression could also be a consequence of the splice site mutation.

Of course, all these considerations are speculative and rigorous studies at the RNA level, e.g. by reverse transcriptase reaction/polymerase chain reaction (RT/PCR) are needed for further elucidation. Moreover, also investigations at the protein level will be necessary in order to detect aberrant splicing products. At any case, this is the first case of a tumour suppressor gene mutation to be reported in the feline lung.

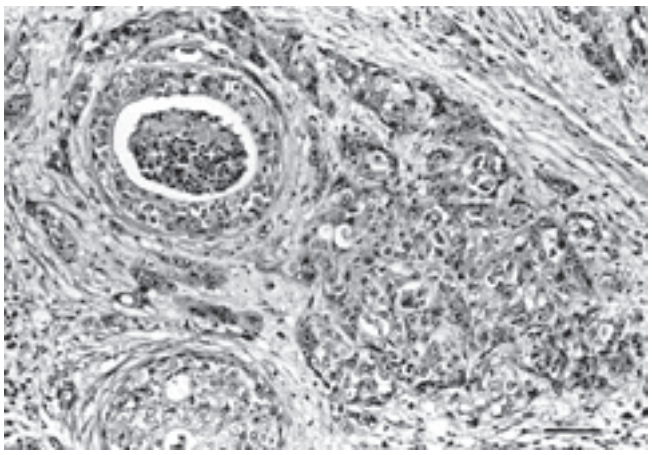

Fig. 1. Tumour of an eighteen years old male Persian cat. Bronchial gland carcinoma; heterogeneous epithelial tumour cells forming solid sheets and gland like structures; cells invading connective tissue of the bronchial wall. Bar represents 60 µm

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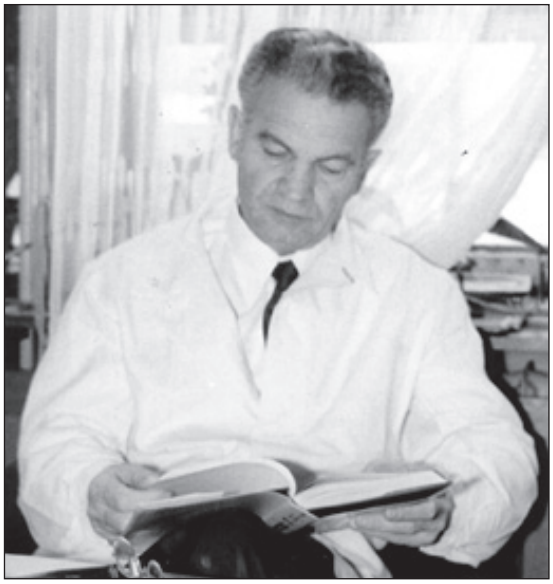
CHRONICLE

THE FIRST RECTOR OF THE VETERINARY COLLEGE IN KOŠICE HAS PASSED AWAY



On 15 November, 2001, the noble heart of Academician Ján Hovor-ka, the first rector of the Veterinary College in Košice, suddenly stopped. The veterinary and academic circles lost its founder, an important pedagogue, scientist, and relentless organizer. Moreover, we all have lost a rare person who left us before reaching his 88th anniversary.

Academician Ján Hovorka was born on February 9, 1914, in Zvolen. In 1939 he graduated from the Veterinary College in Brno. After a short period of military service he practised veterinary medicine as a private veterinarian in the Bratislava region. On the basis of a contest, he was promoted to associate professor and later to deputy professor of the Department of Agricultural Engineering at the Slovak Technical College in Bratislava where he established an Institute of Anatomy and Physiology and read lectures on veterinary subjects untill 1945.

After the College of Agricultural and Forestry Engineering (CAFE) was founded in Košice, he joined the new establishment and, under modest but very creative conditions, helped to build up the agricultural and later on also the veterinary college education.

He took an active part in coping with the problems which our agriculture faced after World War II. There was shortage of veterinarians in Slovakia which called for the establishment of a new veterinary college. Thanks to Prof. Hovorka, a Veterinary College (VC) was established in Košice and he became its first rector. He occupied this post from 1949 untill 1952. In the period of 1955—1982 he was a member of its Scientific Council and the head of the Department of Parasitology.

Academician Ján Hovorka was the founder of parasitology in Slovakia. He became involved in parasitology already in 1947 at the CAFE. He was interested in the parasites of poultry. Already in 1950, at the Department of Parasitology of VC in Košice, he became involved in the research into pneumohelminthoses of ruminants. The serious problems associated with parasitoses brought up the idea of establishing a scientific institute within the prospective Slovak Academy of Sciences (SAS) involved in the research of helminthoses.

His profound interest in building up Slovak national culture, education and science enticed him to join the group of people who laid the basis of the Slovak Academy of Sciences. In 1950 he was a member of the Presidium of SAS and established a Veterinary cabinet within this institution. In 1952, as a chairman of Subcommittee for biological sciences and the preparatory Presidium of SAS, he helped to lay the basis of the SAS. He was a member of the Presidium of SAS in the period of 1962—1965.

Prof. Hovorka acted in many organs of the SAS and Czechoslovak Academy of Sciences (CzAS) as a chairman and member of scientific boards, the editorial board, and similar. He defended his doctoral thesis in 1961 and became a Doctor of Science. He was elected a member correspondent of the SAS (1953), Czechoslovak Academy of Sciences (CzAS) (1962), Academician of the SAS (1964), and Academician of the CzAS (1977). For his unrelenting activities toward the development of science he was awarded many prizes and medals. He was elected an honorary member of seven parasitological societies abroad.

The intention to establish a monothematical institute oriented on the research of helminths acquired a concrete shape in 1953 by establishment of the Helminthological Laboratory of the SAS which was later renamed to Helminthological Institute of the SAS. Academician Hovorka headed the Institute for 35 years during which it gained recognition international the national scope.

His merits and position in world-wide parasitology were acknowledged by electing Academician Hovorka an *honorary member* of nine associations abroad.

The scientific work of Academician Hovorka is closely connected with his publication activities. He is an author of books for students of veterinary medicine and of scientific monographs. He published more than 300 papers at home and abroad and hundreds of specialised contributions.

He founded and edited the journals *Veterinársky zborník (Veterinary Proceedings)* (1952) and *Veterinársky časopis (Veterinary Journal)* (1953), the predecessor of today's *Slovenský veterinársky časopis (Slovak Veterinary Journal),* issued until 1957. He founded also an international journal *Helmithologia* issued up to this day in English. He was a member of seven editorial boards at home and abroad.

Prof. Hovorka participated in the education of generations of veterinarians. He was a very demanding but also understanding teacher. Because of that they remember him with respect and esteem. As a chairman of the Slovak society of veterinarians he acted in this professional institution disregarding the limitations associated with the political system of that time.

Not less important were his scientific-education activities. He was the tutor of 70 candidates of sciences in the branch of parasitology and invasive diseases who hold today various posts at home and abroad.

The unrelenting, demanding and varied activities of the founder, teacher, scientist and organizer so beneficial to the society, science and practice were appraised by the highest state orders, prizes, honorary memberships and also by branch and scientific distinctions. Many of them were awarded by scientific institutions abroad.

The passing away of Academician Ján Hovorka means that our society and the veterinary and scientific circles have lost a great personality, a man who ploughed a deep the furrow in the history of the Slovak nation. He justly deserves deep thanks and good memories of the wide veterinary and scientific community, his pupils, co-workers and friends who came to the Crematorium in Košice on 21 November, 2001, to part from him and to express deep sympathy to his wife, children, and grandchildren.

Your memory will always remain with us, dear Academician Hovorka. Rest peacefully in the Slovak ground that you loved so much.

Cabadaj, R.
the Rector of UVM in Košice
Dubinský, P.
the Director of the Parasitological Institute of SAS in Košice

FOLIA VETERINARIA, 46, 1: 55—56, 2002

THE 22nd CONGRESS OF THE CZECHOSLOVAK MICROBIOLOGICAL SOCIETY (CSMS) IN KOŠICE HELD IN SEPTEMBER 2001

The 22nd Congress of the Czechoslovak Microbiological Society took place at the University of Veterinary Medicine in Košice between 5 and 9 September, 2001. The Organizing committee, headed by Prof. DVM. I. Mikula, DSc., prepared an abundant first-rate scientific and social programme for the participants.

The Congress was opened by Prof. I. Mikula, DSc., and RNDr. J. Spížek, CSc., the chairman of the Society. The opening ceremony took place at the presence of the rector of UVM, Dr.h.c., Prof. DVM. Rudolf Cabadaj, PhD., who was the patron of the Congress, and Valéria Fuleová, the vice-consul of The Czech Republic in Košice. Assoc. Prof. RNDr. J. Jelínková, the chairman of the Society for Epidemiology and Microbiology of the Czech Medical Society of J. E. Purkyně, conveyed greetings to the Congress participants from the sister society. The plenary session of CSMS was opened by Dr. J. Spížek. He presented an evaluation of the past three-year period of 1998—2001 and recapitulated briefly the activities of the Society. He spoke about the structure of the Society and its important scientific activities over the 1998—2000 period, scientific meetings that took place in the past and about collaboration with the Federation of European Microbiological Societies (FEMS).

After the report about the activities of national branches of the Society and a presentation of the report of the audit committee, new honorary members of the CSMS were elected. They were Prof. Hana Kopecká and Prof. I. Lefkovits. The chairman of the Society awarded Patočka's medals to Prof. Ladislav Borecký, Prof. Libor Ebringer, the former chairman of the Society, and Prof. Jiří Stárek in honour of their achievements in the development of Czechoslovak microbiology. The medal commemorating the founder of Czechoslovak microbiology Prof. František Patočka was established several years ago and was awarded for the first time in 2000.

The introductory part of the Congress comprised invited lectures of Prof. L. Borecký and Prof. I. Lefkovits and election of the new Committee of the Society. The new Committee appointed RNDr. J. Spížek, CSc., the vice-director of the Microbiological Institute of the Academy of Sciences of CzR in Prague, to serve another term as the chairman of the Society. An exceptional experience for the participants was the performance of a Romany band from the Intermediate artistic school in Košice. The first day of the Congress ended with a very successful welcome cocktail.

The scientific part of the 22nd Congress of CSMS included 12 plenary sessions and 16 thematic sections. The plenary sessions were held in the Pavilion of morphological disciplines of the UVM. The participants listened to lectures about the present knowledge regarding the basic orientation of medicinal and environmental microbiology. With regard to the increased interest of society in mad cow disease (BSE), the lecture of Prof. Michal Novák, DSc., the director of the Neuroimmunological Institute of SAS in Bratislava, on the theme *"Prionoses and tauonoses of humans and animals"* attracted the biggest attention. The principal theme of the Congress, *"Health and Microorganisms",* resounded through lectures *"Microorganisms and their interactions with the immune system"* (Prof. I. Mikula), *"Do we need new antibiotics?"* (Dr. J. Spížek), *"Molecular factors and mechanisms of bacterial pathogenicity"* (Assoc. Prof. I. Čižnár), *"Immunomodulation effects of targeted cytostatics"* (Prof. B. Říhová), *"Intestinal immunity"* (Prof. H.Tlaskalová), *"Very early and early proteins encoded with herpes viruses, interfering with cellular signalisation and regulation of transcription"* (Assoc. Prof. J. Rajčáni), and *"Why there is no effective herpes vaccine?"* (Dr. G. Russ). Close association between the environmental quality and human health was suggested in the lecture by Dr. J. Gabriel on the theme *"The use of wood-destroying fungi in environmental microbiology"*. One of the most important collections of micro-organisms in the Czech and Slovak Republic was presented by Dr. Z. Páčová from Brno, under the title *"The Czech collection of micro-organisms as a centre of biological resources"*. In the plenary part, the participants from abroad were represented by Prof. Artem Totoljan from Russia who presented a lecture on the theme *"Genetic evidence of IgG-binding proteins participation in poststreptococcal glomerulonephritis"* (APSGN).

The scientific sections included 81 lectures and 231 posters. The sections were formed on the basis of all-year round activities of the Society and discussed the progress and new development in all areas of current microbiology. In six rooms during three Congress afternoons, discussions were held on the following topics: Antibiotics, Probiotics, Biosorption and Biodegradation, Genetics of micro-organisms, Mucosal immunity, Immunological aspects of interaction micro-organism – host, Yeasts and the health, Micro-organisms and human health, Molecular trends in the diagnosis of micro-organisms, Food and micro-organisms, Subviral agents, Factors and mechanisms of bacterial pathogenicity, Virology, Microbiology of water, Companion animals and health, Micro-organisms in the environment. The

majority of lectures were on a high scientific level and it was very satisfying that this applied practically to all the lectures and posters prepared by young authors in the succeeding generation of young microbiologists.

A part of the 22nd CSMS Congress was the abundant social programme. In addition to the concert of the Romany band, the participants could attend a concert in Dom umenia in Košice and a ballet performance of Giselle in the historical building of the East Slovakian theatre of Janko Borodáč. The social programme culminated with a banquet in the Mirror hall of the Institute for Post-graduate Education of Veterinarians and ended with a picnic.

Altogether 380 participants attended this Congress, mostly from Slovakia and the Czech Republic, but also from Croatia, France, Poland, Ukraine and Russia. I believe that despite some minor shortcomings, relating to the organization of the Congress, all participants of the 22nd Congress of CSMS returned from Košice with very strong positive experiences. On their behalf we should thank to the organizers of the Congress headed by Prof. I. Mikula.

RNDr. Jiří Gabriel, CSc.
Scientific secretary of CSMS.
Vice-chairman of the Organising Committee

ADVANCED NOTICE

In endeavouring to bring FOLIA VETERINARIA up to the standard of important European and other international scientific journals, a new arrangement of papers will be found in issue No. 1/2002. New guidelines will be published in issue No. 2/2002.

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