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SUSTAINED VERSUS SHORT-TERM *CAUDA EQUINA* CONSTRICTIONS RESULTING IN A SELECTIVE DAMAGE TO LUMBOSACRAL SPINAL CORD NEURONAL POOLS IN THE DOG

Šulla, I.[1], Vanický, I.[2], Capková, J.[3], Balik, V.[4]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice
[2]Neurobiological Institute of the Slovak Aacademy of Sciences, AD Centre of Excellence, Šoltésovej 4, 040 01 Košice
[3]Department of anesthesiology and intesive care, P. J. Šafárik University School of Medicine, Tr. SNP 1, 040 11 Košice
[4]Department of Neurosurgery, AD Centre, P. J. Šafárik University School of Medicine, Tr. SNP 1, 040 11 Košice
The Slovak Republic

sulla@uvm.sk

ABSTRACT

The aim of the present canine experimental study was to evaluate light-microscopic changes observed in lumbosacral spinal cord segments of dogs following short-time and sustained *cauda equina* constrictions. Fifteen adult mongrel dogs of both sexes, weighing 11–18 kg, were included in the study. The animals were anaesthetized with thiopental administered intravenously at a 4 mg.kg^{-1} dose, intubated with an endotracheal cannula and placed on a volume-cycled ventilator. The anaesthesia was maintained by a mixture of medical oxygen with 1–2 % halothane. The dogs were divided to 3 groups: (1) Sham controls (n = 3). Animals underwent L_7–S_1 laminectomy only; (2) 3-day *cauda equina* constrictions (n = 6). After L_7–S_1 laminectomy 4 surgical ligatures with about 2 mm spacing were applied around the dural sac containing nerve roots of *cauda equina* and surgical wounds were sutured in anatomical layers; (3) 9-day *cauda equina* constrictions (n = 6). *Cauda equina* was constricted by 4 surgical ligatures tightened around the dural sac at the level of L_7–S_1 vertebrae. After completing experimental procedures all animals were placed into separate disinfected compartments, they were offered drinking water *ad libitum* and a full diet. On day 3 (dogs from group 2) and 9 (dogs from groups 1 and 3) post-operation, in a deep thiopental anaesthesia (6 mg.kg^{-1} i. v.), the animals were perfused by 3 000 cc of saline and fixed by 3 000 cc of solution of 3 % paraformaldehyde with 2 % of glutaraldehyde in 0.1 mol phosphate buffer at pH 7.4. The L_3–S_1 spinal cord segments were cut in semi-thick (30 µm) sections and processed by Nauta and Nissl staining methods. No visible argyrophilia was detected in spinal cord specimens of control animals. Following 3 days of *cauda equina* constrictions (dogs from group 2) we observed a distinct axonal argyrophilia affecting postganglionic sacrococcygeal dorsal root afferents. In dogs from group 3 the light-microscopic observations revealed a broad spectrum of spinal cord changes-argyrophilia of dorsal root afferents and neurons located in dorsal horn superficial layers (laminae I–IV); argyrophilia of middle-sized and large neurons in the central part of the intermediate zone (lamina VII); cellular swelling, tigrolysis, milky appearance of cytoplasm and displacement of nucleoli of large motoneurons (lamina IX). *Cauda equina* syndrome caused by sacrococcygeal spinal nerve roots constrictions in dogs was accompanied by distinct histological changes in spinal cord structures. The extent of damage increased with elapsing time.

Key words: *cauda equina* constrictions; dog; spinal cord neuronal damage

INTRODUCTION

Clinicaly manifested *cauda equina* syndrome (CES) in humans is characterised by a saddle hypaesthesia or anaesthesia, low back pain, bilateral sciatica, severe paraparesis or paraplegia of lower extremities, sphincter dysfunction, and sexual impotence, i. e. symptoms thought to result from damage of the motor, sensitive and autonomous components of lumbosacral spinal nerve roots (1, 4, 14, 16–19). Recent experimental studies using models of porcine and canine graded *cauda equina* compression described changes in the intraneural blood flow, pressure changes within the lumbosacral spinal nerve roots after constriction of

the spinal dural sac, intraneural oedema formation, changes in impulse propagation, afferent and efferent conduction, cortical evoked potentials, etc. (3, 7, 15, 19).

Although the extent of the previous studies was almost completely limited to changes of peripheral neurons, the blockade of axoplasmic flow and series of discrete electrophysiological changes were noted with regard to the amplitudes and frequencies of cortical evoked potentials registered in animals subjected to chronic *cauda equina* compression, speaking clearly in favour of the spinal neuronal pools and circuits participation in the pathogenesis of the CES (13–15).

These findings were supported by studies which used the model of peripheral mononeuropathy, e. g. multiple loose sciatic nerve ligations known to cause the transneuronal degeneration of interneurons and spinal cord projecting neurons in lumbosacral spinal cord segments (9, 13). Moreover, studies in which the c-fos gene expression was used to study the central projections of pelvic visceral afferents showed that electrical stimulation of the pelvic nerve or stimulation of afferent pathways in the lower urinary tract or colon, induced c-fos expression in a large group of neurons in the region of the lateral dorsal horn, including the spinopontine, spinohypothalamic neurons, interneurons and in the region of the sacral parasympathetic nucleus (3, 9, 13, 19).

The aim of the present study was to determine, whether the sustained *versus* short-term *cauda equina* constrictions may have any impact on lumbosacral spinal cord neuronal pools and if so, how the segmentally and suprasegmentally organized neuronal circuits do respond to the constriction injury inflicted on the peripheral neurons which form the *cauda equina*.

MATERIAL AND METHODS

The experimental protocols were elaborated in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 and approved by the Regional State Veterinary Administration in Košice (decision No. SK P 53004) and by the Ethical Commission of the Neurobiological Institute of the Slovak Academy of Sciences in Košice.

Fifteen adult mongrel dogs of both sexes, weighing between 11 and 18 kg were included in the study. Since one member of our team (J. C.) was an experienced anaesthesiologist, we decided for a type of anaesthesia routinely and daily used in thousands of patients worldwide in the human surgical practice. The general anesthesia in all animals, experimental as well as control, was induced with thiopental ("Thiopental" – *Thiopentalum natricum cum natrii carbonate sicco*, SPOFA, Praha, the Czech Republic), administered intravenously at a dose of 4 $mg.kg^{-1}$ and further maintained by a mixture of medical oxygen with 1–2 % halothane ("Narcotan" – *Halothanum thymolo 0.01 % stabilisatum*, LÉČIVA, Praha, the Czech Republic). All animals were intubated by a Portex endotracheal cannula (BERCK, Paris, France) 8–12 mm in diameter and placed on a volume-cycled ventilator (Anemat N8, CHIRANA, Stará Turá, Slovakia).

Before the surgical part of the experimental procedure was started, the antibiotic "Ampicilin" was administered to each dog as a preventive measure ("Ampicilin" – *Ampicillinum ut sal natrico*, BIOTIKA, Slovenská Ľupča, Slovakia) in a single dose of 100 $mg.kg^{-1}$ intramuscularly. A catheter was inserted into the right femoral artery and continuous monitoring of the arterial blood pressure (Monitor LMP 150, TESLA, Nové Mesto nad Váhom, Slovakia) and EKG (Monitor LKM 220, CHIRANA, Stará Turá, Slovakia) were carried out during the surgical procedures. The rate of ventilation was adjusted to maintain arterial pO_2 between 10.6 and 13.3 kPa (80–100 mm Hg) and pCO_2 at about the normal canine level (5.06 kPa, i. e. 38 mm Hg). Arterial blood gases were periodically analyzed by Automatic Gas Check (AGC 995-Hb, CMI, Wien, Austria) to secure the required levels of pO_2 and pCO_2. With an aim to suppress pain sensations after recovery from anaesthesia the dogs were administered analgetica – anodyna (Tramadol AL 100 – *Tramadoli hydrochloridum* – ALIUD PHARMA GmbH Co KG, Laichingen, Germany, 3-times in a dose of 4 $mg.kg^{-1}$ i. m. during the first 24 hours). The animals were allowed free access to food and drinking water before as well as after surgery. The dogs were divided to three groups as follows:

Group 1 (n = 3). In dogs of this group, serving as sham controls, only L_7–S_1 laminectomy, strictly observing basic principles of *asepsis* and *antisepsis*, was performed. The dorsal lumbosacral area was shaved, skin disinfected by iodine solution ("Betadin" – *Povidonum iodatum*, EGIS PHARMACEUTICALS, Budapest, Hungary), and a dorsal midline incision was made from the spinous process of the 5th lumbar to the spinous process of the 2nd sacral vertebra. Then laminectomy of L_7 and S_1 vertebrae was carried out to gain access to the lumbosacral part of the spinal dural sac (at the level of L_7–S_1 vertebrae) containing roots of *cauda equina*. After inspection of situation in the lumbar canal, without any manipulation with neural structures, the surgical wound was sutured in anatomical layers. Following surgery the animals were treated in exactly the same way as members from groups 2 and 3 and were let to survive for nine days.

Group 2 (n = 6). Following the laminectomy L7-S1 the constrictions of the entire *cauda equina*, including bilateral L_7 roots were produced by placing four ligatures with about 2 mm spacing circumferentially around the dural sac (Fig. 1) thereby creating multiple protracted *cauda equina* constrictions (MPCEC). After placement of the constrictive ligatures the surgical wound was sutured and all dogs from this group were let to survive for three days. As a consequence of MPCEC, all postganglionic sacrococcygeal and L_7 lumbar spinal nerve roots were constricted.

In **group 3** (n = 6) the same MPCEC procedures were made but the animals were allowed to survive for nine days.

At the completion of the experiments, i. e. after elapse of pre-determined time interval, all dogs (including the members of the control group) were deeply anaesthetized with thiopental (6 $mg.kg^{-1}$ i. v.), their hearts were exposed by thoracotomy through the 5th intervertebral space and they were perfused transcardially with 3 000 ml of heparinized saline ("Heparin" – *Heparinum natricum*, ZENTIVA, Praha, the Czech Republic, at a dose of 5 000 u. i./3 000 ml of *Infusio natrii chlorati isotonica*, IMUNA PHARM a. s., Šarišské Michaľany, Slovakia). Following perfusion, the animals were euthanised by the same volume

of fixative containing 3 % of paraformaldehyde and 2% of glutaraldehyde in 0.1 mol phosphate buffer at pH 7.4.

The precise location of surgical intervention and constrictive ligatures were verified using the position of dorsal root ganglia as a basic criterion. After overnight fixation *in situ* L1–L7 and S1–S3 segments, the corresponding dorsal root ganglia, C1–C3 segments, the region of the dorsal column nuclei of *medulla oblongata* and ventrobasal complex of the *thalamus* were removed, divided into small blocks, cut by freezing microtome to semithin (30 μm thick) sections and processed separately for light-microscopic observations, using routine (Nissl) neurohistological staining and highly sensitive impregnation procedure introduced by Nauta (10, 12).

RESULTS

No visible argyrophilia (in specimens processed by Nauta method), cellular swelling and displacement of nucleoli (in specimens processed by Nissl histological staining) was detected in spinal cord specimens of dogs from group 1 (Fig. 2).

Neither transneuronal, nor retrograde damage could be observed after application of short-term (3-day) *cauda equina* constrictions, and only an early phase of anterograde degeneration of the sacrococcygeal and L_7 dorsal root afferents was noted in the superficial layers of the dorsal horn in S_1–S_3 segments (Figs. 3, 4 and 5).

The combination of Nissl neurohistological staining with Nauta impregnation procedure revealed in semithin (30 μm thick) sections a broad spectrum of neurohistopathological changes induced in a wide variety of neurons in the lumbosacral spinal cord segments by exposure to sustained (9-day) *cauda equina* constrictions.

A fully developed anterograde degeneration seen as a typical drop-like axonal disintegrations affecting all postganglionic sacrococcygeal and L_7 dorsal root afferents was found in S_1–S_3 segments. The dorsal column in S_1–S_3 segments was densely packed with dark degenerated axons (Figs. 3 and 4). Thin isolated bundles of degenerated fibres penetrated into the superior layers of the dorsal horn and concentrated bilaterally in the medial portion of lamina VII where a typical pattern of terminal degeneration was clearly discernible. The extent of the degenerated gracile fascicle is depicted in S_1–S_3 and L_7 segments. At higher segmental level a narrow band forming the sacrococcygeal portion of the gracile fascicle was identified in the lumbar, thoracic, and cervical segments (Figs. 5 and 6). At lower *medulla oblongata* level the signs of terminal degeneration were identified in the gracile and also in the lateral cervical nuclei, located in C_1–C_2 segments.

Along all sacrococcygeal and lower lumbar segments, but most prominently pronounced in S_1–S_3 ones, a transneuronal degeneration appeared, affecting two different subgroups of the spinal cord neurons. The first subgroup of middle-sized (15–25 μm in diameter) and larger multipolar (≥ 25 μm) argyrophilic neurons with broad dendritic arbors was found in the midportion of the dorsal horn (lamina IV) and then very often in the central core of the gray matter (laminae V–VII) which penetrated between numerous argyrophilic fragments of the dorsal afferents described above (Fig. 7). A surprisingly large proportion of these argyrophilic neurons, mainly those located in lamina IV, was in close contact with the fragments of terminal degeneration. Considering the laminar distribution, soma size and the pattern of dendritic branching, it seemed probable that these elements belonged to the category of sensory projection neural cells of wide-dynamic-range neurons (WDR) and proprioceptive (PR) type neurons located predominantly in the deep dorsal horn layers (laminae V–VI) and the intermediate zone (lamina VII).

A quite different subset of transneuronally degenerating neurons was detected in the ventralmost part of the anterior horn (lamina VII and IX) in S_1–S_3 segments. Some of these small, darkly impregnated neurons were found in the border zone of the anterior horn, close to normally appearing motoneurons located in lamina IX. Considering the cell body position and the existence of two to five heavily argyrophilic dendrites, emerging from the slightly bulging pericarya, it is possible to compare them with the occurrence of small dark shrunken neurons located in the same position in the ventral spinal cord horn.

Bilateral retrograde degeneration affecting the motoneurons in the ventrolateral portion of the anterior horn in S_1–S_3 segments was detected. Motoneurons, undergoing retrograde degeneration, appeared round or oval due to the acute swelling. The displacement of the nucleus in the cytoplasm, which was more or less opaque and has a light blue, milky appearance, was the most easily detectable light-microscopic sign of the retrograde damage of these important cells (Fig. 8).

DISCUSSION

Our study presents an experimental model for multiple protracted nerve root constriction injury of the canine *cauda equina*. Detailed neurohistopathological analyses showed, that sustained *cauda equina* constrictions may have a deleterious effect on lumbosacral spinal cord neuronal pool and circuitry, rendering them selectively vulnerable and inducing the neuronal damage expressed in the form of a lightmicroscopic triad, characterised by an anterograde degeneration of sacrococcygeal dorsal root afferents in S_1–S_3 segments, a transsynaptic degeneration of two subsets of neurons occuring in coccygeal and S_1–S_3 segments, and finally a retrograde degeneration of motoneurons located in the ventrolateral portion of the ventral horns in S_1–S_3 segments.

Functional disturbances resulting in an almost complete damage to the gracile fascicle at sacrococcygeal level can be explained by the fact, that the vast majority of this bundle's primary afferent axons come from rapidly

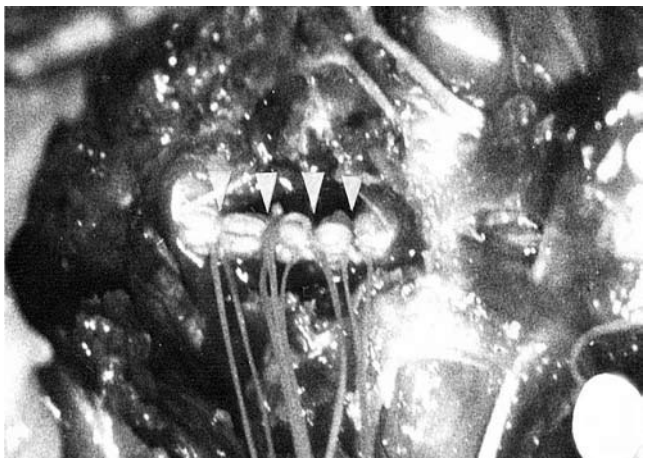

Fig. 1. Intraoperative photograph following L_7–S_1 laminectomy shows position and extent of four constrictive ligatures located circumferentially around the spinal dural sac containing roots of the *cauda equina* (MPCEC procedure)

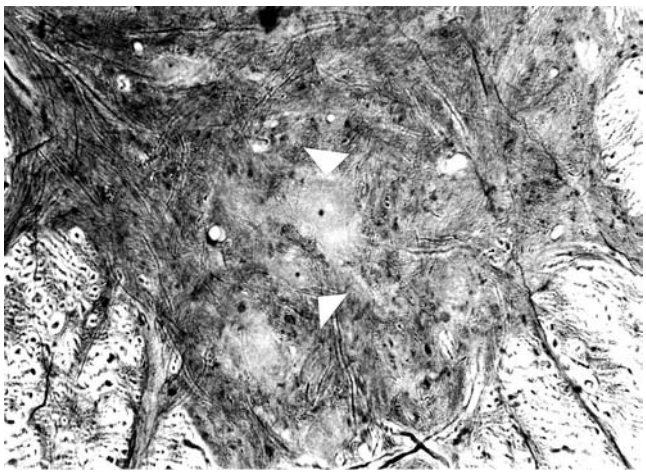

Fig. 2. Microphotograph of ventral spinal cord horn of dog from group 1 (sham controls). No histopathological changes could be detected in neural structures of Rexed's lamina IX. Nauta impregnation procedure. Magn. × 80

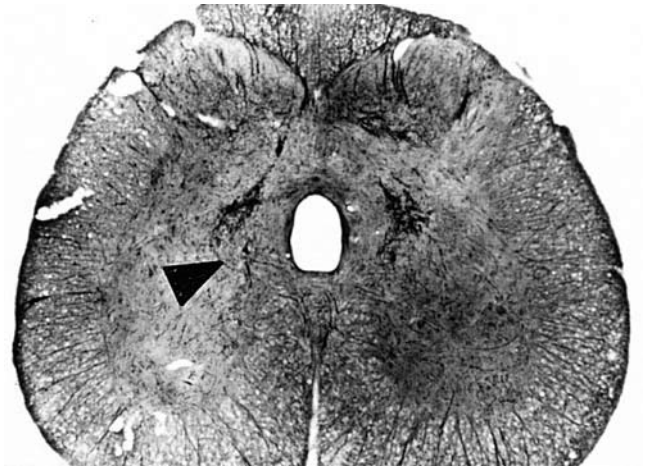

Fig 3. The pattern of anterograde degeneration in sacral and lower lumbar segments in a dog surviving 3 days with the *cauda equina* constriction. Dense bilaterally occurring anterograde degeneration (arrowhead) seen in the intermediate zone (Rexed's lamina VII) of S_3 spinal cord segment and dorsal columns. Magn. × 9

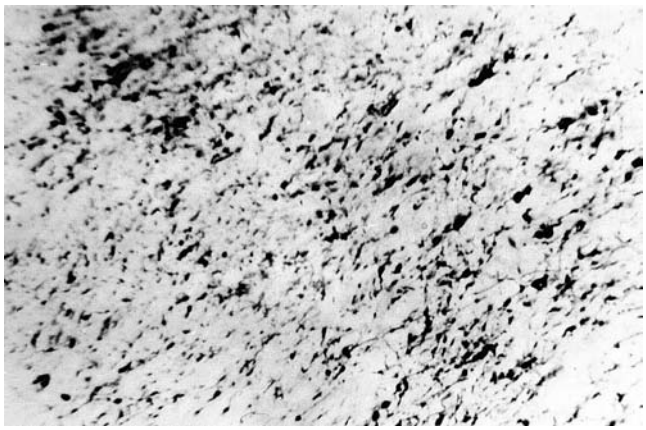

Fig. 4. Microphotograph showing damaged (argyrophilic) axons located in dorsal spinal cord columns. Nauta impregnation method. Magn. × 80

adapting cutaneous mechanoreceptors and Paccinian corpuscles (10, 11). Only a small proportion of axons of the gracile fascicle come from slowly adapting joint receptors and muscle stretch receptors (3, 10, 14).

It should be noted that a small proportion of the degenerated gracile fascicle fibers may belong to the postsynaptic dorsal column neurons, located normally in laminae III and IV of the lumbosacral enlargement (10), since clearly descernible middle-sized argyrophilic neurons were detected in the same laminar position. Most of these neurons are wide-dynamic-range or multi-convergent neurons activated by convergent inputs from both mechanoreceptors and nociceptors (10, 13). Similar functional characteristics, i. e. activation by a homogenous set of mechanoreceptors or by convergent inputs from both sensitive mechanoreceptors and nociceptors, are known in connection with the spinocervical tract (3, 10, 13). Terminal degeneration of this pathway in the lateral cervical nucleus was found in all animals subjected to sustained *cauda equina* constrictions surviving for nine days.

Moreover, transneuronal degeneration of middle-sized and large multipolar neurons found in our experiments and expressed in the form of somatodendritic argyrophilia was detected mainly in laminae IV–VII, i. e. in those layers, where the majority of the wide-dynamic-range (WDR) and proprioceptive (PR) neurons can be identified (10, 11). It was repeatedly confirmed, that WDR

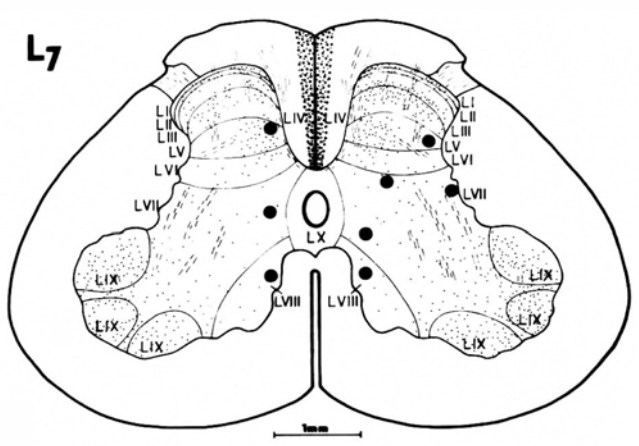

Fig. 5. Schematic drawing of the spinal cord neuronal pools of dog 3 days following the MPCEC. The early phase of anterograde degeneration is demonstrated Bar = 1 mm

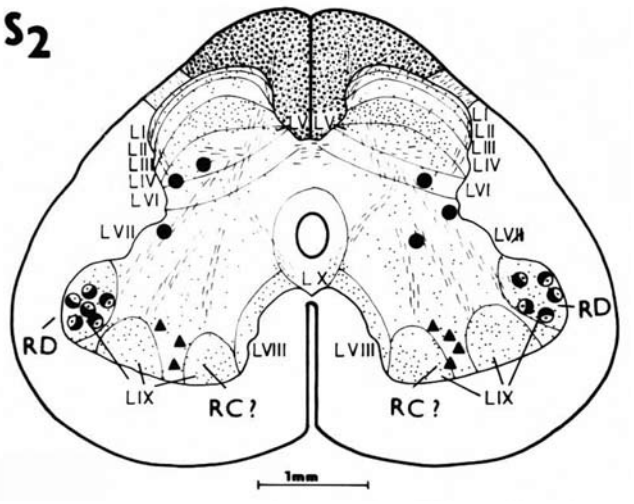

Fig. 6. Schemmatic drawing of the spinal cord neuronal pools of experimental animals 9 days following the MPCEC. Dorsal spinal cord columns are densely packed with dark degenerated axons. LI–IX (Rexed's laminae), RC? = suspect Renshaw cells Bar = 1 mm

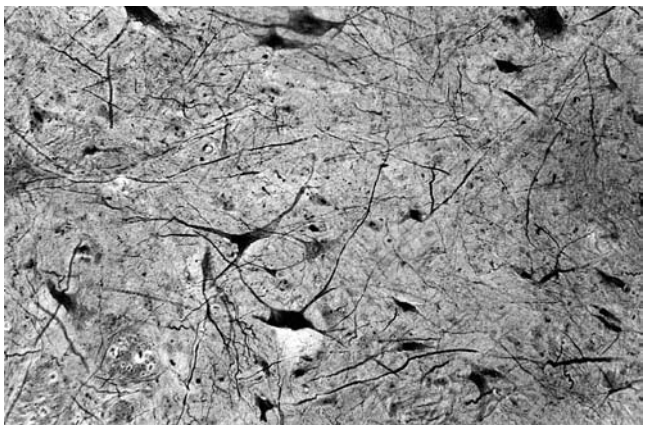

Fig. 7. Microphotograph showing degenerated middle-sized (15–25 μm) and larger (≥ 25 μm) argyrophilic multipolar neurons in midportion of the dorsal horn. Nauta impregnation method. Magn. × 80

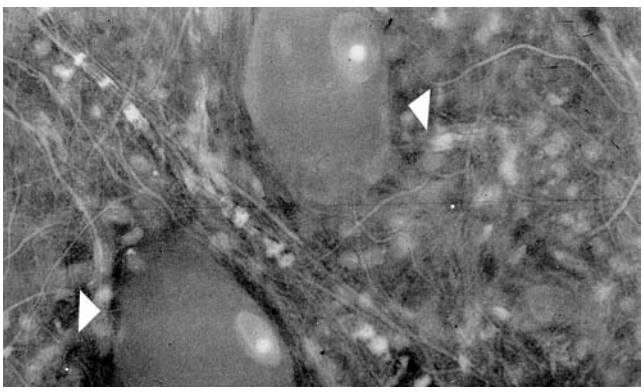

Fig. 8. Microphotograph of a specimen from S_3 spinal cord segment of a dog from the group 3 (9 day MPCEC) shows two swelled motoneurons with milky cytoplasm and nucleoli displaced to the periphery of the cell. Nissl stainig method. Magn. × 150

neurons received excitatory afferents from impulses in both large-diameter (Aß) sensitive mechanoreceptive cutaneous afferents and in small-diameter (Aδ and C) nociceptive cutaneous afferents (10, 13). Therefore WDR neurons are massively stimulated by a monosynaptic input from large diameter fibers and a polysynaptic input from C-fibers (3, 13).

Chronic *cauda equina* constrictions used in the present study are undoubtedly accompanied by a sustained stimulation of all sacrococcygeal dorsal root afferents, a phenomenon which may result in a functional overloading of WDR neurons and generation of long-term afterdischarges known for significantly longer duration and greater magnitude in a model of chronic constriction injury of the sciatic nerve (9), finally resulting in cell death of some neuronal population in the dorsal horn (13).

It is possible to admit that the origin of some symptoms accompanying CES, such as saddle anaesthesia, low-back pain, or radicular pain may originate not only from the compressed or constricted sacrococcygeal dorsal root afferents, but may also have a direct relation with the damaged sacral WDR neurons, described in this study (2, 5, 15–19). Moreover, it is known that approximately two-thirds of WDR neurons participate in the formation of the primate spinothalamic tract, carrying pain impulses, and terminating in the ventral posterolateral nucleus of the ventrobasal thalamic complex (3, 10). It is tempting to suggest an idea, that small degenerated argyrophilic neurons found in the ventralmost part of lamina VII in S_1–S_3 segments could be the Renshaw cells (RC), damaged by overstimulation of afferent neurons participating in spinal cord circuits. It should be noted, that a long-term *cauda equina* constrictions (for nine

days; in dogs of group 3) caused much more complex damage of spinal cord neuronal pools, than short-term (for three days; in dogs of group 2).

Results of the present experimental study could explain the positive effect of an early surgical decompression of lumbosacral spinal nerve roots in humans with *cauda equina* syndrome, which developed as a consequence of intervertebral disc disease (1, 5–8).

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MORPHOLOGICAL AND MORPHOMETRICAL STUDY OF THE SUPERFICIAL LYMPH NODES OF KAGANI GOAT (*CAPRA HIRCUS*) IN JAMMU REGION

Sarma, K., Devi, J., Srivastava, A. K.

**S. K. University of Agricultural Sciences and Technology, Division of Anatomy and Histology
Faculty of Veterinary Sciences, R. S. Pura, Jammu-181102
India**

kamalsarma73@yahoo.com

ABSTRACT

The investigation was conducted on eight adult apparently healthy Kagani goats, aged 15 to 22 months (irrespective of sex). The animals were embalmed in 10 per cent formalin solution after induction of proper anaesthesia and the superficial palpable lymph nodes (parotid, mandibular, superficial cervical, prefemoral, popliteal and inguinal) were dissected out from both the sides and their biometry were recorded for length, width, thickness, volume, organ weight and shape indices of the right and left sides separately. Tissue pieces from these lymph nodes were used for histomorphological and histomorphometrical studies. The parotid lymph nodes were single on either side in all animals under study. The mandibular lymph nodes were single on either side and no bilateral variations pertain119ing to different values were significant ($P < 0.05$) except for their weight and shape which showed a significant variation among the nodes of either side. The width of the superficial cervical lymph nodes on both sides varied significantly ($P < 0.05$) but no variation was recorded in other biometrical parameters. The popliteal lymph node showed a highly significant difference ($P < 0.05$) in width, thickness and shape index among the nodes of either side. The highest values in terms of all parameters were recorded for superficial cervical lymph node and the lowest for supramammary lymph node, except for the shape index. Collagen fibres were abundant in both capsule and trabeculae but the elastic fibres were scant and were restricted only to the central part of the trabeculae. The reticular fibres of various sizes predominated in both the capsule and trabeculae and were thickly populated on the inner aspect of the capsule forming an *inner lamina*. The germinal centres of the secondary lymphatic nodules had a fine distribution of reticular fibres. The flat squamous cell lining of the trabecular sinus showed interruption. A number of post capillary venules were detected in the paracortical region, some of them contained migrating lymphocytes. Not all values pertaining to different parameters under study showed a significant bilateral variation. The capsule and trabeculae were the thickest in the superficial cervical lymph node and thinnest in the mandibular lymph node.

Key words: *Capra hircus*; histomorphology; histomorphometry; Jammu region (India); lymph nodes

INTRODUCTION

Lymph nodes are vital organs of the defence mechanism of the body against the invasion of foreign bodies. Moreover, these lymph nodes, particularly the superficial lymph nodes that can be palpated easily, are significant indications of certain diseases processes in animals, thus helping in ante mortem and postmortem diagnosis of these diseases mostly during clinical practice and meat inspection. Studies were conducted to investigate the gross, histological and micrometrical aspects of superficial lymph nodes in cattle (3), sheep (9), goat (11), rabbit (10), crossbred calves (8), Assam goat (12) and pigmy hog (14).

Kagani goat is the most versatile native breed of goat of Jammu and Kashmir state of India with strongly built body. This animal is mostly kept for meat and it is strongly felt that detailed anatomical data should be established pertaining to its superficial lymph nodes as these play an important

and indispensable role in the process of meat inspection and diagnosis of different disease processes.

Therefore the aim of this investigation, the first one of this kind on Kagani goat, was to elucidate the gross biometrical, histomorphological and micrometrical aspects of certain superficial palpable lymph nodes in this animal.

MATERIAL AND METHODS

Topographical, gross and biometrical studies

The investigation was conducted on eight adult apparently healthy Kagani goats, aged 15 to 22 months (irrespective of sex), procured from Sheep Breeding Farm, Panthal, Jammu province of Jammu & Kashmir state of India. The animals were embalmed in 10 per cent formalin solution after induction of proper anaesthesia. The superficial palpable lymph nodes, i.e. parotid, mandibular, superficial cervical, prefemoral, popliteal and inguinal, were dissected out from both sides and their biometry was recorded for length, width, thickness, volume, organ weight and shape indices for the right and left sides separately. The length, width and thickness were recorded using a digital slide callipers. Volume and organ weight were recorded by water displacement method and digital balance. The shape index was calculated using the formula as per Barnwal and Sinha (2).

$$\text{Shape index} = \frac{\text{Width}}{\text{Length}} \times 100$$

Histomorphological studies

The tissues were fixed in 10 percent neutral buffered formalin (NBF) solution and subsequently processed for paraffin sections (5). The sections were cut at 5 µ thickness using a Spenser type Rotary Microtome (Leica) and were stained with Mayer's haematoxylin and eosin, Mallory's stain for collagen, Gomori's method for reticulum and Hart's method for elastic fibres. Tissue pieces were also processed by Bielschowsky block impregnation technique (4) for demonstration of nerve fibres. Gross biometry of the lymph nodes was recorded using digital slide callipers and the micrometrical parameters of different histomorphological compartments of the lymph nodes were recorded under Carl Zeiss compound microscope using Zeiss ocular micrometer calibrated with Zeiss stage micrometer under 10X objective. The data obtained in this study were analyzed statistically (16).

RESULTS

Topographical, gross and biometrical studies

The parotid lymph nodes in adult Kagani goat were bean shaped while the mandibular nodes were elongated and oval in shape. The superficial cervical lymph node was massive and the prefemoral and superficial inguinal lymph nodes were elongated. The popliteal lymph node was oval in outline. The parotid lymph nodes were single on either side in all animals under study. The various dimensions of the parotid lymph nodes (length, width and volume) varied significantly among the nodes on the right and left side and the were significant with respect to their shape indices (Table 1). The mandibular lymph nodes were single on either side and no bilateral variations pertaining to different values were significant ($P < 0.05$) except for their weight and shape which varied significantly among the nodes on either side. The width of the superficial cervical lymph nodes on both sides varied significantly ($P < 0.05$) but no variation was recorded in other biometrical parameters.

Statistical analysis revealed that the thickness and shape index of superficial inguinal lymph nodes varied significantly ($P < 0.05$) among its right and left counterparts. The popliteal lymph node showed a significant difference ($P < 0.05$) with respect to width, thickness and shape index among the nodes on either side. Similarly the difference in values of right and left superficial inguinal lymph nodes pertaining to thickness was significant ($P < 0.05$) so was the weight and shape index ($P < 0.05$). This shows marked bilateral variations of various lymph nodes among their right and left counterparts in the Kagani goats.

The highest values in terms of all parameters were recorded in superficial cervical lymph node and the lowest in supramammary lymph node, except for the shape index (Table1). The shape index reached maximum in the left parotid lymphoid and minimum in the right superficial cervical lymph node.

Histomorphological and micrometrical studies

The capsule and trabeculae of the lymph nodes under study were composed of dense white fibrous connective tissue. However, smooth muscles predominated in the trabeculae rather than in the capsule. Collagen fibres were abundant in both capsule and trabeculae but the elastic fibres were found to be scant and were limited only to the central part of the trabeculae. The reticular fibres of various sizes predominated both in the capsule and trabeculae and were thickly populated especially on the inner aspect of the capsule forming an *inner lamina*. The reticular fibres also formed a meshwork in the subcapsular sinus as well as in the trabecular sinus, the later being wider. The flat squamous cell lining of the trabecular sinus was interrupted. Nerve fibres were richly distributed in the capsule and cortical trabeculae whereas the medullary trabeculae showed poor distribution of nerve fibres.

The cortex had thickly arranged cell population which formed primary and secondary lymphatic nodules. Secondary nodules were mostly found at the periphery of the cortex. The germinal centre of the secondary lymphatic nodules revealed a diversified cell population – mostly lymphoblasts, few macrophases, plasma cells and reticular cells. Occasional neutrophils were also found (Fig. 4). Primary lymphatic nodules contained mostly small matured lymphocytes.

A number of post capillary venules were detected in the paracortical region, some of them contained migrating lymphocytes.

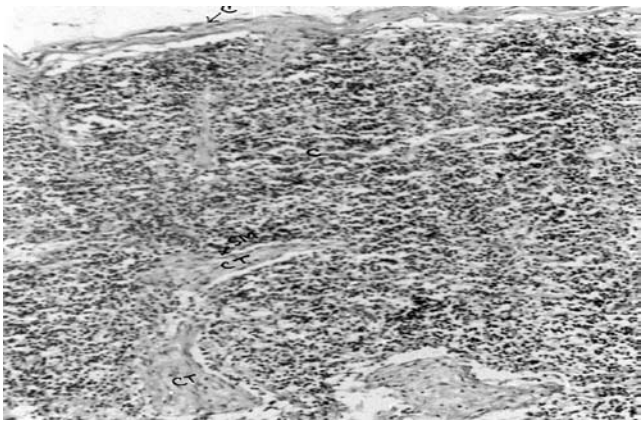

Fig. 1. Photomicrograph of the superficial lymph node of Kagani goat of Jammu region showing capsule (C), cortex (CR), cortical trabeculae (CT) and smooth muscle cells (SM)

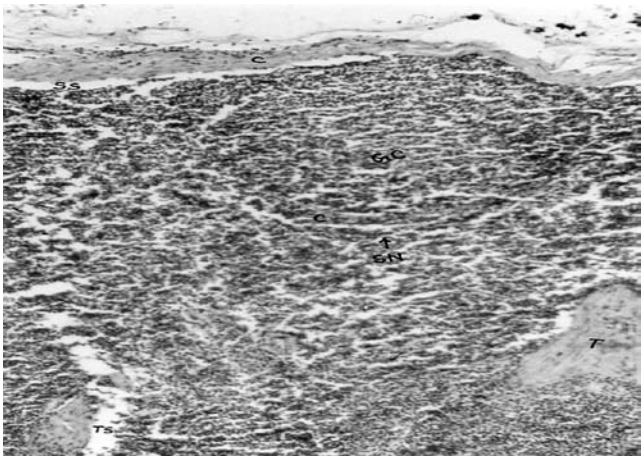

Fig. 2. Photomicrograph of the superficial lymph node of Kagani goat of Jammu region showing capsule (C), subcasular sinus (SS), cortex (CR), germinal centre(GC) and secondary nodule (SN)

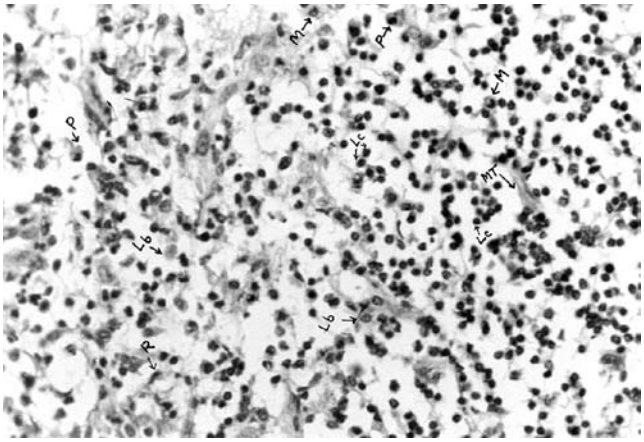

Fig. 3. Photomicrograph of the superficial lymph node of Kagani goat of Jammu region showing lymphoblasts (Lb), macrophases (M), plasma cells (P), neutrophils (N) and reticular cells (R)

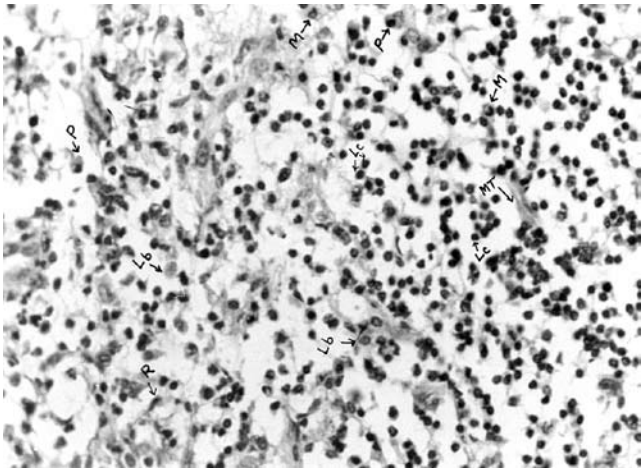

Fig. 4. Photomicrograph of the superficial lymph node of Kagani goat of Jammu region showing lymphoblasts (Lb), lymphocytes (Lc), plasma cells (P), neutrophils (N), reticular cells (R) and medullary trabeculae (MT)

The medulla of the lymph nodes was composed of medullary cords, medullary trabeculae and medullary sinuses. The cords consisted of lymphocytes, macrophases, reticular cells, plasma cells and occasional neutrophils. The medullary trabeculae had histological structure similar to that of the cortical trabeculae.

Reticular fibres and large reticular cells formed the reticulum of all lymph nodes. The reticular fibres were evenly distributed within the primary lymphatic nodules whereas peripheral distribution was observed in the secondary nodules. The germinal centres of the secondary lymphatic nodules had a fine distribution of reticular fibres.

The micrometrical studies were conducted on sections stained with Mayer's haematoxylin and eosin. The micrometrical parameters recorded are shown in Table 2.

All the values pertaining to different parameters under study showed no significant bilateral variations. The capsule and trabeculae were the thickest in the superficial cervical lymph node and thinnest in the mandibular lymph node. The diameters of germinal centres were the biggest in superficial cervical lymph node while the lowest values were recorded in the inguinal lymph node.

DISCUSSION

The parotid lymph nodes in the adult Kagani goat were bean shaped, but the mandibular ones were elongated and oval in shape.The superficial cervical lymph node was massive and the prefemoral and supramammary lymph nodes were elongated in shape. But the popliteal lymph node was oval in outline. Similar description regarding the shape of these lymph nodes was presented also for adult Assam local goat (13). However, the mandibular lymph nodes were roughly quadrilateral in shape in the pigmy hog (14).

Table 1. The means and standard deviations for the measurement of various biometrical parameters of the superficial palpable lymph nodes in Kagani goat

Sample No	Lymph node	Side	Biometrical parameters					
			Length (cm)	Width (cm)	Thickness (cm)	Volume (cm)	Organ wt. (gm)	Shape index
1	Parotid	Left	1.45 ± 0.02	1.14* ± 0.05	1.0 ± 0.29	0.66* ± 0.07	0.97 ± 0.04	91.46** ± 1.49
		Right	2.03 ± 0.03	1.12 ± 0.04	0.64 ± 0.05	0.79 ± 0.09	0.93 ± 0.02	58.47 ± 1.59
2	Mandibular	Left	2.04 ± 0.24	0.984 ± 0.07	0.64 ± 0.04	1.14 ± 0.18	0.97* ± 0.10	49.29* ± 2.66
		Right	1.82 ± 0.18	1.20 ± 0.14	0.64 ± 0.04	1.21 ± 0.29	1.83 ± 0.13	61.33 ± 1.63
3	Superficial cervical	Left	3.52 ± 0.30	1.62* ± 0.04	0.87 ± 0.03	2.9 ± 0.13	3.14 ± 0.24	46.78 ± 1.90
		Right	3.72 ± 0.19	1.52 ± 0.03	0.90 ± 0.08	2.89 ± 0.27	2.91 ± 0.24	40.34 ± 0.99
4	Prefemoral	Left	2.08 ± 0.29	0.93 ± 0.03	0.49* ± 0.01	0.69 ± 0.14	0.84 ± 0.15	55.46* ± 7.52
		Right	2.49 ± 0.07	1.09 ± 0.09	0.57 ± 0.03	0.71 ± 0.12	0.81 ± 0.11	42.56 ± 4.79
5	Poplitial	Left	1.63 ± 0.01	1.15** ± 0.04	0.80** ± 0.03	0.89 ± 0.10	0.66 ± 0.15	70.88** ± 1.48
		Right	1.61 ± 0.48	0.85 ± 0.04	0.66 ± 0.04	0.71 ± 0.09	0.87 ± 0.09	60.54 ± 1.39
6	Superficial inguinal	Left	1.18 ± 0.06	0.90 ± 0.01	0.37** ± 0.02	0.29 ± 0.02	0.53* ± 0.02	75.06* ± 1.07
		Right	1.25 ± 0.01	0.83 ± 0.03	0.45 ± 0.02	0.27 ± 0.01	0.64 ± 0.02	66.86 ± 1.38

* – significant at the level of $P < 0.05$
** – significant at the level of $P < 0.01$

Table 2. The means and standard deviations for the measurement of various micrometrical parameters of the superficial palpable lymph nodes in Kagani goat

Parameter	Parotid		Mandibular		Superficial cervical		Prefemoral		Popliteal		Superficial inguinal	
Side	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Capsular thickness (μ)	39.72 ± 3.649	35.26 ± 2.921	51.25 ± 3.911	50.179 ± 4.117	75.33 ± 8.325	72.66 ± 7.945	65.22 ± 4.521	65.17 ± 4.749	56.32 ± 5.721	54.79 ± 5.239	57.32 ± 5.227	57.49 ± 5.016
Thickness of trabeculae (μ)	47.25 ± 5.972	47.53 ± 6.115	65.52 ± 6.259	63.61 ± 5.977	112.52 ± 10.117	109.52 ± 10.273	88.92 ± 7.725	87.05 ± 7.011	75.32 ± .924	72.11 ± 5.059	87.11 ± 8.015	85.26 ± 7.959
Diameter of secondary lymph nodule (μ)	325.11 ± 14.057	325.01 ± 14.539	405.27 ± 15.672	400.97 ± 15.237	541.72 ± 42.155	538.52 ± 41.972	490.521 ± 35.523	485.56 ± 34.592	465.11 ± 30.175	460.43 ± 29.521	305.82 ± 20.527	300.13 ± 20.115
Diameter of primary lymph nodule (μ)	275.52 ± 12.159	273.59 ± 12.005	340.17 ± 13.592	338.21 ± 12.992	465.35 ± 15.197	463.23 ± 15.001	415.11 ± 17.175	413.52 ± 17.115	390.25 ± 15.517	387.32 ± 15.172	242.32 ± 10.152	242.57 ± 10.062

The parotid lymph nodes were single on either side in all animals under study in contrast to the occasional presence of two nodes on each side in the adult goat as reported by Tanudimadja and Ghoshal (17).

Our investigations showed a marked bilateral variation of the various lymph nodes among their right and left counterparts in the Kagani goats. Contrary to the present study, no significant variations were observed among various lymph nodes in the adult Assam local goat (13). The variation recorded in the present study might be due to specific breed characteristic and further study is needed in this respect.

The highest values in terms of all parameters were recorded in our study in the superficial cervical lymph

node and the lowest in the supramammary lymph node with the exception of shape index (Table 1.). The shape index reached maximum in the left parotid lymphoid and minimum in the right superficial cervical lymph node. Sarma *et al.* (14) reported that the superficial cervical lymph node exhibited the highest value for various dimensions except for length and thickness and the lowest mean values for all dimensions were exhibited by the parotid lymph node as also observed in the pigmy hog.

The composition of the capsule and trabeculae with dense white fibrous connective tissue found in the present study was in agreement with the findings of Banks (1) in ruminants and Singh *et al.* (15) in buffalo calves. Reticular fibres were seen in the capsule and trabeculae and in the capsule they formed an inner lamina. Similar pattern of distribution of reticular fibres were also reported in buffalo calves (15) and in Assam goats (12). The endothelial cell lining of the cortical and sub cortical sinuses showed an interruption which may facilitate adequate and free percolation of lymph to the parenchyma of the lymph nodes for providing a free access of lymph borne antigens to parenchymatous cells (6).

The capsule and trabeculae had rich nerve supply. However, nerve fibres could not be detected in the parenchyma due to large and thick cell population as described by Dellmann and Brown (6).

In this study the secondary lymphatic nodules were mostly found towards the periphery of the cortex. This might be due to the fact that as the development of secondary lymphatic nodules was mediated by antigenic stimulation (8), the high concentration of antigen in the subcapsular sinus might attract the formation of secondary nodules near the subcapsular sinus. The variety of cell population recorded in the present study was also reported in buffalo calves (15) and Assam goats (12).

Numerous post capillary venules were recorded in the paracortical region, some containing lymphocytes. This migration of lymphocytes through the walls of the post capillary venules was also well documented by Tizzard (18).

The reticular fibres were evenly distributed within the primary lymphatic nodules, whereas in the secondary lymphatic nodules they had a peripheral distribution. Similar findings were also reported by Singh *et al.* (15) in buffalo calves and Sarma *et al.* (12) in Assam goat. This might be a factor for distinguishing primary and secondary lymphatic nodules.

No bilateral variation of the values of various micrometrical parameters was recorded in the present study. Similar findings were also reported in the Assam goat (12). However, all the values recorded in this study were greater than those reported for the same lymph nodes in the Assam goat (12). This might be due to breed variation. The trabeculae were thicker than the capsule in all lymph nodes under study which was in agreement with the findings of Sarma *et al.* (12) in the Assam goat but in contrast to those of Gadre *et al.* (7) in bovines. This peculiarity might serve as a specific species characteristic differentiating caprines from bovines.

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IN VITRO ANTIMICROBIAL ACTIVITY OF ENROFLOXACIN AGAINST CLINICAL ISOLATES FROM DOGS AND CATS IN BULGARIA

Popova, T. P., Todorov, T. A.

Faculty of Veterinary Medicine, Forestry University, Sofia, Bulgaria

ttodorow@yahoo.com

ABSTRACT

Antimicrobial activity of enrofloxacin against 60 strains of Gram-negative and 40 strains of Gram-positive bacteria, isolated from dogs and cats with different tissue infections was studied.

Results of the susceptibility testing, carried out by the classical agar disc-diffusion technique, demonstrated significant enrofloxacin activity. The mean diameter of inhibition zone for all isolates tested was 29.006 ± 1.193 mm. It was larger for the Gram-positive strains (32.009 ± 0.471 mm) in comparison with the Gram-negative strains – 26.003 ± 1.596 mm ($P < 0.05$). Determination of the minimum inhibitory concentrations (MICs) confirmed enrofloxacin efficacy. Mean values of MIC_{90} for Gram-positive bacteria (0.214 ± 0.042) were lower than MIC_{90} for the Gram-negative strains (0.446 ± 0.104), but the difference was insignificant ($P > 0.05$).

According to MICs, 93% of clinical isolates were defined as susceptible to enrofloxacin, 3% were intermediately susceptible and 4% were resistant. Only strains of *P. aeruginosa* and *S. enteritidis* constituted the last two categories.

Key words: antibacterial activity; cat; dog; enrofloxacin

INTRODUCTION

Enrofloxacin is a synthetic, second-generation fluorinated quinolone acid derivative, developed for veterinary use only. It possesses a wide spectrum of antimicrobial activity against many Gram-negative and Gram-positive bacteria, including multiresistant strains, mycobacteria and mycoplasms (3, 4, 19). By inhibiting the subunit A of the bacterial DNA-gyrase and thus interfering with supercoiling of the chromosomal material, fluoroquinolones are rapidly bactericidal (1). In order to reach their target of action, fluoroquinolones easily penetrate into cells and accumulate in them (17). However, cell penetration could be effectively antagonized by the presence of magnesium, calcium and other divalent cations in the medium (14).

The primary sources of bacterial resistance against fluoroquinolones to date are low-frequency chromosomal mutations, but Gram-positive bacteria possess an energy-dependent efflux transport system which pumps the quinolones out of the cells (4). Although fluorinated quinolones exert a strong bactericidal effect at very low concentrations, they are minimally toxic to mammalian cells with few side effects at therapeutic doses (4).

Because of its safety and wide spectrum of antimicrobial activity enrofloxacin is recommended for treatment of respiratory, urinary and digestive tract infections, infections of the CNS, ears, eyes and joints, osteomyelitis and mycobacterial diseases in dogs and cats (4, 18, 20).

However, fluoroquinolone efficacy could be significantly reduced at concentrations much higher as well as lower than MICs (8). This phenomenon is among the primary causes for bacterial resistance (12). Taking into consideration the substantially increased population of stray and domestic dogs and cats in Bulgaria, their close contact to man and the alarming rate of bacterial resistance selection (9), current information about susceptibility of clinical isolates from dogs and cats to enrofloxacin is clearly needed.

MATERIALS AND METHODS

Antimicrobial agent. Antibacterial activity of enrofloxacin (Biofloxavet 5 %, Biovet) was studied**.** The dilutions were prepared in sterile phosphated buffered saline (PBS) with pH 7.2.

Bacterial strains. Pure cultures of 100 clinical isolates from dogs and cats were used in the study – 60 strains of Gram-negative and 40 starains of Gram-positive bacteria: *E. coli* (13), *S. enteritidis* (7), *K. pneumoniae* (9), *K. oxytoca* (2), *E. agglomerans* (4), *S. marcescens* (4), *P. vulgaris* (2), *P. aeruginosa* (12), *P. fluorescens* (4), *P. cepacia* (3), *S. aureus* (13), *S. epidermidis* (3), *S. haemolyticus* (3), *S. pyogenes* (8), *S. pneumoniae* (4); *L. monocytogenes* (3), *B. subtilis* (6)**.** Control strains were also used (*E. coli* and *S. aureus,* TSA MRSA).

Antimicrobial tests. The study was carried out using the classic agar-diffusion method (2) according to recommendations of NCCLS (1997). Bacterial suspensions were inoculated at a dose of 10^6 cells.ml^{-1} onto Mueller-Hinton's agar (Scharlau, Antisel), pH 7.2 – 7.4, of thickness 4 mm, in Petri dishes of diameter 9 cm. After 30 min adsorption of microorganisms, discs of chromatographic paper 6 mm in diameter, loaded with standard concentration (5 μg) of enrofloxacin were applied to the agar. Diffusion for 40 min at room temperature was followed by cultivation at 35 – 37 °C during 18 – 24 h.

Results were recorded by measuring the diameters of inhibitory zones in mm, including disc diameter, with a lucent ruler outside of the bottom of the Petri dishes. Inhibitory zones were evaluated by a three-degree categorization system of Bauer-Kirby. High susceptibility of the microorganisms was established at growth inhibition zones ≥ 21 mm, intermediate susceptibility – at zones with diameters from 16 to 20 mm, and resistance – at zones ≤ 15 mm (15, 16).

Determination of the minimum inhibitory concentrations (MICs) was performed using two-fold serial dilutions from 0.016 to 8 μg.ml^{-1} on Mueller-Hinton's agar with pH 7.2 – 7.4 (6). Bacterial suspensions were inoculated at a dose of 10^6 cells.ml^{-1}. The number of colonies developed was evaluated after incubation at 35 – 37 °C for 18 – 24 h. Minimal concentrations (μg.ml^{-1}) of enrofloxacin, reducing the number of colonies with 50 % and with 90 % in comparison with untreated controls, as well as diapason of growth inhibition (D) – minimal concentrations causing full growth inhibition, were calculated. Bacteria with MIC_{90} ≤ 1.0 μg.ml^{-1} were defined as susceptible and those with MIC_{90} ≥ 2.0 μg.ml^{-1} as resistant (16).

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.

RESULTS

Results from disc-diffusion susceptibility testing are presented in Table 1. High inhibitory effect of enrofloxacin was established in 88 % of the micro-organisms tested. Zones of growth inhibition indicating intermediate sensitivity were measured in 7 % of the isolates – 2 strains of *E. coli*, 2 strains of *S. enteritidis* and 3 strains of *K. pneumoniae*. Small sterile zones (6 – 15 mm), indicating bacterial resistance, were established in 5 % of microorganisms – for one strain of each *S. pyogenes*, *E. coli*, *S. enteritidis*, *K. pneumoniae* and *K. oxytoca*. Mean diameter of zone inhibition was larger for Gram-positive strains – 32.009 ± 0.471 mm in comparison with Gram-negative – 26.003 ± 1.596 ($P < 0.05$). Except for one strain of *S. pyogenes,* all Gram-positive bacteria demonstrated high susceptibility to enrofloxacin. With Gram-negative bacteria the largest sterile zones were measured for *E. agglomerans* – 37.250 ± 1.750, and the smallest for *S. enteritidis* – 20.200 ± 2.375 ($P < 0.05$).

Table 1. Susceptibility (inhibitory zones) to enrofloxacin of clinical isolates from dogs and cats

Microorganisms	No. of strains	Inhibitory zones (mm) ($\bar{x} \pm S\bar{x}$)	Susceptibility
E. coli	13	30.133 ± 3.112	S
S. enteritidis	7	20.200 ± 2.375	I
K. pneumoniae	9	23.308 ± 2.953	S
K. oxytoca	2	26.333 ± 8.089	S
E. agglomerans	4	37.250 ± 1.750	S
S. marcescens	4	27.800 ± 3.967	S
P. vulgaris	2	26.250 ± 5.089	S
P. aeruginosa	12	21.154 ± 3.166	S
P. fluorescens	4	21.800 ± 4.363	S
P. cepacia	3	25.800 ± 4.841	S
Gram (-)	**60**	**26.003 ± 1.596**	**S**
S. aureus	13	32.038 ± 0.995	S
S. epidermidis	3	31.833 ± 2.857	S
S. haemolyticus	3	31.667 ± 1.542	S
S. pyogenes	8	29.733 ± 2.754	S
S. pneumoniae	4	32.143 ± 3.888	S
L. monocytogenes	3	32.833 ± 3.563	S
B. subtilis	6	33.818 ± 2.123	S
Gram (+)	**40**	**32.009 ± 0.471**	**S**
Total	**100**	**28.476 ± 1.193**	**S**

Results from MICs determination are shown in Table 2. Cumulative curves of MIC_{90} for Gram-negative and Gram-positive bacteria are presented in Fig. 1 and Fig. 2, respectively. The mean value of MIC_{90} for Gram-positive bacteria (0.214 ± 0.042) was lower than that for Gram-negative (0.446 ± 0.104), but the differences were insignificant($P > 0.05$). MIC_{90} of all Gram-positive isolates did not exceed 1 μg.ml^{-1}. Among the Gram-negative isolates tested, the lowest MICs were established for *K. oxytoca* (MIC_{90} – 0.125 ± 0.063. Intermediate susceptibility was established in two strains of *P. aeruginosa*, with MIC_{90} values 1.5 and 1.25 μg.ml^{-1} and one strain of *S. enteritidis* with MIC_{90} equal to 1.25 μg.ml^{-1}. From among all bacterial strains tested, resistance to enrofloxacin was detected in 3 strains of *P. aeruginosa*, with MIC_{90} 2.5, 2.5 and 2.25 μg.ml^{-1}, respectively, and one strain of *S. enteritidis* with MIC_{90} equal to 2.25 μg.ml^{-1}.

Table 2. Susceptibility (MICs) to enrofloxacin of clinical isolates from dogs and cats

Microorganisms	No. of Strains	MIC_{50} ($\bar{x}\pm S\bar{x}$)	MIC_{90} ($\bar{x}\pm S\bar{x}$)	D ($\bar{x}\pm S\bar{x}$)
E. coli	13	0.095 ± 0.030	0.189 ± 0.061	0.399 ± 0.122
S. Enteritidis	7	0.435 ± 0.134	0.871 ± 0.268	1.778 ± 0.527
K. pneumoniae	9	0.092 ± 0.027	0.191 ± 0.048	0.423 ± 0.128
K. oxytoca	2	0.063 ± 0.032	0.125 ± 0.063	0.312 ± 0.187
E. agglomerans	4	0.219 ± 0.059	0.625 ± 0.072	1.250 ± 0.144
S. marcescens	4	0.092 ± 0.041	0.184 ± 0.082	0.367 ± 0.164
P. vulgaris	2	0.250 ± 0.062	0.500 ± 0.125	1.000 ± 0.250
P. aeruginosa	12	0.537 ± 0.133	1.074 ± 0.265	2.148 ± 0.530
P. fluorescens	4	0.109 ± 0.057	0.219 ± 0.115	0.437 ± 0.229
P. cepacia	3	0.239 ± 0.130	0.479 ± 0.261	0.958 ± 0.522
Gram (-)	**60**	**0.213 ± 0.051**	**0.446 ± 0.104**	**0.907 ± 0.205**
S. aureus	13	0.035 ± 0.004	0.264 ± 0.054	0.588 ± 0.113
S. epidermidis	3	0.033 ± 0.001	0.250 ± 0.062	0.500 ± 0.125
S. haemolyticus	3	0.102 ± 0.039	0.407 ± 0.159	1.042 ± 0.397
S. pyogenes	8	0.084 ± 0.034	0.217 ± 0.077	0.479 ± 0.162
S. pneumoniae	4	0.031 ± 0.014	0.071 ± 0.029	0.141 ± 0.057
L.monocytogenes	3	0.076 ± 0.038	0.186 ± 0.102	0.453 ± 0.209
B. subtilis	6	0.043 ± 0.016	0.106 ± 0.046	0.295 ± 0.144
Gram (+)	**40**	**0.058 ± 0.011**	**0.214 ± 0.042**	**0.500 ± 0.106**
Total	**100**	**0.149 ± 0.035**	**0.350 ± 0.068**	**0.739 ± 0.135**

DISCUSSION

Results of both the methods used to assess bacterial susceptibility to enrofloxacin were similar. Based on MIC values, 93 % of the clinical isolates were defined as susceptible to enrofloxacin, 3% were intermediately susceptible and 4 % were resistant. Only strains of *P. aeruginosa* and *S. enteritidis* constituted the last two categories, witch is in agreement with results reported in other studies (19). The mean MIC values for these strains were higher compared to the rest of the microorganisms tested, but within the sensitivity range. The low MICs, established for Gram-positive microorganisms, point to high susceptibility to enrofloxacin. Some of the MICs are equal or even lower than the values reported by Scheer (19). Comparatively higher MIC values were established for *Staphylococcus* spp., especially for *S. haemolyticus.* In comparison with the results of other studies (19), the higher susceptibility of *Streptococcus* spp. and *Bacillus* spp. was also an interesting finding.

Enrofloxacin was synthesized in the mid-eighties for veterinary use only. In comparison with other quinolone acid derivatives used in veterinary medicine (flumequine), enrofloxacin has considerably lower MIC values and better activity against pseudomonads, streptococci and mycoplasms (19). In the case of severe multiresistant Gram-negative infections, Rutgers *et al.* (18) recommended enrofloxacin as a good alternative to aminoglycoside antimicrobials.

The results of the present study show that in spite of almost 20 years of its use in animals, enrofloxacin is still one of the most effective chemotherapeutic agents in veterinary medicine. The high level of activity could be explained by the slow rate of development of bacterial resistance due to prevalence of chromosomal mutations (10) compared to plasmid-mediated resistance (4). The alternative pathway of bacterial resistance selection is based on impaired penetration of quinolones through cell membranes or active efflux out of bacterial cells (13).

Regardless of the relatively low level of bacterial resistance there is a tendency toward decline in efficacy of enrofloxacin in poultry and pig populations (5, 7, 21). This could be ascribed to predominant medication of these species through water and feed which cannot always ensure plasma concentrations above MICs and explains better results of individual treatment of pet animals.

On the other hand, the high level of bacterial susceptibility found in our study could also be due to a relatively limited administration of fluorquinolones in pet animals. Data from various EU countries revealed that quinolones are among the antimicrobials with a relatively low level of administration. According to official information from DANMAP for 2002, the antimicrobial agents with greatest market share in Denmark are tetracyclines – 24 420 kg, followed by penicillines – 17 421 kg, macrolides – 16 203 kg and aminoglycosides – 12 126 kg (9). Only 97 kg of fluoroquinolones were used, which amounts to 0.1 % of the total amount of the antimicrobials used in veterinary medicine.

Regarding the rate of administration of chemotherapeutic agents in small animal practice, the share of the fluoroquinolones is the lowest too (≅ 1.4 %). In Finland, out of 2719 pet animals only 3 – 5 % were treated with quinolones. β-lactams were the first choice antimicrobials used in up to 78 % of pet population (11).

In order to avoid the risk of antimicrobial resistance, enrofloxacin administration should strictly meet the criteria of antimicrobial treatment. The low dose administration and short course of treatment are thought to be among the main causes of selection for bacterial resistance (12).

In conclusion, the results of the present study show that regardless of the long standing administration, en-

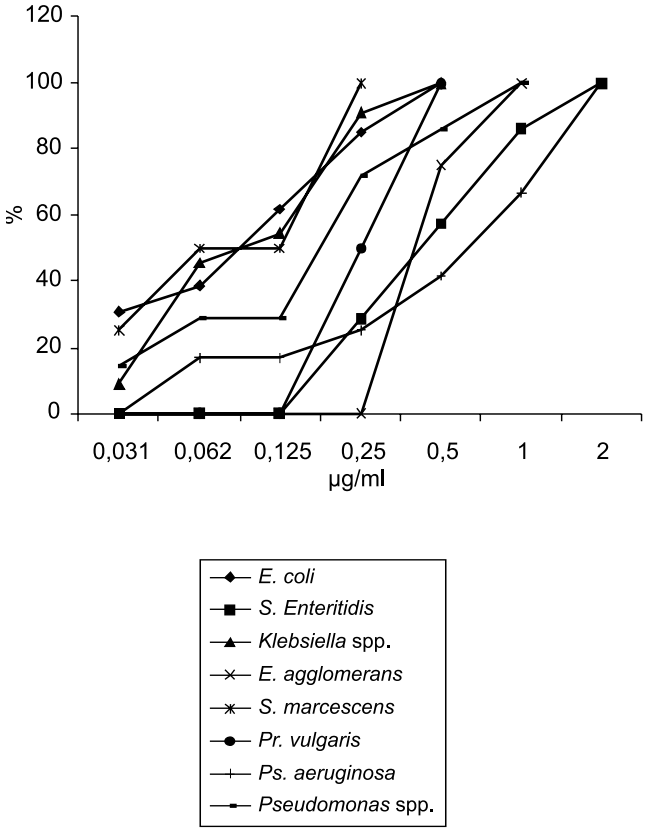

Fig. 1. Cumulative curves of MIC_{90} for Gram-negative bacteria

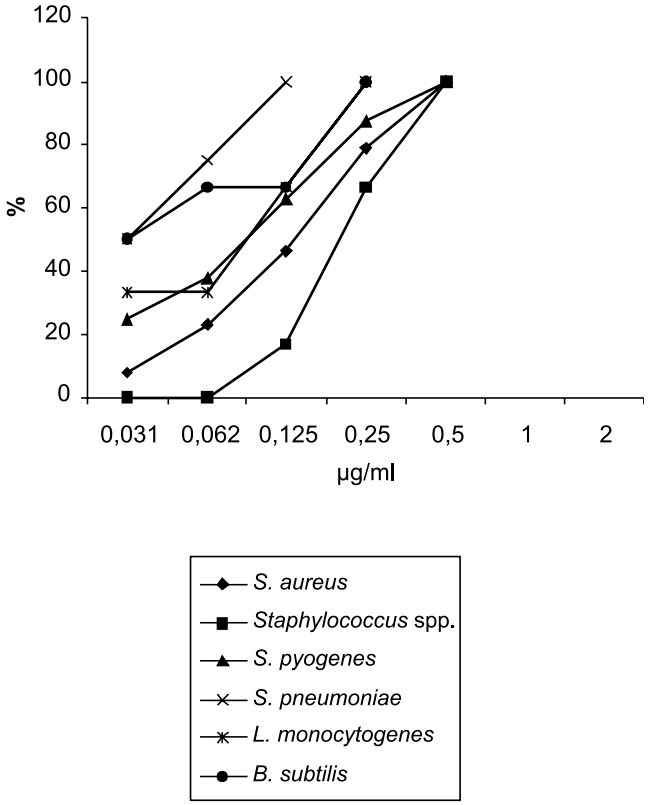

Figure 2. Cumulative curves of MIC_{90} for Gram-positive bacteria

rofloxacin is still the chemotherapeutic agents showing high activity against wide spectrum of Gram-negative and Gram-positive bacteria. Its efficacy against many enteric bacteria, staphylococci and streptococci is high but is lower against some strains of *P. aeruginosa* and *S. enteritidis*.

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REVIEW ARTICLE: BIOCHEMICAL MARKERS OF AQUATIC POLLUTION IN FISH – GLUTATHIONE S-TRANSFERASE

Slatinská, I., Smutná, M., Havelková, M., Svobodová, Z.

University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1-3, 612 42 Brno The Czech Republic

iveta.slatinska@email.cz

ABSTRACT

The aim of this review is to summarize the up-to-date knowledge on the function of the glutathione S-transferase conjugation enzyme (EC 2.5.1.18) and its potential use as a biochemical marker of aquatic pollution. Attention is also given to its chemical structure, classification and function in both the endogenous metabolism and the metabolism of xenobiotics, as well as to methods of glutathione S-transferase assay in biological materials. The main emphasis is placed on the possibility of using glutathione S-transferase as a biochemical marker in fish under laboratory and natural conditions.

Key words: biochemical marker; fish; glutathione S-transferase

BIOCHEMICAL MARKERS

It is well-known that the aquatic environment is contaminated with many types of organic and non-organic pollutants (31) of municipal, industrial, agricultural and mining industry origin (21).

Such pollutants affect the integrity of ecosystems and physiological functions of animals (31) as well as of people as consumers (28).

Harmful effects on population are sometimes difficult to prove in wild animals because many of these effects tend to manifest themselves only after a rather long period of time (36). Chemical analysis can measure only a fraction of contaminating substances present in polluted water and, moreover, chemical monitoring does not indicate their generally negative impact on organisms. The need to detect and determine the impact of contamination on the quality of the environment has prompted the study of, and research into, the biochemical and molecular markers of biological effects of pollutants on aquatic organisms (8). Biomarkers may be defined as changes in the biological response that are linked to the exposure to, or toxic effects of, chemical substances in the environment Analyzed in body fluids, cells or tissues, they are sensitive indicators demonstrating the penetration of a toxic substance into the organism and its distribution among tissues. They therefore are the decisive indicators of the toxic effect (36).

Fish have been used as aquatic contamination indicators for many years. In the case of an environmental disaster, they are unable to leave the site affected (13), bioaccumulate toxic substances (1) and because they are the last link in the food chain in the aquatic environment, they may negatively influence the safety of food and raw materials of animal origin (fish and fish products) (13). Understanding the reaction and response to the exposure to toxic substances in fish may be very important from the environmental point of view (36). An early detection of the sublethal effect may be the basic element in deciding about biodegradation and revitalization methods at the site investigated (8).

One of intensely investigated biochemical markers in fish is the glutathione S-transferase conjugation enzyme. The glutathione S-transferase activity assay as a response of the fish organism to aquatic contamination has been described in many papers from all over the world (21, 8).

GLUTATHIONE S-TRANSFERASE

Knowledge of the metabolism of xenobiotics is essential because it may offer an insight into the basic elements of the intoxication mechanism and determine toxicological risks of damage following an exposure to similar substances (5). Besides, special biotransformation processes may transform chemical substances to substances more, equally, or less toxic than the original substances (5). Metabolism of xenobiotics takes place in two stages. In the first stage of biotransformation, in which hydrolytic, reduction and oxidation enzymes play a role, a more polar product is produced by introduction or exposure of substituents. They are able to react with conjugation enzymes in the second stage of the metabolism of xenobiotics. The second stage enzymes catalyze the conjugation of activated xenobiotics and endogenous compounds in the internal environment of the organism by hydrophilic biomolecules, such as the reduced glutathione (GSH), UDP-glucuronic acid or glycine. The resulting conjugate is usually a very polar substance that cannot be resorbed and it is therefore excreted from the organism (18).

The glutathione S-transferase enzyme (GST) is an important intracellular enzyme of the second stage of xenobiotic metabolism (7). Its main function is to catalyze the conjugation of tripeptide glutathione and electrophilic substances of exogenous origin that might be one of the etiological factors of carcinogenesis and the development of degenerative diseases. Glutathione conjugation also participates in the metabolism of endogenous biomolecules, such as prostaglandins and steroids (35). Glutathione S-transferase also acts as an intracellular binding and transport protein in the metabolism of bilirubin in hepatocytes (33). Recently, glutathione S-transferase and its types are the centre of attention in connection with their ability to detoxify organic lipoperoxides (38). Those enzymes exhibit activity similar to glutathione peroxidases, but because it does not contain the atom of selenium, the activity is called Se-independent glutathione peroxidase. The enzymes mentioned are found in practically all organisms including bacteria, plants and animals (7).

According to their localization, the GST may be divided into cytosolic and microsomal. Cytosolic GSTs are dimeric proteins made up of two identical subunits or two different subunits, i.e. homodimers or heterodimers (26), the molecular weight of which is approximately 25 kDa. Each enzyme subunit has an active site composed of two regions performing a different function: the hydrophilic binding G-site, which binds the physiological basis of glutathione, and the neighbouring binding H-site, which provides hydrophobic environment for the binding of structurally diverse electrophilic substances. The G-site is very frequent among all GST as a result of its high specificity for glutathione, while the H-site may be very different in different GST, and exhibits a broadly variable binding specificity (7). This structural variability within the GST group provides for its ability to conjugate with a broad spectrum of substances (19).

Contrary to cytosolic enzymes, microsomal GSTs include a group of small (16 to 18 kDa) membrane-bound proteins that are active as trimmers with identical units (22). Their localization is bound to the endoplasmic reticulum membrane where potential substrates are produced by the cytochrome P450 system and where an accumulation of hydrophobic xenobiotics and their metabolites is expected. Compared with cytosolic GST, they provide for a more effective elimination of xenobiotics (22). The membrane-bound GST are also known as the Membrane Associated Proteins (in eicosanoides and glutathione metabolism) (MAPEG), and are composed of six proteins, i.e. MGST 1, MGST 2, MGST 3, leukotrien-C4-synthase, 5 lipoxygenase-activating protein and prostaglandin-E synthase. MAPEG takes part in the interaction with lipid derivates and plays a role in the transformation of reactive lipids into local hormones (leukotrienes and prostaglandins) or to unreactive products (lipid alcohol or hydroxyalkenal conjugates).

GST CLASSIFICATION AND NOMENCLATURE

At first, glutathione S-transferases were named on the basis of their common carbon nucleus of substrates or the functional group leading to their expression as glutathione alkyltransferases, glutathione S-aryltransferases, glutathione S-epoxidtransferases, glutathione S-alkentransferases, etc. Subsequent tests showed that they are different forms of GSTs that are capable of responding at a certain stage in all the reactions monitored (16).

Glutathione S-transferases are divided into a constantly growing number of classes based on varied and not clearly defined criteria including amino acid and nucleotide sequences, and immunologic, kinetic and structural characteristics (32). A majority of GST classes share a typical crystal structure. Structural differences are concentrated mainly around the active site, and along the borderline between subunits (9). As a general rule, GST identity within a class is greater than 60 % and GSTs with less than 30% identity are classified to different classes (32).

To date, five classes of GSTs have been described in mammals: alpha class GST (A1 – A4), mu class GST (M1 – M5), pi class GST (P1), theta class GST (T1 – T2) and zeta class GST (Z1). Sigma class GST has been described in invertebrates (7). Alpha-, pi- and theta-like GST isoforms have also been identified in fish (19). The distribution of individual GSTs is tissue-specific. For instance, GSTM-3 is found in large quantities in testicles, GSTM-2 in brain, members of the GST class are also found in the lens. Large quantities of theta and alpha GSTs are also found in the liver, and pi GSTs in the intestines. Pi class GSTs are localized in larger quantities in proliferating cells rather than in fully differentiated cells (35).

GLUTATHIONE S-TRANSFERASE FUNCTIONS

The main GST function is to catalyze the nucleophilic atack of tripeptide glutathione (gama-glu-cys-gly) on the electrophilic centre of the appropriate substrate (6). The reaction below is a simplified description of this catalytic function of GSTs:

$$GSH + R\text{-}X \xrightarrow{GST} GSR + HX$$

The GSTs facilitate the nucleophilic attack of glutathione on electrophilic substrate by binding glutathione onto the active G-site, and electrophilic substrate onto the active H-site, thus transferring substrate to the immediate vicinity of glutathione (7). At the same time, the SH group of GSH, which participates in the reaction, is activated. In the course of the conjugative reaction, a thioether bond is formed between the cysteine remains of glutathione and the electrophilic substance, and the result usually is a less reactive and a more readily soluble product (7). This reaction between glutathione and the GST catalyzed by electrophilic substrate is considered the first step towards the biosynthesis of mercapturic acids that facilitate the elimination of exogenous substances (6). Although GSTs are primarily involved in the detoxification process, they in some instances also catalyze reactions that activate certain chemical substances including toxic and carcinogenic substance (7). For instance, a large number of halogenated alkenes become nephrotoxic following glutathione conjugation (25).

In addition to their catalytic function, GSTs have been demonstrated to also have a binding function. GSTs bind a broad range of lipophilic substances at the binding site that do not participate in the catalytic reaction (20) and thus they facilitate their transport. The quantity of GSTs and their broad specificity for binding large amounts of ligands suggest the existence of similarities with serum albumin functions in blood plasma, and is the reason for the name "ligandin". In a proper nomenclature, ligandin corresponds to the alpha GST A1-1 class with the contribution of the heterodimer GST A1-2. Members of the class of GSTs bind bilirubin, thus preventing bilirubin reflux from cytoplasm to blood and increasing the level of bilirubin excreting by the liver (20). Members of the theta class of GSTs bind hemin and are involved in its transport from mitochondria to the endoplasmic reticulum where it is incorporated to apoprotein of cytochrome b5. Other classes of GSTs participate in the binding of some other endogenous substances, such as bile acids and steroid hormones (20).

Large amounts of chemical substances including xenobiotics and products of oxidative stress may serve as a GST substrate. The most frequently used standard substrate for almost all GSTs (except GST T1) is 1-chloro-2,4-dinitrobenzen (CDNB). An increased activity towards CDNB represents a total activity of various GST isoforms, and for that reason it is not possible to differentiate between effects of substances on individual GST isoforms (15). And so while the total specific GST activity may show little or no variation following pollution exposure, greater variations may be found in individual samples of each of the isoenzymes provided a good environmental biomarker is used (28). Other frequently used substrates for the determination of GST activity are 3,4-dichloronitrobenzene (DCNB) as a specific substrate for mu class GSTs, ethacryninc acid (EA) as a specific substrate for pi class GSTs and 4-nitrobenzyl chloride (NBC) as a specific substrate for theta class GSTs (10, 11, 15).

Very few GST inhibitors have been identified to date. Such inhibitors must be sufficiently liposoluble to be absorbed by cells, and they must be metabolized slowly. GSTs are inhibited by a bond between either the inhibitor and the active binding G-site or the active binding H-site, or a completely different GST site. Very strong GST inhibitors are the dibromated analogue of the ethacrynic acid and derivates of glutathione (e.g. S-(4-bromo-2,3-dioxybutyl)glutathione and S-(phenylsulphonyl) glutathione) (25).

GST ACTIVITY ASSAYS

The most frequent method for the determination of total GST catalytic activity is spectrophotometric detection of conjugate production between reduced glutathione and the substrate common for all isoforms of glutathione S-transferase, 1-chloro-2,4-dinitrobenzene (14).

Sample preparation

After they are collected, fish tissue samples (liver, kidneys, gills, etc) are immediately frozen and kept in liquid nitrogen. Ten per cent homogenate is made of individual tissues with chilled phosphate buffer (0.1 M, pH 7.4), and centrifuged in a chilled centrifuge at 10 500 g for 20 min at 4 °C. The postmitochondrial supernatant obtained is used for further analysis (27).

Spectrophotometric analysis

The total GST activity is assayed by the spectrophotometric method according to Habig *et al.* (14) with minor modifications (27). The reaction mixture is made up of 0.1 $mol.l^{-1}$ phosphate buffer (pH 7.4), 1 $mmol.l^{-1}$ GSH, 1 $mmol.l^{-1}$ 1-chloro-2-dinitrobenzene (CDNB) and 10 % postmitochondrial supernatant of a total volume of 2 ml. Absorbance variations are read at 340 nm wavelength. The resulting total GST catalytic activity is expressed as nmol. $min.^{-1}$ mg^{-1} protein. Protein volumes are measured by the method according to Bradford (3) or by the bicinchoninic acid assay (BCA).

The methods that can be used to assay the activity of individual isoenzymes include the Western blot procedure (21), high-performance liquid chromatography (HPLC) (28, 26) or the SDS polyacrylamide gel electrophoresis (SDS PAGE) (2, 15).

GLUTATHIONE S-TRANSFERASE AS AN AQUATIC POLLUTION INDICATOR

In fish, GSTs are used primarily as a biomarker indicating aquatic environment pollution with wastewater of municipal, industrial, agricultural or mining origin. In fish liver, the main organ of xenobiotic metabolism (34), GST may represent up to 10 % of total liver cytosolic proteins (20).

GST activity has been studied in various tissues of different fish species both under laboratory and under natural conditions.

GST ACTIVITY UNDER LABORATORY CONDITIONS

The most attention has focused on the study of GST activity in the liver as the major detoxification organ.

Increased total GST activity has been demonstrated in liver tissue following the exposure of the African catfish *(Clarias gariepinus)* to a mixture of 17α-ethynylestradiol and benzo(a) pyrene. After a 3-day exposure to benzo(a)pyrene only and to a mixture of the two substances, 3 times higher GST activity was demonstrated. GST activity increased twofold following a 6-day exposure to 17α-ethynylestradiole only (24).

Total GST activity in liver tissue following exposure to dimethoate and β-naphthoflavone has also been studied in the guppy *(Poecilia reticulata)*. While a 96-hour exposure to dimethoate resulted in a decrease in GST activity, a 96-hour exposure to β-naphthoflavone increased GST activity in fish liver samples (9). Total activity in liver tissue examined in the chub *(Leuciscus cephalus)* following exposure to β-naphthoflavone and benzo(a)pyrene showed only a minimum increase in total GST activity at low concentrations of the two xenobiotics. Higher concentrations of xenobiotics resulted in a decrease of the total GST activity. This can be explained by an accumulation of metabolites of the first or the second detoxification stage that may cause the inhibition of the total GST activity (17).

An increase in the total GST activity by a factor of 1.6 was also demonstrated in the liver of the brown bullhead *(Ameiurus nebulosus)* after 14-day exposure to ethoxyquine. Exposure of the brown bullhead *(Ameiurus nebulosus)* to ethoxyquine resulted in an increase in GST activity by a factor of 1.2 towards theta GST NBC specific substrate (p-nitrobutyl chlorine), but no GST activity increase towards the specific pi GST ECA substrate (ethacrynic acid) was found (15).

From the liver of the European plaice *(Pleuronectes platessa)*, three GST isoforms (GST A, GST A1 and GST A2) have been isolated. Following an exposure of the European plaice to the perfluoro-octanoic acid (PFOA), an increased expression of the GST A and GST A1 isoforms was demonstrated (19).

GST activity has also been studied in freshly isolated hepatocytes of the rainbow trout *(Oncorhynchus mykiss)*. Using the HPLC method, Perez-Lopez *et al.* (29) demonstrated the presence of the pi GST isoform in the hepatocytes. Liver cells of the rainbow trout were used also for the assessment of water samples taken from the inflow and the discharge sections of the wastewater treatment plant in Nice. Inflow samples showed higher levels of PAHs and PCBs than discharge samples. Total GST activity levels were determined after a 48-hour exposure of liver cells of the rainbow trout to various dilutions of inflow and discharge water samples. Higher total GST activity levels were found in the case of higher dilutions of both influx and discharge samples, which may be interpreted as an early adaptation response to the oxidative stress induced by test water samples (8).

In addition to the liver, other exposed fish tissues, such as the gills, olfactory organs and the intestines, have been studied. Fish ingest large amounts of exogenous substances with their feed and, together with the feed, the exogenous substances pass through the digestive tract of the fish. Intestinal mucous membrane of the fish is thus the first barrier that comes into contact with exogenous compounds. For that reason, the role of GST in the intestines has been investigated by many authors. In the catfish, e.g., higher total GST activity in the proximal segment of the intestines than in the distal one has been demonstrated. This suggests higher capabilities for the biotransformation of electrophilic pollutants in the proximal than in the distal segment of the intestines. It seems that the principal GST isoform in the intestines of the catfish is a class connected with the mammalian pi class GST. Activity of GST similar to alpha class GST was demonstrated only in the distal segment of catfish intestines.

The presence of GST similar to human alpha class GST in the distal segment of the intestines only is explained by the response to substances that pass more slowly through this segment. That suggests that different expression of GST resembling human alpha class GST in the distal segment of the intestines might serve as a biomarker of PAH and PCB (11). GST activity in the intestines has also been studied in the European plaice *(Pleuronectes platessa)*, where increased expression of the isoform of GST A following exposure to β-naphtoflavone (BNF) was demonstrated (19).

Another very much exposed organ are the gills. Total GST activity in the gills following exposure to dimethoate and β-naphthoflavone has also been studied in the guppy *(Poecilia reticulata)*. While a 96-hour exposure to dimethoate resulted in a decrease in GST activity, a 96-hour exposure to β-naphthoflavone increased GST activity in fish gill samples (9).

GST activity has also been studied in freshly isolated gill epithelium cells of the rainbow trout *(Oncorhynchus mykiss)*. The cells were cultivated and GST activity decreased with the length of cultivation. Five-day gill epithelium cells were cultivated at the presence of PAH-β-naphtoflavone (BNF) and 3-methylholanthrene (3-MC) for 48 h and for 7 days. While the 48-hour cultivation at the presence of BNF and 3-MC increased the total GST activity by a factor of 2 and 1.5, respectively, no increase in the GST activity was found after the 7-day cultivation, which may have been caused by poor physical condition of the cells (30).

GST activity in the gills has also been studied in the European plaice *(Pleuronectes platessa)*, where increased GST A and GST A1 activity following exposure to perfluoro-octanoic acid (PFOA) was demonstrated (19).

Total GST activity has also been demonstrated in the olfactory organ of the rainbow trout *(Oncorhynchus mykiss)*. The presence of pi class GST isoform was demonstrated using the Western immunoblot method. The imunofluorescence method demonstrated GST in the dendritic and perinuclear regions of the olfactory receptor neurons, no activity was demonstrated in the axons. That points to the involvement of olfactory organs in detoxification defence systems (33).

GST ACTIVITY UNDER FIELD CONDITIONS

When studied outside the laboratory, GST activity is usually studied in liver samples collected from various fish species. Total GST activity was determined in liver samples of the chub *(Leuciscus cephalus)* from the river Sava. The chub were captured at five different sites along the river. At the same time, PCB and PAH levels in the liver and muscle tissue samples were determined. At the site least contaminated with PCBs and PAHs, the total GST activity was comparable with

control site levels. At the site most contaminated with PCBs and PAHs, however, the total GST activity level was lower compared with that at the control site (17).

Total GST activity was determined in liver samples of male and female brown bullhead *(Leuciscus cephalus)* from the Lake Apopka March. Chemical analysis showed high concentrations of organochlorinated pesticides (OCPs) and mainly DDT metabolites (e.g. DDE, p, p', DDD, p, p') in muscle tissue of the fish. PCB concentrations in muscle tissue of the fish were also monitored and found comparable with those found at a control site at the Lake Woodruff. While GST activity found in liver samples from male brown bullhead from the Lake Apopka March was comparable with levels found at the control site at the Lake Woodruff, GST activity found in liver samples from female brown bullhead from the Lake Apopka March was significantly lower than that found at the control site.

Besides the lower GST activity, lower expression of GST protein in female brown bullhead liver samples compared with the controls was demonstrated by the Western blotting method. The results suggest that some xenobiotics are capable of reducing the expression of GST proteins, which may be connected with the damage to the toxicokinetics and metabolism by exogenous and endogenous substances. Significantly higher androgen concentrations in female brown bullhead plasma were demonstrated compared with the control site. There may be a relationship between higher androgen concentrations in the plasma of brown bullhead females and lower GST activity in the fish liver samples (12).

PCB concentrations in muscle tissue and total GST activity in liver were determined in samples taken from the nase *(Chondrostoma nasus)*, roach *(Rutilus rutilus)* and grayling *(Thymallus thymallus)* captured at three sites along the Rhone. Muscle samples of fish captured at the Miribel site had higher PCB concentrations compared with other those from other sites. At the same site, higher GST activity was recorded compared with the rests of the sites (23).

PCB, PAH and OCP concentrations were studied in bottom sediment samples and muscle tissue samples of roach *(Rutilus rutilus)* from two Amsterdam lakes. In the lake where higher PCB, PAH and OCP concentrations in sediments and higher PCB and OCP concentrations in roach liver samples were demonstrated, significantly lower GST activity was found (37).

Total GST activity was determined in liver samples from the red mullet *(Mullus barbatus)* captured at five sites along the French and Spanish Mediterranean coast.

Higher GST activity compared with the rests of the sites was recorded at the site where higher PAH concentrations in the sediment were demonstrated (4).

CONCLUSIONS

Glutathione S-transferase is one of intensely investigated conjugation enzymes of the second stage of xenobiotic detoxification. It is very often used as a biochemical marker of aquatic environment contamination with exogenous substances. Many laboratory experiments have demonstrated increased levels of GST activity following exposure of various fish species to organic substances commonly occurring in the environment. However, results of laboratory tests do not always coincide with results obtained under field conditions. The differences may be caused by the fact that fish under natural conditions are exposed to a constantly changing composition of chemical substances.

When evaluating aquatic environment pollution, we should always take into account a combination of several biochemical markers in our final assessment together, of course, with the results of classical chemical monitoring, because only a combination of all this information will give us the most objective picture of the status of the environment monitored.

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BIOLOGICAL EFFECTS OF ROSEMARY *(Rosmarinus officinalis* L.*)* ESSENTIAL OIL (A Review)

Faixová, Z.[1], Faix, Š.[2]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice
[2]Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4, 040 01 Košice
The Slovak Republic

faixova@uvm.sk

ABSTRACT

Many herbs and plant extracts are added to the diet not only for their aromatic properties but they have been identified as a source of various phytochemicals, many of which possess an important biological activity. Results of many experiments showed that rosemary essential oil had antimicrobial, antioxidant, anti-carcinogenic, cognition-improving and certain glucose level lowering properties which makes it useful as a natural animal feed additive. This review describes the most important biological activities of rosemary (*Rosmarinus officinalis* L.) essential oil in animals and humans. *In vitro* and *in vivo* effects of rosemary essential oil are discussed.

Key words: essential oil; metabolism; rosemary

INTRODUCTION

The use of antibiotics as growth promoters in animal feed is facing reduced social acceptance due to the appearance of residues and resistant strains of bacteria; antibiotic use has been banned in the European Union since January 2006 (Regulation 1831/2003/EC).

Many herbs and plant extracts have antimicrobial activities and antioxidant properties which make them useful as natural animal feed additives. Si *et al.* (28) reported than cinnamon has antimicrobial activity against *Salmonella* serotype *Typhymurium* DT 104 of swine *in vitro* study. These findings are in agreement with the reports of Prabuseenivasan *et al.* (25). They reported that cinnamon clove, geranium, lemon, orange and rosemary oils exhibited significant antibacterial properties against both gram-negative and gram-positive bacteria *in vitro.*

Antioxidant effect of aromatic plants is due to the presence of hydroxyl groups in their phenolic compounds (30).

Most essential oils are classified as Generally Recognized as Safe (GRAS) and have been approved for food and beverage consumption by US Food and Drug administration (31).

Plant extracts (anise oil, cinnamon oil, and garlic oil) may allow one to manipulate with rumen microbial fermentation by decreasing total volatile fatty acid concentration and reducing ammonia N concentration (6).

Essential oils derived from sage, rosemary, thyme and other herbs were shown to inhibit osteoclast activity and increase bone density *in vitro* (26).

Recently it was reported that some essential oils, such as cinnamon, have the potential to influence favourably the insulin system and affect beneficially blood glucose and lipid metabolism in people with type 2 diabetes (17).

The greatest attention from among all herbs and spices acting as biologically active compounds has been focused on rosemary.

The objectives of the current review were, therefore, to describe biological activities of rosemary *(Rosmarinus officinalis L.)* essential oil in animals and humans.

Essential oil composition

The essential oil of rosemary contains several compounds at a rather different concentrations. It is characterized by two or three major components at fairly high concentrations (20 to 70 %) compared to other compounds present in traces.

Generally, these major components determine the biological properties of the essential oil and can act in synergic manner or regulate one another.

Debersac *et al.* (8) reported that the major component of dried leaves of *Rosmarinus officinalis* (L.), supplied by Robertet (Grasse, France), was monoterpene oxide 1,8 cineole (36.1 %). Many monoterpene and sesquiterpene hydrocarbons were present in large amounts (32.2 %). The authors identified α-pinene, β-pinene, β-caryophylene, camphene, limonene, myrcene and p-cymene. They also detected the monoterpene ketone camphor (12.8 %) and monoterpene alcohols (9.6 %), such as borneol. All these compounds amounted to 93.4 % (w/w) of essential oil. All remaining substances were minor volatile compounds and six of them were still unidentified.

In the dichlormethane extract (DCME) from rosemary leaves only 28.1 % of the compounds present were quantified. The main compounds characterized in DCME were flavonoids and phenolic diterpenes. Two flavones were identified as cirsimaritin and genkwantin. Six structures of phenolic diterpenes were detected in that extract, namely carnosic acid (14.7 %) and its methylated form (7.4 %), carnosol (3.8 %), rosmanol (1.4 %), epirosmanol methyl ether (0.8 %) and epirosmanol (traces). Dichlormethane extracted also other lipophilic chemicals, probably corresponding to pigments, sterols and hydroxylated fatty acids (8).

Water-soluble extract from rosemary leaves was rich in rosemarinic acid (1.3 %) and flavonoids (3 %). A wide range of oxygenated monoterpenes were identified: oxides, such as 1,8 cineole, and alcohols, such as borneol and α-terpineol.

Chemical composition of rosemary essential oil can vary between regions and it depends mostly on climate, soil composition, plant organ, age and stage of vegetative cycle.

Cytotoxicity of rosemary essential oils

Oxygenated monoterpenes of essential oil seem to exhibit a variable degree of cytotoxicity. As typical lipophilic substances, they pass through the cell wall and cytoplasmic membrane and disrupt their structure. In bacteria, the membrane damage is related to the loss of ions and reduction in membrane potential, collapse of the proton pump and ATP depletion. This cytotoxic property is used to treat individuals affected by some human or animal pathogens or parasites (20).

Bozin *et al.* (5) tested antimicrobial and antioxidant activities of the essential oils of rosemary *(Rosmarinus officinalis* L.*)* and sage *(Salvia officinalis* L.). Their antimicrobial activity was tested against 13 bacterial strains and 6 fungi, including *Candica albicans* and 5 dermatocytes. The highest antibacterial activity of both essential oils was expressed on *E. coli*, *Salmonella typhi*, *S. enteritidis* and *Shigella sonei*. Essential oil of rosemary exhibited a significant rate of antifungal activity. Fu *et al.* (10) reported that essential oils from clove and rosemary alone and in combination exerted a significant antimicrobial effect against *Staphylococcus epidermidis*, *Escherichia coli* and *Candica albicans*.

The cytotoxic effect of rosemary is of great importance in preservation of agricultural or marine products. The antimicrobial efficacy of plant essential oils depends on food composition. Gutierrez *et al.* (14) found out that essential oils might be more effective against food-borne pathogens and spoilage bacteria when applied to ready to use food containing a high protein level at acidic pH, as well as lower levels of fats or carbohydrates.

Antioxidant properties

Free radicals/reactive oxygen species are associated with many biological phenomena, such as inflammation, aging, and carcinogenesis. The antioxidant activity of polar extracts of rosemary is related to the content of phenolic compounds (i. e. carnosol, carnosic acid). Constituents in rosemary have shown a variety of pharmacological activities for cancer chemoprevention and therapy in *in vitro* and *in vivo* models (29).

Cigarette smoke, containing reactive oxygen species, may activate the second step of benzo(a) pyrene metabolic way and thus may be partially responsible for the formation of the critical lung tumorigenic adduct. Alexandrov *et al.* (1) reported that cigarette filter containing rosemary extract caused higher than 70 % decrease in benzo(a) pyrene adducts levels arising due to cigarette smoke in MCF cells. This approach may reduce the lung cancer risk in addicted smokers.

Fahim *et al.* (9) found that rosemary ethanolic extract (0.15 g/100 g b. w.) given to rats for 3 weeks showed hepatoprotective and antimutagenic effects attributed to the presence of a relatively high percentage of phenolic compounds with high antioxidant activity.

Cheung and Tai (15) studied anti-proliferative properties of crude extracts of rosemary *(Rosmarinus officinalis* L.*)* in several human cancer cell lines and their anti-oxidative properties *in vitro* in a mouse RAW 264.7 macrophage/monocyte cell line. The study showed that the crude ethanolic rosemary extract had anti-proliferative effect on human leukemia and breast carcinoma cells.

The body possesses various antioxidative systems (free radical scavenging activity, FRSA) that prevent oxidative stress, for example saliva exhibits such an activity.

Atsumi and Tonosaki (2) measured the total salivary FRSA induced after sniffing lavender and rosemary essential oils which are widely used in aromatherapy. Salivary FRSA values were increased by stimulation with low concentrations (1 000 times dilution) of lavender or by high concentrations (10 times dilution) of rosemary. In contrast, both lavender and rosemary stimulations decreased cortisol levels. A significant inverse correlation was observed between the FRSA value and the cortisol levels with each concentration of rosemary stimulation. These findings show that lavender and rosemary enhance FRSA and decrease the stress hormone, cortisol, protecting body from oxidative stress.

Plant extracts rich in polyphenols (PERP) could provide an important alternative antioxidant but little is known about their use in ruminants since the antioxidant capacity of PERP could be altered by digestive processes.

Gladine *et al.* (12) reported that a single acute dose of PERP, given directly into the rumen highly susceptible to lipoperoxidation, enhanced the plasma total antioxidant status. The digestive processes of PERP *in vivo* are beneficial by improving the biological effect of polymeric proanthocyanidins.

It was found that addition of rosemary extract delayed the oxidation of lipid fraction of minced meat balls during storage in the freezer. The antioxidative effect was related to the concentration of the ethanol rosemary extract in the product (16). Similar results were reported by Lopez-Bote *et al.* (19).

In contrast to the above, other experiments have reported little or no antioxidant activity of rosemary.

Galobart *et al.* (11) found that dietary supplementation with 500 or 1 000 mg/kg of a commercial rosemary extract had no effect on the lipid oxidative stability of eggs enriched with omega-3 fatty acids.

Effect on rumen microbial fermentation

In dairy cattle, the use of antibiotics as feed additives, such as ionophore antibiotics, has proved to be a useful tool to reduce energy (in the form of methane) and nitrogen (in the form of ammonia) losses from the diet. However, the use of antibiotics as feed additives in dairy cows is banned in the European Union. The use of plant extracts appears as one of the most natural alternatives to the antibiotic use in animal nutrition.

Castillejos *et al.* (7) studied the effects of 10 essential oils (rosemary, hyssop, sage, tea tree, clove leave, lavandin, lavender, thyme, oregano and savor), administered *in vitro* at three doses (5, 50 and 500 mg/l), on rumen fermentation of high concentrate, feedlot-type diet. The majority of the essential oils modified rumen microbial fermentation and may allowed one to manipulate with rumen fermentation to improve animal performance.

Effect on bone

Mühlbauer *et al.* (22) tested the effects of some common herbs (rosemary, thyme and sage) and their constituent essential oils and monoterpenes on bone resorption in ovariectomized rats. Bone resorption was inhibited by the addition of 1 g of powdered leaves of each herb, and the essential oils extracted from sage and rosemary had similar inhibitory effects. Recently our results showed that diet supplementation with graded proportions of rosemary essential oil (0; 0.1; 0.05 and 0.025 %) caused a significantly lower plasma calcium level in broiler chicks and the effect of rosemary essential oil on plasma calcium levels tended to be dose-dependent (Faixová *et al.* 2008, unpublished data).

Antidiabetic properties

Rosemary *(Rosmarinus officinalis)* is used in traditional Turkish folk medicine for the treatment of hyperglycaemia.

Bakirel *et al.* (4) investigated potential effect of ethanolic extract of *Rosmarinus officinalis* leaves on glucose homeostasis in rabbits. Results of this study showed that ethanolic extracts of leaves of *Rosmarinus officinalis* (L.) reduced blood glucose level in normoglycaemic and glucose-hyperglycaemic rabbits. Repeated administration of rosemary extract to alloxan-diabetic rabbits led to a decrease in blood glucose level and significant increase in serum insulin level. However, results of our experiment with dietary graded levels of rosemary essential oil in broiler chicks did not confirm the above mentioned findings (Faixová *et al.,* 2008, unpublished data).

Effect on immunity

There is a lack of data regarding the effects of dietary rosemary extract on immunity.

The results of Babu *et al.* (3) indicate that rosemary dietary extract might not result in general enhancing of the immune system in young rats and will probably be effective under some stress condition, such as protein or antioxidant deficiency.

Cognition-enhancing properties

Several non-toxic European herbal species have pan-cultural traditions as treatment for cognitive deficit, including that associated with ageing. Particularly promising candidate species include sage, lemon and rosemary.

The essential action of rosemary essential oil is in stimulation of the nervous system under sympathetic control resulting in improved memorizing and concentrating abilities (27).

Moss *et al.* (21) assessed the olfactory impact of essential oils of lavender and rosemary on cognitive performance and mood of healthy volunteers. They reported that rosemary produced a significant enhancement of performance in terms of overall quality of memory and secondary memory factors, but also impaired the speed component of memory compared to the control.

Essential oils of peppermint and rosemary have been found to increase the activity level of mice (18) and diffusion of rosemary into the environment encouraged the dogs to spend more of their time alert (i. e. standing, moving) than any other olfactory conditions (lavender, chamomile) (13).

Rosemary essential oil was found to cause moderate inhibition of acetylcholinesterase (24). The anti-acetylcholinesterase activity of rosemary essential oil is explained by synergic interaction between 1,8 cineole and 2-pinene in rosemary essential oil. Inhibition of acetylcholinesterase is considered one of the treatment strategies against several neurologic disorders, including Alzeimer's disease, senile dementia and myasthenia gravis.

Adverse effects of rosemary

Nusier *et al.* (23) studied the effect of dietary rosemary (250 and 500 mg/kg b. w.) on fertility of adult rats over the period of 63 days. They reported that sperm motility and density were significantly decreased in the *cauda epididymidis* and in the testes of rosemary-treated male rats in group 2. In addition, the treatment markedly increased the number of cases of foetal resorption in female rats impregnated by group 2 males, thereby reducing their fertility.

CONCLUSION

Many *in vivo* and *in vitro* experiments investigated effects of rosemary essential oil in order to show that its constituents have important biological properties. Results of the relevant studies showed that there are biologically active compounds in rosemary essential oil exhibiting cytotoxic, antioxidant, anti-carcinogenic and cognition-enhancing properties. They also have the potential to influence glucose level in diabetic patients,

modify rumen microbial fermentation and enhance bone resorption. Rosemary essential oil, however, does not enhance immune response and there are reports about its adverse effects on fertility. Essential oils, despite their wide use and being familiar to us as fragrances, constitute effective alternatives or complements to synthetic compounds produced by chemical industry without showing the same side effects.

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EFFECT OF CONVERSION FROM CONVENTIONAL TO ORGANIC DAIRY FARM ON MILK QUALITY AND HEALTH OF DAIRY COWS

Hanuš, O.[1], Brychtová, J.[2], Genčurová, V.[3]
Pešl, J.[2], Hulová, I.[1], Vyletělová, M.[1], Jedelská, R.[3], Kopecký, J.[3]

[1]Agriresearch Rapotín, Výzkumníků 267, 788 13 Vikýřovice
[2]Bemagro, Malonty
[3]Research Institute for Cattle Breeding, Rapotín, Výzkumníků 267, 788 13 Vikýřovice
The Czech Republic

oto.hanus@vuchs.cz

ABSTRACT

Organic farming (O) is an alternative for friendly use of the environment in time of presupposed global climate changes. Possible impacts of O on raw cow-milk quality, composition and properties, as compared to conventional farming (C), were evaluated in this paper. The C milk was used as reference *versus* O. There is still a lack of knowledge in this field. Bulk milk samples (BMSs) were investigated in conventional and conversion (to O) period in one Holstein (H) herd. Milk yield (MY) was reduced by 14.6 and 28.5 %, respectively, so was the crude protein (CP) (3.17 < 3.25 %). Lower milk urea in O farming (22.02 < 28.56 mg.100 ml^{-1}; by 22.9 %) is a normal phenomenon. Milk freezing point was a little better (C : O; –0.52504 < –0.52309 °C) in C farming. H herd BMSs from winter and summer seasons of one year were investigated in four O herds (n = 16) and in one C herd (n = 36). O herds grazed during summer and were fed total mixed rations (TMR) during winter, C herd was supplied TMR throughout the year. Nutrition was typical under the Czech Republic (CR) conditions. Average MY of O herds was 7037 ± 422 and of C herd 8900 kg per lactation. Significantly lower were fat (3.79 < 4.06 %), CP and true protein (3.09 < 3.17 %) and casein (2.54 < 2.66 %) in O herds. Average lactose (4.92 > 4.82 %) and whey protein were significantly higher. These facts together with significantly lower urea (19.53 < 40.39 mg.100 ml^{-1}) and higher acetone (6.8 > 1.6 mg.l^{-1}) indicated permanent energy and protein deficiency in O herd nutrition. Citric acid in O herds was insignificantly lower (8.45 < 8.76 mmol.l^{-1}). It is necessary to improve the energy supply to the O herd. Somatic cell count (SCC) was significantly lower in C herd, both (C and O) had SCC at extra milk quality level (< 300 ths.ml^{-1}). Significantly higher Ca and Mg levels were detected in O milk (1257 > 1172 and 112.0 > 107.4 mg.kg^{-1}; Hanuš *et al.*, 2008). Additional research is necessary to make more detailed assessment of O milk benefits.

Key words: acetone; casein; conventional and organic dairy farm; dairy cow; fat; lactose; raw milk; somatic cell count; true protein; urea; whey protein

INTRODUCTION

Global aspects of organic farming

Recently many authors (2, 3, 8, 10) investigated various aspects related to confirmation, development and simulation of possible impacts of global warming and greenhouse effect on the global climate, air, soil, water, vegetation and animals round the world. The reason is the general increase in global contamination of the environment due to human activities, increased exploitation of natural resources, chemical pollution and erosion of Earth's surface. Geller *et al.* (8) studied the effect of transportation. The decrease in particulate matter mass or number of emission factors, resulting from various engine configurations, was non-linearly related to the decrease in overall redox activity of particulate matter.

According to the results presented (1, 2, 3, 7, 10, 27, 30, 32, 41) it is possible to anticipate future changes and take them into account. Goubanova and Li (10) predicted, on the basis of three model simulations, a warmer climate with less total precipitations but more intense precipitation events for the Mediterranean basin.

In general, this fact can have an impact on agriculture including the range, way and outcomes of keeping of farm animals. One of these reactions could be also greater expansion of organic farming and dairying. In historical sense, current organic farming is in fact an old-new alternative under new conditions. Kirner *et al.* (22) analysed the situation on the biomilk market in Austria and concluded that the interest in bioproducts is growing up abroad.

Reasons for organic farming

People, i. e. consumers, wish to have an alternative in terms of the enhancement of food chain safety and environmental protection on a long-term basis. Development related to this demand was initiated due to some well known negative historic-social experiences and technological development concerning the environment. The modern trend in European agriculture is the implementation of economical activities in the so-called LFA (less favourable area). It is related to the legislatively defined system of organic farming and the so-called low input system of animal farms with narrow link to grazing. This is the general response to the anthropogenic load on the environment. A biologically friendly approach to milk yield limits was highlighted in various papers (11, 12, 13, 14, 26) which also provided answer to the topical question: *"Only it is economic in the long term, what is ecological*". Rosati and Aumaitre (34) mentioned that organic milk production, practised according to European and national regulations on organic farms in recent years, had impact on livestock systems, animal feeding, forage management, reproduction behaviour and animal health in particular. They themselves discussed animal welfare, product quality, environmental issues, and economic results of organic dairy farming. Müller *et al.* (31) mentioned that the problem of milk yield level predicated only very little about production efficiency and that less could mean sometimes more. They mentioned also the low input system of dairy cow keeping which was characterized by an effort to make maximal use of pasture as the cheapest source of roughage.

Hajšlová *et al.* (15) mentioned significantly higher nitrate load on potatoes from conventional (C) agriculture in comparison to those grown on organic farm. Conventional potatoes supplied less vitamin C as well. Leifert (28) reported that the risk of mould occurrence was by 8 % higher at application of mineral fertilizers (NPK) and pesticides to wheat on C farms compared to the O farms which did not use them. An increase in deoxynivalenole load as fusarium mycotoxine in perennial wheat (by 8 or 18 $\mu g.kg^{-1}$) was also mentioned.

Some results of organic dairy farms in the Czech Republic

Organic dairy farming is a potential form of friendly approach to society development and exploitation of natural resources in agriculture. However, in the Czech Republic, the number of organic farms has been reduced by 33 % (36) in the recent period. C herds were characterised by better longevity (6.0 > 2.5 lactation), reproduction (98.7 < 124.3 days of service period) and lower percentage of eliminated milk due to secretory disorders and lower antibiotic therapy (3.0 < 4.6 %). There was also lower concentration of nitrates (16, 17) in water sources (10.5 < 19.0 mg/l, nevertheless both levels corresponded to good quality). Leifert (28) mentioned lower percentage of animals which were treated against mastitis (O : C, 3.3 < 6.2 %; $P < 0.05$). On the other hand, the decrease in protein content was insignificant (3.35 < 3.40 %) but higher acetone concentration (5.3 > 2.6 mg/l) was found in milk (16, 17). The typical cattle breed on O dairy herds is Czech Fleckvieh (59.8 %) in contrast to Holstein breed (18.8 %).

There was a different ratio in C herds (46.7 : 47.5 %). The O farms reported 83 % milk yield compared to C herds (36). Similar to milk yield, Louda *et al.* (29) reported that cow fertility was not affected negatively by conversion to O system. However, the average multiparity percentage was higher in organically managed cows (33). Rosati and Aumaitre (34) stated that few experimental data comparing biological, technical, and economical traits between the two systems, O and C, were available in the literature. On the other hand, detailed information was published in several countries abroad (Switzerland, Austria, France, etc.).

The aim of the paper

The general aim of organic farming is to contribute to better prospective of global development. The objective of this paper is to investigate the economical aspect of change (from C to O) on primary milk production in terms of quality of milk as a raw material for food industry and on some health indicators of dairy cows.

MATERIALS AND METHODS

Location of the organic dairy farm

The experimental location (evaluation 1) of conversion from C to O farming was characterized by the following climate parameters: altitude 650 m; total annual precipitation 800 mm; mean annual temperature 7.0 °C. The heard comprised 384 dairy cows of Holstein breed. Before the conversion period (CPE; two years), during conventional (C) farming, the mean daily milk yield (MY) was 25.3 kg per day (7 706 kg per standard lactation on average; 2005, 2006). During CPE the MY was reduced to 21.6 kg per day (6 577 kg per lactation; 2007). In terms of the environment but not the breed and size of the herd the farm was a typical organic farm in CR, situated usually in LFA (less favourable area).

1) Conditions during conversion period (CPE) from C to O farming

Dairy cows were housed in new free house with individual resting boxes and milked in a herringbone milking parlour. During spring, summer and autumn they were kept in a run overnight (about 6 hectares of grass-covered land). The cows were supplied hay in this run. During CPE, a homoeopathic prophylactic treatment was carried out to prevent inflammatory illnesses, such as mastitis, lameness and/or endometritis (similar to organic farms in Germany, Switzerland and Austria: 21, 23, 24, 25, 38, 39, 40, 42, 43).

Clinical mastitis was treated by antibiotics. In addition to that a salve based on animal fat and medicinal herb (*Hypericum perforatum*) was used to treat mastitis. The feed efficiency was

reduced during CPE. It caused the mentioned decrease in MY by 14.6 %. Further reduction in MY was anticipated (18.1 kg per day and 5 521 kg per lactation, reduction by 28.5 %; 2008). C (2005, 2006) summer feed rations (kg per day and cow), fed according to MY of respective groups and standard demands, consisted on average of the following components: red clover-grass silage 24 kg; maize silage 12 kg; hay 2 kg; green-red clover and grass 20 kg (fodder crops); concentrate 3.7 kg; dry matter intake 21.0 kg; as total mixed ration (TMR).

Feeding in 2006 was a little below the impact of higher mycotoxin (in ppb) occurrence in red clover-grass silage: DON 157.2; OTA 3.1; ZEA 69.2; FUM 58.9; AFL 5.8. Similarly CPE and O (2007) summer feed rations consisted of the following: red clover-grass silage 20 kg; maize GPS 6 kg; hay 2 kg; green-red clover and grass including pasture 20 kg (fodder crops); concentrate 3.0 kg; dry matter intake 18.2 kg; mostly as TMR.

Bulk milk samples (BMSs) were obtained monthly for the first six months of every year during both periods C (one year, 6 BMSs) and CPE (two years, 12 BMSs). Milk quality was investigated using basic milk indicators as mentioned below. The total mesophilic bacterial count (TMBC) was determined by a Bactoscan instrument (Foss Electric, Denmark) as part of the routine milk quality control.

2) Monitoring of milk quality in selected dairy cow herds

BMSs (each from eight dairy cows) were obtained regularly every two weeks in winter and spring seasons of one year in one conventional (C) Holstein (H) herd (150 cows; n = 36 BMSs, 8 cows in one BMS) and four organic dairy herds (O; n = 16 whole BMSs) for one year two times in winter and summer. The altitude was 361.5 ± 121.2 m for O herds and 257 m for the C herd. The average total annual precipitation was 750 ± 86 mm for O farms and 468 mm for the C farm. The average annual temperature was 6.05 ± 1.21 °C for O farms and 9.35 °C for the C farm. There were from 25 to 384 H dairy cows in the four investigated O herds. The cows in O herds were fed in a way typical of CR farms (alfalfa, clover and grass silage, hay, concentrate and mineral mixtures in the form of TMR according to relevant milk yield and standard demands, similarly to evaluation 1, O rations).

The O herds were housed in free stables and grazed during summer as stipulated by law on organic farming in CR. The C herd was kept in a free stable and fed TMR throughout the year (corn silage 15.0 kg per head and day, alfalfa silage 10.0 kg, LKS 5.0 kg, alfalfa hay 1.0 kg, brewer's yeast 3.0 kg, dried whey 0.3 kg, concentrate and mineral mixtures 5.0 kg, according to relevant milk yield (MY) and standard demands). Dry matter intake was 20.2 kg per head and day on average (crude fibre 3.47 kg per head and day; PDIE 2.14 kg; PDIN 2.64 kg; ADF 4.35 kg; NDF 8.61 kg; NEL 151.7 MJ per head and day). All cows were milked twice a day. Other farm characteristics related to MYs and herds were noted as well.

Analytical procedures used

The quality of drinking water from the milking parlour of farm in conversion (from C to O) was examined. The analysis was performed regularly by an accredited laboratory in Rapotin (No. 1340, EN ISO 17025, Accreditation certificate No. 124/2004) according to current standard operation procedure. The milk quality was evaluated by the same laboratory. The following abbreviations and units were used for the investigated milk indicators: F = milk fat content (%); L = lactose content (monohydrate; %); SNF = solids non-fat content (%); SCC = somatic cell count (ths.ml^{-1}); F/CP = ratio; U = urea (mg.100 ml^{-1}); A = acetone (mg.l^{-1}); CA = citric acid (mmol.l^{-1}); MFP = milk freezing point (°C); EC = electrical conductivity (mS.cm^{-1}); SPM = specific mass (g.cm^{-3}); CP = crude protein (%); TP = true protein (%); CAS = casein %); WP = whey protein (difference TP-CAS; %); NNM = non-protein nitrogen (N) (%); UNPN = ratio of urea N in non-protein N (%); CN-CP and CN-TP = casein level for CP and TP (%); RIS = residues of inhibitory substances (+/–; by microbiological test Delvo-test). The following methods were used:

F, L and SNF indicators were measured by an instrument MilkoScan 133B (Foss Electric, Denmark), which was calibrated regularly according to the reference method results (standard ČSN 57 0536, by the Röse-Gottlieb method for F content, Kjeldahl method for CP content and polarimetric and gravimetric methods for L and SNF contents, according to the standard ČSN 57 0530). The instrument was included in the proficiency testing with regularly successful results.

The extensive combined result uncertainties (1.96 times the combined uncertainty as a standard deviation with probability level of 95 %) were: ± 2.77 % for F (± 0.101 % for original unit), ± 2.59 % for CP (± 0.085 %) and ± 2.77 % for L (± 0.115 %); the protein fractions CP, TP and CAS were determined by the reference Kjeldahl method using an apparatus of Tecator line with Kjeltec Auto Distillation unit 2 200 (Foss-Tecator AB, Sweden) according to ČSN 57 0530. The apparatus was included in the international proficiency testing (AFEMA, APLAC and ICAR-CECALAIT) regularly with mostly successful results. The extensive combined result uncertainty of measurement was ± 1.7 % for CP (± 0.057 %);

The SCC was determined by Fossomatic 90 apparatus (Foss Electric, Denmark) according to ČSN EN ISO 13366-3. The apparatus was included in the proficiency testing with good results obtained regularly. The extensive combined result uncertainty was ± 9.3 % for SCC ≤ 900 thousands.ml^{-1}.

The MFP values were analysed by top cryoscopic instrument Cryo-Star automatic (Funke-Gerber, Germany). The selected measurement mood was Plateau Search (with parameters: interval = 23 seconds and delta t = 0.4 m °C). The instrument was calibrated regularly by standard NaCl solutions (Funke-Gerber) and was included in proficiency testing with successful results regularly. The extensive combined result uncertainty of measurement was ± 0.00608 °C, i. e. ± 1.18 %.

The U was determined by a spectrophotometric method at 420 nm. The specific reaction solution was prepared as an acidic mixture with p-dimethylaminobenzaldehyde. Spekol 11 (Carl Zeiss Jena, Germany) was calibrated using six samples on the scale with U increase from 6 to 60 mg.100 ml^{-1}.

The A was investigated by a spectrophotometric method at 485 nm. Acetone was absorbed in alkaline solution of KCl with salicylaldehyde during 24 hours of microdiffusion in special vessels (at 20 °C in darkness). Spekol 11 was subjected to 5-point

Table 1. Differences in bulk milk sample indicators in conventional (C) and organic (O) period of one H herd (evaluation 1)

MI	Unit	Period		t	Statistical significance
		2005 and 2006, C x ± sx	2007, O x ± sx		
CP	%	3.26 ± 0.020	3.17 ± 0.083	2.36	*
F	%	3.82 ± 0.082	3.90 ± 0.120	1.23	Ns
CAS	%	2.51 ± 0.122	2.545 ± 0.044	0.55	Ns
TMBC	ths. CFU.ml^{-1}	6.73 ± 1.807	5.05 ± 0.084	2.08	Ns
log TMBC	–	0.8163 ± 0.1091	0.7032 ± 0.0071	2.31	*
SCC	ths. ml^{-1}	282.19 ± 24.484	254.37 ± 18.519	2.03	Ns
log SCC	–	2.4491 ± 0.0389	2.4045 ± 0.0308	2.01	Ns
U	mg.100 ml^{-1}	28.56 ± 4.58	22.02 ± 3.38	2.61	*
SNF	%	8.74 ± 0.046	8.77 ± 0.094	0.64	Ns
MFP	°C	525.04 ± 1.019	523.10 ± 1.198	2.76	*

Valid also for Tab. 2 and Figures: MI = milk indicator; x = arithmetical mean; ag = geometric average; sd = standard deviation; vx = variation coefficient (%); Statistical significance of differences: *, ** and *** = $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, resp.; ns = $P > 0.05$; F = milk fat; L = lactose, monohydrate; SNF = non-fat solids; SCC = somatic cell count; TMBC = total mesophilic bacteria count; CFU = colony forming unit; F/CP = ratio; U = urea; A = acetone; CA = citric acid; MFP = milk freezing point; EC = electrical conductivity; SPM = specific mass; CP = crude protein; TP = true protein; CAS = casein; WP = whey protein; NNM = non-protein nitrogen; UNPN = ratio of urea N in non-protein N; CN-CP and CN-TP = casein number

calibration on the scale with A increase from 1 to 2 mg.l^{-1}.

The CA was determined by spectrophotometric measurements at 428 nm. Milk was coagulated with trichloracetic acid and the filtrate allowed to react with pyridin and acetanhydride (30 minutes at 32 °C). Reaction of citric acid with pyridine produced a yellow-coloured complex in the acetanhydride medium. The Specol 11 was subjected to 6-point calibration with concentration ranging from 1.5 to 20.0 mmol.l^{-1} (from 0.03 to 0.36 %).

The EC was measured by OK 102/1 (Radelkis, Hungary) conductometer at 20 °C (in mS.cm^{-1}) with the help of geometrical exactly defined bell glass electrode with platinum ring contacts. The instrument was calibrated by the relevant salt (KCl) solution (10.2 mS.cm^{-1}) before measurement of each milk sample set.

The SPM was determined employing Moh's hydrostatic scale.

Statistical evaluation

The results obtained were evaluated by basic statistical methods and conventional *t*-test. In this way the average parameters of quality of dairy cow herd milk were compared between conventional (C) and conversion period (CPE) in one herd and between one conventional (C) and four organic (O) herds. C milk results were used as a reference for O milk. Before statistical evaluation the values of milk quality indicators with abnormal frequency distribution (TMBC, SCC, A) were transformed logarithmically.

RESULTS AND DISCUSSION

1) Changes during conversion from conventional to organic farm

During CPE the drinking water had the following quality: electrical conductivity 7.2 mS.m^{-1}; hardness 0.4 mmol.l^{-1}; nitrates 2.72; nitrites 0,31; ammonium < 0.05; chemical oxygen demand (COD) 0.33; free Cl_2 0.0; chlorides 1.42; sulphates 9.1; K 1.5; Na 2.6; Fe 0.2; Cu 0.01; Mn 0.029; Zn 0.56; Pb 0.006; Ni 0.001 (mg.l^{-1}); pH 7.39; odour and flavour acceptable; colour 6 mg.l^{-1} Pt; turbidity 1 FU; coliform bacteria 3; *E. coli* 0; enterococci 0 (CFU.100 ml^{-1}); mesophilic bacteria 60; psychrotrophic bacteria 120 (CFU.ml^{-1}). Water was obtained from municipal water supply. The results indicate very good quality of water with the exception of mesophilic bacterial count. The quality of drinking water used on organic dairy farms was evaluated in other papers (16, 17, 18). Genčurová *et al.* (9) observed significant correlation (–0.39; $P < 0.01$) between herd location altitude and nitrate concentration in water sources supplying dairy herds in the CR.

Table 2. Results of bulk milk sample indicators in conventional (C) herd and organic (O) herds (evaluation 2)

MI	Unit	C				O				Stat. signif.
		x	Ag	sd	vx	x	ag	sd	Vx	
F	%	4.06		0.418	10.3	3.79		0.310	8.2	*
L	%	4.82		0.073	1.5	4.92		0.118	2.4	***
SNF	%	8.77		0.114	1.3	8.70		0.170	2.0	Ns
SCC	ths.ml^{-1}	141		58.001	41.1	245		114.88	46.9	***
log SCC		2.1186	131	0.1613		2.3347	216	0.2363		***
U	mg.100ml^{-1}	40.39		5.409	13.4	19.53		5.739	29.4	***
A	mg.l^{-1}	2.03		1.106	54.5	7.77		4.177	53.8	***
log A		0.2042	1.60	0.3717		0.8341	6.82	0.2254		***
CA	mmol.l^{-1}	8.76		1.228	14.0	8.45		2.349	27.8	Ns
F/CP		1.21		0.108	8.9	1.18		0.099	8.4	Ns
EC	mS.cm^{-1}	4.31		0.338	7.8	3.60		0.414	11.5	***
MFP	°C	-0.5320		0.0050	0.9	-0.5288		0.0116	2.2	Ns
SPM	g.cm^{-3}	1.0324		0.0013	0.1	1.0313		0.0010	0.1	**
CP	%	3.36		0.114	3.4	3.21		0.118	3.7	***
CAS	%	2.66		0.088	3.3	2.54		0.094	3.7	***
TP	%	3.17		0.120	3.8	3.09		0.104	3.4	*
WP	%	0.51		0.052	10.2	0.55		0.060	10.9	*
NNM	%	0.19		0.039	20.5	0.11		0.053	48.2	***
UNPN	%	62.81		13.351	21.3	59.66		31.482	52.8	Ns
CN-CP	%	79.01		1.007	1.3	79.26		1.960	2.5	Ns
CN-TP	%	83.83		1.262	1.5	82.18		1.787	2.2	***

All RIS investigations of BMSs provided negative results. No antibiotics were detected. The milk yield decline between C and O periods (by 14.6 and 28.5 % respectively) is a typical phenomenon related to conversion. The limits on the use of concentrates in dairy feed rations on organic farms reduce milk yield throughout Europe (34). In a Denmark experiment conducted on organic farms, reduction in dry matter intake through cow feed rations and reduction in concentrate in feed rations from 38 % to 19 % resulted in reduction of both the milk yield and milk protein content and in simultaneous increase in free fatty acids concentration (37). There were no indications of health problems associated with the reduced feeding level. Organic husbandry proved more efficient than did the conventional husbandry in converting roughage into milk (33).

There were significant differences ($P < 0.05$) in raw milk composition and properties between C and O farming period of one H cow herd regarding protein content (CP), log TMBC, urea concentration and milk freezing point (Tabs. 1 and 2). The CP content was reduced in O farming period (3.17 < 3.25 %; Fig. 1). TMBC was only a little better on O farms with excellent results of milk hygiene in both periods. This fact should not be necessarily ascribed to C or O management system. Lower U concentration on O farms (22.02 < 28.56 mg.100 ml^{-1}; by 22.9 %; Fig. 2) is a quite common phenomenon (16, 17, 18) caused by typical nutritional changes between the C and O period.

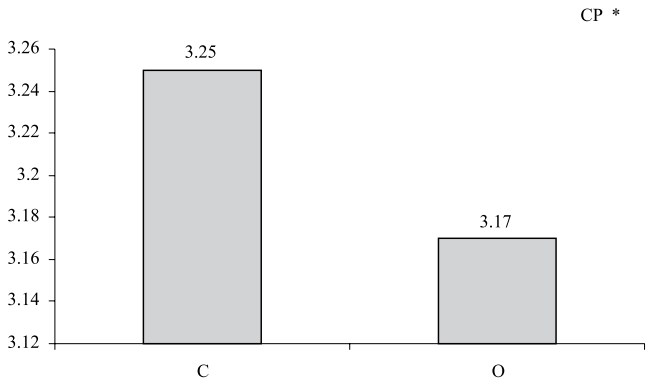


Fig. 1. Milk crude protein content (%) change in conversion from C to O farming in one H cow herd (evaluation 1)

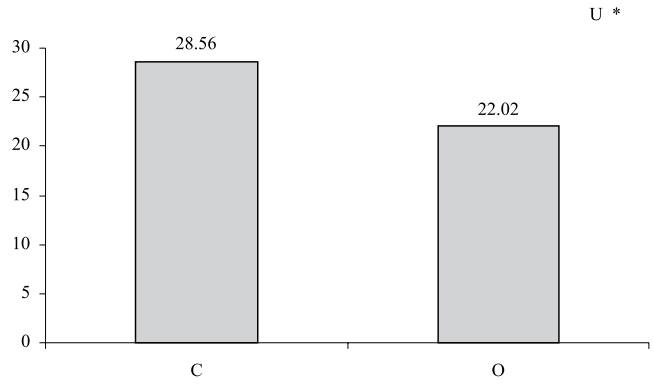


Fig. 2. Milk urea concentration (mg.100ml^{-1}) change in conversion from C to O farming in one H cow herd (evaluation 1)

The proportion of concentrate in feed rations on organic dairy farms is limited. This is typical of organic farms. For example Reksen *et al.* (33) presented Norwegian demands. Organic dairy production is characterized by low proportion of concentrate in feed rations because, on the basis of regulations, only 30 % of the energy requirements of a cow can be met by concentrate feed. MFP was a little better (C : O; –0.52504 < –0.52309 °C) during C period, probably as a result of previously mentioned changes. Nevertheless, both MFP averages showed good raw milk quality as compared to standard quality limits determined by legislation (< –0.520 °C). The remaining raw milk quality indicators (F, CAS, SCC and SNF) were not influenced (P > 0.05) by typical herd management changes during conversion from C to O farming (Tab. 1).

2) Differences between conventional and organic milk

The recent European regulations on organic dairy farming did not change requirements on the quality of dairy products (34). The more natural dairy management on organic farms is felt to improve animal welfare and animal health. Greater use of pasture versus more intensive dairy system in conventional herds certainly improves animal welfare, but because of the limited drug use in organic systems the animal health is not always enhanced.

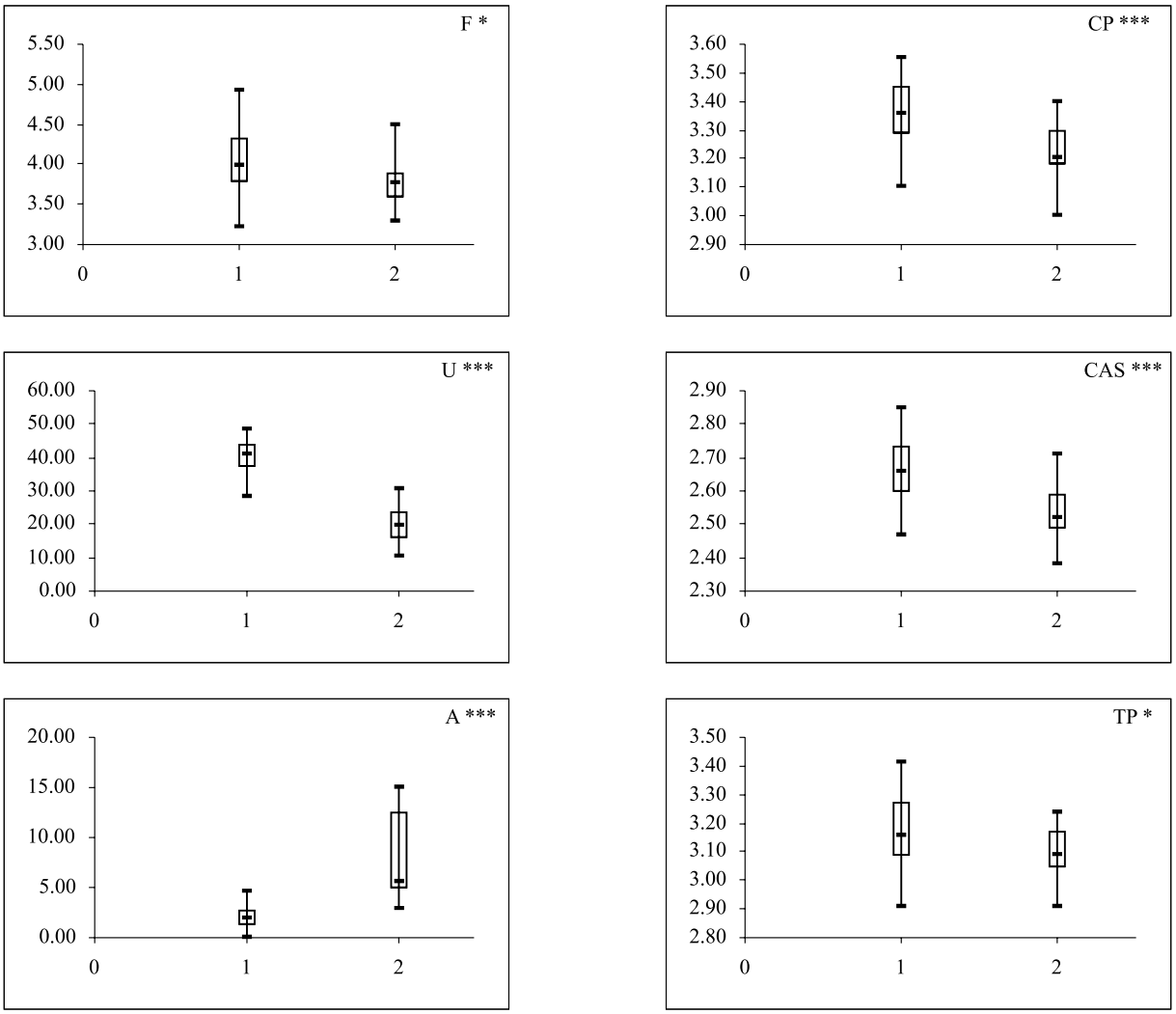
Organic farming systems are more environmentally friendly than conventional management, especially due to lower eutrophication potential of organic herds. The average multiparity percentage was higher in organically managed cows (33). Rozsypal *et al.* (36) were interested in herds which produced milk under organic conditions in the Czech Republic from the point of view of principal characteristics. Louda *et al.* (29) described the effect of conversion from conventional to organic regarding MY and cow reproduction indicators. However, there is a lack of detailed data on milk composition and properties related to conversion.

The C herd milk yield (MY) was 8900 kg and average O herd MY was 7037 ± 422 kg per standard lactation. The significantly lower content of some principal milk components, such as F (3.79 < 4.06 %; Fig. 3), CP, TP (3.09 < 3.17 %; Fig. 3) and CAS (2.54 < 2.66 %; Tab. 2; Fig. 3) were presented for O herds compared to C herd. Also milk specific mass was significantly lower in this context. The average content of L (4.92 > 4.82 %) and WP was significantly higher and that was the reason why SNF content was lower only insignificantly. Also the casein number based on TP was lower (82.18 < 83.83 %; Tab. 2). These facts, together with significantly lower U concentration (19.53 < 40.39 mg.100 ml^{-1}; Fig. 3) and non-protein nitrogen (0.11 < 0.19 %) and approximately four times higher A concentration (6.8 > 1.6 mg.l^{-1}; P < 0.001; Tab. 2; Fig. 3) did not indicate any significant permanent energy and protein deficiency in O dairy cow nutrition. This generally corresponds to previous results (evaluation 1) and former conclusions (16, 17, 18). As the mean CA concentration in O herds was only insignificantly lower (8.45 < 8.76 mmol.l^{-1}, Tab. 2) some measures should be taken to adjust cow nutrition.

In the Denmark experiment on organic farm, reduction in dry matter intake through feed rations and reduction in concentrate in feed from 38 % to 19 % resulted not only in reduction of the milk yield (production of energy corrected milk) but also milk protein content and in simultaneous increase in free fatty acids (37). There were no indications of health problems associated with the reduced feeding levels.

The mean SCC was significantly lower in the C herd (Tab. 2) as compared to O herds, nevertheless the O herds showed SCC still in the category of extra milk quality (< 300 ths.ml^{-1}) and the O values were lower (18) in comparison to C herds (35) in screening conducted throughout the Czech Republic. EC was significantly higher in the C herd (Tab. 2) and MFP differed insignificantly. All RIS investigations in BMSs were negative.

With respect to fodder crops feeding a hypothesis exists (20) that the generally changed regimen of soil fertilization (reduction) in the Czech Republic resulted in ecologization of agricultural production, in particular of milk production (including the conventional (C) milk production) during the last fifteen years. The nutrient load resulting from the use of artificial fertilizers was



Box graph: median (the central short horizontal line); top edge of 1st and 3rd quartile (the tetragon); variation range, maximum – minimum (the vertical line); 1 = C and 2 = O)

Fig. 3. Example of data distribution in sets of F (%), U (mg.100ml^{-1}), A (mg.l^{-1}), CP (%), CAS (%) and TP (%) in conventional (C) herd and organic (O) herds (evaluation 2)

reduced radically. The subsequent change in the ratio of nutrients in soil can influence the mineral composition of green parts of plants and also of milk. Decrease in potassium supply to fodder crops can result in an increase in the magnesium and calcium levels in milk back to the previously encountered level. In this relation the milk Mg level could serve as a potential indicator of ecologization of milk production. This hypothesis has a real basis. Significantly higher mean calcium and magnesium levels were found in organic herd milk (1257 > 1172 mg.kg^{-1} and 112.0 > 107.4 mg.kg^{-1}; 19).

CONCLUSION

The general energy and protein malnutrition of organic dairy herds, indicated by some metabolic milk indicators, should be addressed to by appropriate measures in cow nutrition. The purchase of certified feedstuff dry matter should not be strictly limited (by zero) by the relevant legislation as this can ensure full-value nutrition of organic herds. Nevertheless, the previous results showed that due to low NPK fertilization of fodder the organic milk could be a better source of calcium and magnesium for humans at milk yield of organic cows comparable to that of conventional herd. However, there is still lack of results to be able to draw final conclusions. Additional research should contribute to better understanding and assessment of organic milk benefits.

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THE EFFECT OF GOAT UDDER HEALTH ON COMPOSITION AND PROPERTIES OF RAW MILK

Hanuš, O.[1], Genčurová, V.[2], Vyletělová, M.[1]
Landová, H.[2], Kopecký, J.[1], Jedelská, R.[2]

[1]Research Institute for Cattle Breeding, Rapotín, Výzkumníků 267, 788 13 Vikýřovice
[2]AgriResearch Rapotín, Výzkumníků 267, 788 13 Vikýřovice
The Czech Republic

oto.hanus@vuchs.cz

ABSTRACT

Consumption of goat milk has not been a tradition in the Czech Republic but has increased recently. Goat population also showed some changes. The curd yield and milk quality depend on udder health. It is important to improve knowledge in this area. Sixty samples of bulk milk (4 animals in sample), obtained from goats (White short-haired) in the first two thirds of lactation (as lactation stage) in winter and summer, were investigated. The herd was kept at an altitude of 572 m above sea level with total precipitation of 1 200 mm and mean air temperature 3.7 °C. Somatic cell count (SCC) was 3646 as geometric mean and 4267 ± 2279 ths.ml^{-1}. Significance of relationships between log SCC and other milk indicators were investigated: between log SCC and fat (0.34); lactose (–0.46); non-fat solids (0.25); urea concentration (0.34); rennet coagulation time (0.40); curd quality (–0.26); whey volume (–0.53); specific weight (–0.36); fat/crude protein ratio (0.33); Na (0.50) and Zn (0.25). Higher SCCs were linked with higher fat, non-fat solids and urea level. 11.7 % of log SCC variations were explained by urea variation. It was caused by higher permeability of mammary gland secretory epithelium for blood components during mastitis. Lactose was reduced by higher log SCC in cows (–0.43; P < 0.001) and sheep (–0.58; P < 0.001). 21.1 % of variations in lactose was caused by variations in log SCC in goats. Cheeseability was negatively influenced by deteriorated udder health. With higher log SCC the rennet coagulation time was prolonged, whey volume was reduced (27.6 % of variations in whey volume was explainable by log SCC variations). The relationships between log SCC and the observed milk indicators was insignificant for 26 of them. This was higher ratio (70.3 %) than in cows and sheep (32.4 %). It is possible to improve monitoring and prevention of milk secretion disorders and increase protein yield in goats by means of relevant interpretation of relationship between SCC and other milk indicators.

Key words: chemical composition; electrical conductivity; goat milk; less favourable area; macroelement; microelement; protein; somatic cell count; technological properties; urea

INTRODUCTION

Consumption of goat milk has not been a tradition in the Czech Republic. However, importance of the so-called alternative goat milk in human nutrition has been increasing recently, particularly in groups of people more susceptible to health problems related to specific nutrition and metabolic demands. General goat milk properties were described in detail recently (14, 15).

During the past two decades goat keeping in the Czech Republic underwent dramatic changes. An obvious renaissance of keeping goats for milk was noted in 1995. Goats were kept extensively during both world wars, mostly as small family herds. After the Second World War the goat population was reduced considerably. It increased again twelve years ago (up to 44 993 goats and bucks in 1995) along with increased size of herds and use of modern technology. This occurred due to public recognition of alternative systems and health importance of goat milk. Unfortunately, not long time ago the goat population was reduced again (down to 14 402 heads in 2006). This

is reduction by 68.0 % compared to 1995. The current average goat milk yield in the Czech Republic is 731 (280 lactation days) kg of milk per lactation.

Good health of goat mammary gland is essential for good quality of milk products. After the period of pronounced farming changes it is important to recognize the effect of mammary gland health on composition and properties of milk.

The aim of this paper was to evaluate the impact of udder health on the range of milk quality indicators in goats in the Czech Republic.

MATERIALS AND METHODS

Animals and bulk milk samples

Bulk milk samples (one sample from 4 to 8 animals) were collected from one goat herd (W; White short-haired; n = 60 bulk milk samples). The animals were grouped at random for milk sampling and samples were taken during spring and summer for three years (2005–2007). General climate conditions in the Czech Republic were described in our previous paper (9). The goat herd was kept at an altitude of 572 m above sea level with total precipitation 1 200 mm and mean air temperature 3.7 °C, i. e. under typical climate conditions of submountaineoous area in the Czech Republic – less favourable area. Animals were investigated during the first two thirds of lactation. The goats grazed on natural grass and herb pasture and received grain supplement at a dose of 0.3 kg per head (mixture of barley, maize, wheat and rape seed-oil and mineral components). All animals were in the first half of lactation with milk yield of 1.75 kg per day and were milked twice a day in a milking parlour. The animals were kept in a free stable. Similar investigations were performed recently in cows and sheep (5).

Milk indicators and their determination

Goat milk was analysed by an accredited testing laboratory in Rapotin. The following abbreviations were used for milk indicators: F = fat (g $100\,g^{-1}$, %); L = lactose (monohydrate %); TS = total solids (%); SNF = non-fat solids (%); SCC = somatic cell count (thousand.ml^{-1}); U = urea concentration (mg.$100\,ml^{-1}$); A = acetone concentration (mg.l^{-1}); CA = citric acid concentration (mmol.l^{-1} or %); EC = electrical conductivity (mS.cm^{-1}); MFP = milk freezing point (°C); SW = specific weight (mass; g.cm^{-3}); AS = alcohol stability (ml of 96 % ethanol at milk titration (5 ml) up to visible precipitation); TA = titration acidity (in ml × 2.5 mmol.l^{-1} NaOH solution); pH = active acidity; RCT = rennet coagulation time (second); CF = curd firmness (depth of penetration of plastic stick into a curd cake in mm after its fall under standard conditions - firmness in the contrary sense); CQ = curd quality (grading from 1 = good to 4 = poor); WV = whey volume (in ml; whey released during rennet curd cake formation for 60 minutes); CP = crude protein content (Kjeldahl, total N × 6.38, %); CAS = casein content (casein N × 6.38, %); TP = true protein content (protein N × 6.38, %); WP = whey protein content (difference TP-CAS, %); NPN = non-protein nitrogen (CP nitrogen-TP nitrogen × 6.38, %); UNR = urea N/non-protein N ratio in %; F/CP = fat/crude protein ratio; the casein numbers were calculated on the basis of CP and TP = CAS-CP and CAS-TP in %; macroelements, such as Ca, P, Na, K and Mg (in mg.kg^{-1}); microelements, such as I (in μg.l^{-1}) and Mn, Fe, Cu, Zn and Ni (in mg.kg^{-1}); residues of inhibitory substances in milk = antibiotic drugs (positive, negative).

The investigated milk indicators, such as lactose, total solids and non-fat solids, were measured by a MilkoScan 133B instrument (Foss Electric, Denmark), which was regularly calibrated according to the reference method results (in %). F was measured by Gerber's method (in %). Nitrogen protein fractions, such as CP, TP and CAS, were determined by a reference Kjeldahl's method (in %) using an instrument of Tecator line with Kjeltec Auto Distillation unit 2200 (Foss-Tecator AB, Sweden). SCC was determined by Fossomatic 90 equipment (Foss Electric, Denmark).

Occurrence of residues of inhibitory substances was investigated by microbiological Delvo-test with *Bacillus stearothermophillus* var. *calidolactis*. The milk samples were analysed for milk freezing point values by a cryoscopic instrument Cry-Star automatic Funke-Gerber (Germany), measurement mode Plateau Search. It was calibrated by standard NaCl solutions (Funke-Gerber). Electrical conductivity was measured by a conductometer Radelkis OK 102/1 with glass bell electrode and standard NaCl solution for calibration. The milk urea concentration was determined spectrophotometrically at 420 nm using p-dimethylaminobenzaldehyde. Specol 11 instrument (Carl Zeiss Jena, Germany) was calibrated on a scale from 1 to 10 mmol.l^{-1}. The milk acetone content was measured spectrophotometrically at 485 nm using alkaline solution (KOH) with salicylaldehyde for absorption (24 hours of microdiffusion at 20 °C in the darkness). The Specol 11 was calibrated on a scale from 1 to 20 mg l^{-1}. The milk citric acid concentration was determined by spectrophotometric measurement at 428 nm. Milk was coagulated by trichloracetic acid and the filtrate was allowed to react with pyridine and acetanhydride (30 min. at 32 °C). CA generates a yellow-coloured complex in acetanhydride medium. The Specol 11 was calibrated from 1.5 to 20.0 mmol l^{-1}. The pH was measured by a pH-meter CyberScan 510 (Eutech Instruments, Netherlands) at 20 °C. It was calibrated by standard buffer solutions (pH 4.0 and 7.0 Hamilton Duracal Buffer, Switzerland) at each sample set measurement. The TA was determined by titration of milk (100 ml) with alkaline solution to a light pink colour.

The macro- and microelement milk concentrations (except P) were determined (after mineralization) by atomic absorption spectroscopy using a Spectrometer Solaar S4 (Thermoelemental, England). The P content was determined as molybdenum-blue (with ammonium, ascorbic and sulphuric acid). Specol 11 was calibrated on a scale from 2 to 20 mg.l^{-1} (at 750 nm).

Statistical processing of results included calculation of basic statistical parameters, regression analysis and correlation coefficients using Excel software.

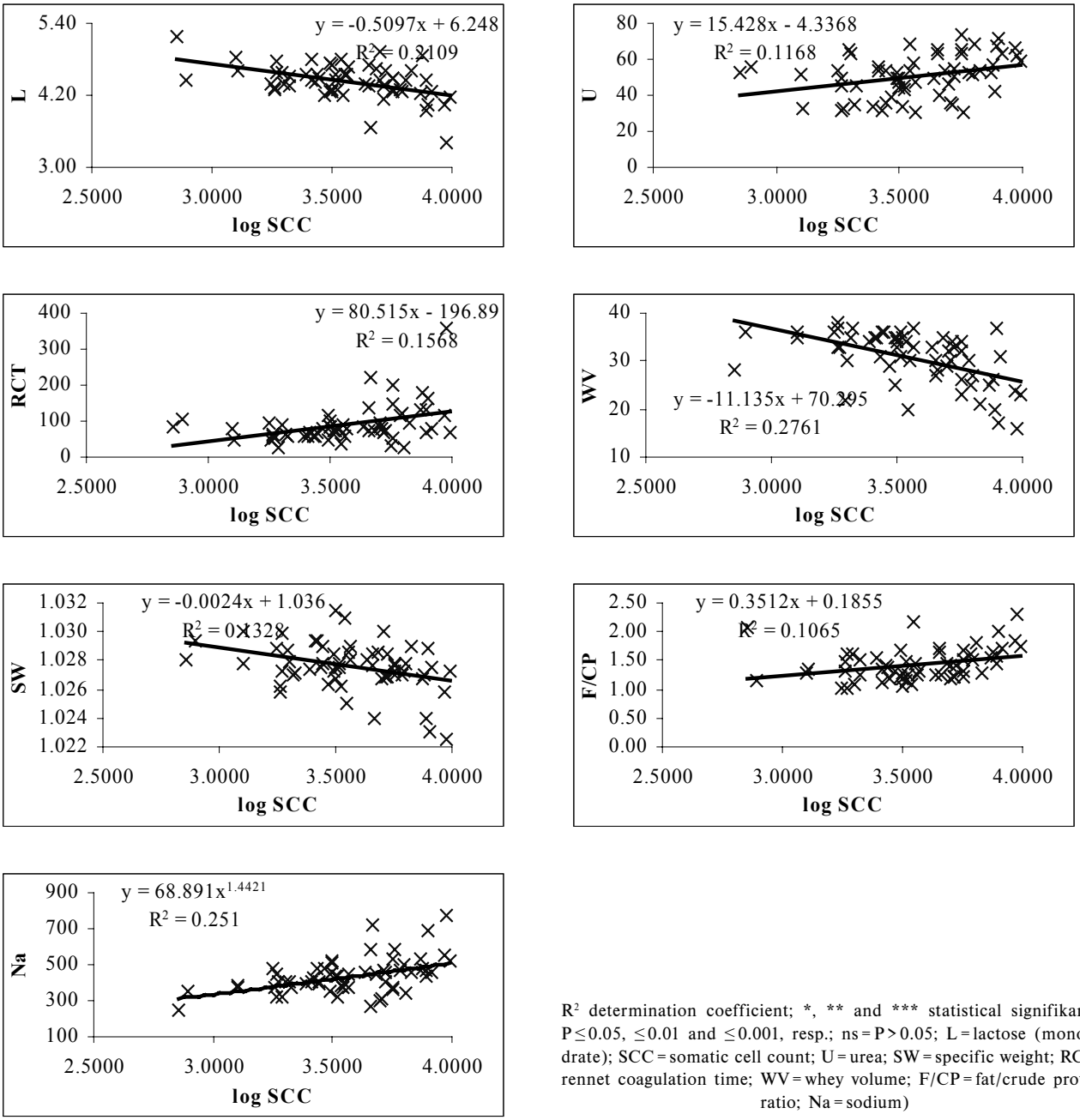
RESULTS AND DISCUSSION

Basic relevant statistical characteristic of goat milk indicators were shown in previous papers (5, 9). In a short survey the somatic cell count was 3646 as geometric mean and 4267 ± 2279 ths.ml^{-1}. These were higher than

Table 1. Regressions and correlations between log SCC and other milk indicators in goats

Milk indicator	Regression equation	R^2	r	Significance
F	y = 1.2563x + 0.1009	0.1131	0.34	**
L	y = -0.5097x + 6.248	0.2109	−0.46	**
SNF	$y = 9.9304e^{-0.0528x}$	0.0646	0.25	*
TS	y = 0.8373x + 9.8341	0.0395	0.20	ns
U	y = 15.428x - 4.3368	0.1168	0.34	**
A	y = 1.2513x + 1.6386	0.0025	0.05	ns
AS	y = -0.1054x + 1.1234	0.0071	−0.08	ns
TA	y = −1.4546x + 12.833	0.0467	−0.22	ns
EC	y = 0.1913x + 4.986	0.0155	0.12	ns
pH	y = −0.1885x + 7.2491	0.0234	−0.15	ns
MFP	y = −0.0215x - 0.4778	0.0360	−0.19	ns
RCT	y = 80.515x - 196.89	0.1568	0.40	**
CQ	y = −0.594x + 5.6492	0.0667	−0.26	*
CFV	y = 0.0219x + 1.7771	0.0091	0.10	ns
WV	y = −11.135x + 70.295	0.2761	−0.53	***
SW	y = −0.0024x + 1.036	0.1328	−0.36	**
CP	y = 0.0739x + 2.9214	0.0061	0.08	ns
CAS	y = −0.0191x + 2.4734	0.0005	−0.02	ns
TP	y = 0.0229x + 2.8272	0.0008	0.03	ns
WP	y = 0.044x + 0.346	0.0107	0.10	ns
NPN	y = 0.0501x + 0.0988	0.0416	0.20	ns
UNR	y = 6.2729x + 31.606	0.0250	0.16	ns
F/CP	y = 0.3512x + 0.1855	0.1065	0.33	*
CAS-CP	y = −2.6641x + 84.989	0.0415	−0.20	ns
CAS-TP	y = −1.6307x + 88.484	0.0121	−0.11	ns
CA	y = −0.9274x + 10.455	0.0203	−0.14	ns
Ca	y = 26.84x + 1128.3	0.0030	0.05	ns
P	y = −71.138x + 1296	0.0353	−0.19	ns
Na	$y = 68.891x^{1.4421}$	0.2510	0.50	***
Mg	y = 3.017x + 120.37	0.0029	0.05	ns
K	y = −172.88x + 2629.5	0.0487	−0.22	ns
I	y = −46.254x + 291.05	0.0520	−0.23	ns
Mn	y = −0.0168x + 0.1184	0.0091	−0.10	ns
Fe	$y = 0.4406e^{0.1519x}$	0.0074	0.09	ns
Cu	y = −0.0633x + 0.3284	0.0455	−0.21	ns
Zn	y = −2.4493Ln(x) + 7.4043	0.0643	−0.25	*
Ni	y = −0.0053x + 0.045	0.0712	−0.27	ns

R^2 determination coefficient; r correlation coefficient; *, ** and *** statistical significance $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, resp.; ns = $P > 0.05$; F = fat; L = lactose (monohydrate); TS = total solids; SNF = non-fat solids; SCC = somatic cell count; U = urea concentration; A = acetone concentration; CA = citric acid concentration; EC = electrical conductivity; MFP = milk freezing point; SW = specific weight (mass); AS = alcohol stability; TA = titration acidity; pH = active acidity; RCT = rennet coagulation time; CF = curds firmness; CQ = curds quality; WV = whey volume; CP = crude protein; CAS = casein; TP = true protein; WP = whey protein; NPN = non- protein nitrogen; UNR = urea nitrogen ratio in non protein N; F/CP = fat/crude protein ratio; casein numbers CAS-CP and CAS-TP; macroelements Ca, P, Na, K and Mg; microelements I, Mn, Fe, Cu, Zn and Ni



R^2 determination coefficient; *, ** and *** statistical signifikance: $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, resp.; ns = $P > 0.05$; L = lactose (monohydrate); SCC = somatic cell count; U = urea; SW = specific weight; RCT = rennet coagulation time; WV = whey volume; F/CP = fat/crude protein ratio; Na = sodium)

Fig. 1. Relationships between log SCC (SCC in ths ml^{-1}) and lactose (%) content, urea (mg 100 ml^{-1}) concentration, rennet coagulation time (s), whey volume (whey volume; ml), specific weight (g.cm^{-3}), fat/crude protein ratio and Na (mg.kg^{-1}) in goat milk

those stated by some other authors (3, 6, 17) although their values were also higher in comparison to cow milk standard (400 ths.ml^{-1}). These SCCs were periodically higher than 1000 ths.ml^{-1}, even in non-infected goats (17) and in another case (3) 1320 ths.ml^{-1} was reported as a geometric mean. The mineral contents were comparable to those mentioned by other authors (1, 2, 12). For instance, the average Ca content 1223.9 ± 125.8 mg. kg^{-1} was markedly higher than that mentioned for goats by Kuchtík and Sedláčková (13), 930 ± 192 mg l^{-1}. Antunac *et al.* (1, 2) and Hejtmánková *et al.* (11) reported Ca levels between 1100 and 1270 mg.kg^{-1} (Alpine and Saanen goats) and between 1100 and 1740 mg.kg^{-1} according to season from April (maximum) to October (White short-haired) in the Czech Republic.

The relationships between log SCC and other milk indicators in goats are summarized in Table 1. They were determined between log SCC, as indicator of udder health, and the following: fat (0.34); lactose (–0.46); non-fat solids (0.25); urea (0.34); rennet coagulation time (0.40); curd quality (–0.26); whey volume (–0.53); specific weight (–0.36); fat/crude protein (0.33); Na

(0.50); Zn (0.25). Higher SCCs were linked with higher fat, non-fat solids and urea content. Similar relationship for urea was observed also in sheep (10). 11.7 % of log SCC variations could be explained by variations in urea concentration. Urea increased significantly along the log SCC scale (0.34; $P < 0.01$; Tab. 1; Fig. 1). This could have a physiological background due to higher permeability of mammary gland secretory epithelium for blood components during mastitis disorders. In general, milk urea and acetone concentrations are approximately two times higher in goats and ewes than in cows (5, 9, 13, 14).

Lactose level in goat milk was reduced by subclinical and clinical mastitis and in this respect by higher log SCC value (–0.46; $P < 0.01$; Tab. 1; Fig. 1) similarly to cows (–0.43; $P < 0.001$) and sheep (–0.58; $P < 0.001$; 10). This finding was confirmed by Hanuš *et al.* (8), Gajdůšek *et al.* (4) and Strzalkowska *et al.* (16). This means that 21.1 % of variability in L content was caused by variability in log SCC in goats. Previously (7) a correlation equal to –0.28 ($P < 0.01$) was reported.

Regular measurement and combination of values of the mentioned indicators in milk could improve the control and prevention of occurrence of milk secretory disorders in goat herds. Cheeseability was negatively influenced by poor udder health. At higher log SCC the rennet coagulation time was longer and whey volume was reduced (27.6 % of variations in whey volume was explainable by variations in log SCC). The significant relationship between log SCC and fat/crude protein ratio (0.33; $P < 0.05$) is similar to that in sheep milk (0.45; $P < 0.01$; 10). It means that 10.7 % of variations in F/CP ratio were caused by variations in log SCC. Higher urea and F/CP ratio could mean lower energy level in rations and higher risk of ketosis. Higher occurrence of secretory disorders (higher log SCC) could be linked to lower maintenance energy of goats. Deteriorated mammary gland health, i.e. higher SCC, was logically linked to increased Na and decreased Zn. For instance, Gajdůšek *et al.* (4) failed to observe significant relationship between SCC and Na (0.07).

We observed a number of insignificant ($P > 0.05$) relationships between log SCC as udder health indicator and other milk indicators (components and properties): total solids; acetone concentration; alcohol stability; titration acidity, electrical conductivity; pH; milk freezing point; curd firmness; crude protein; casein; true protein; whey protein; non-protein nitrogen; UNR; CAS-CP; CAS-TP; citric acid concentration; Ca; P; Mg; K; I; Mn; Fe; Cu; Ni (Tab. 1; 26 indicators). Such results were rather surprising. It is an opposite situation in comparison with sheep (10) and cow milk.

There were also markedly significant relationships between SCC and other milk indicators (components and properties). In similar evaluation concerning sheep only 32.4 % of investigated milk indicators was independent (dependent insignificantly) on SCC while our data in goats showed insignificant relationship in 70.3 %. The correlation between SCC and other milk indicators was not as strong in goats as in sheep or cows. Very surprising were the results obtained for nitrogen (protein) components in milk, such as crude protein, casein, true protein, and whey protein and also electrical conductivity and milk freezing point. In general, it is possible that such an unexpectedly low degree of dependence of milk indicators on SCC (log SCC), i.e. on udder health, could be caused and also explained by generally higher SCCs in goat milk samples, determined in our study, and also by higher SCCs in comparison to sheep and particularly to cows (5).

CONCLUSION

Routine analytical systems of milk quality and composition monitoring, interpretation and subsequent prevention of production disorders are not as sophisticated in milked goats and ewes as in cows. Results obtained in our study may contribute to explanation of some selected relationships between goat milk indicators and support the practical interpretation of results obtained by monitoring of the quality and composition of milk. Regular measurement and combination of values and relationships of the mentioned milk indicators could improve the control and prevention of milk secretory disorders in goat herds. Cheeseability was influenced negatively by impaired udder health. However, the relationships between somatic cell count and other milk indicators were less pronounced in goats in comparison to sheep or cows. This is a rather disadvantageous for practical monitoring. It is possible to improve the rules for monitoring and prevention of milk secretory disorders and to improve also protein yield and cheesemaking in goats by means of relevant interpretation of the relationship between SCC and other milk quality indicators.

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A COMPARISON OF SELECTED MILK INDICATORS IN ORGANIC HERDS WITH CONVENTIONAL HERD AS REFERENCE

Hanuš, O.[1], Vorlíček, Z.[2], Sojková, K.[1], Rozsypal, R.[3], Vyletělová, M.[1] Roubal, P.[4], Genčurová, V.[1], Pozdíšek, J.[1], Landová, H.[1]

[1]Research Institute for Cattle Breeding Rapotín, Výzkumníků 267, 788 13 Vikýřovice
[2]Forage Crops Research Institute Troubsko, Zahradní 1, 664 41 Troubsko
[3]EPOS, Brno, V. Nezvala 977, 675 71 Náměšť nad Oslavou
[4]Dairy Research Institute Prague, Ke Dvoru 12a, 160 00, Praha 6
The Czech Republic

oto.hanus@vuchs.cz

ABSTRACT

In a historical sense, current organic farming is an old-new alternative under changed world conditions. Organic dairying (O) is an alternative of friendly use of the environment in time of presupposed global climate changes. Potential impact of organic farming on raw cow-milk quality, composition and properties, as compared to conventional milk production (C), were evaluated in this paper on the basis of selected milk indicators (MIs). Total solids, whey volume, pH of milk fermentation ability (FAM-pH), FAM streptococci, FAM noble lactic acid bacteria, I and Cu were higher in C milk ($P < 0.05$). The alcohol stability (AS), titration acidity, curd firmness (CF), FAM titration acidity (FAM-T), Ca, P, Mg, K and Fe were higher in O milk ($P < 0.05$). No differences ($P > 0.05$) were observed in pH, rennet coagulation time, curd quality, FAM lactobacilli and streptococci/lactobacilli, Na, Mn and Zn. In general, the differences were a little more advantageous for O milk from both technological and nutritional point of view, particularly because of AS (0.46 < 0.58 ml, C vs. O), CF (1.88 > 1.81 mm), FAM-T (27.3 < 33.8 ml of 0.25 mol.l^{-1} NaOH.100ml^{-1}), FAM-pH (5.1 > 4.6), Ca (1172 < 1257 mg.kg^{-1}), P (950 < 1004 mg.kg^{-1}) and Mg (107.4 < 112.0 mg.kg^{-1}) results. Organic milk can also produce better environment for yoghurt fermentation. Nevertheless, the results obtained should not be overestimated as both sources produced milk of good quality. Additional results are needed to prove organic milk benefits.

Key words: conventional farming; cow; environment; Holstein; macroelement; microelement; milk yield; organic farming; technological property

INTRODUCTION

In a historical sense, current organic farming is an old-new alternative under changed world conditions. The importance of organic farming in less favourable areas was described by Šarapatka *et al.* (18). Organic dairying (O) is an alternative ofr friendly use of the environment in time of the presupposed global climate changes (1, 2, 3, 4, 5, 6, 13, 14, 15, 19). There is a lack of information about quality of raw milk from organic farms. One opinion is that milk quality has not changed under environmentally friendly, natural dairy management on organic farms (16). It has been felt that organic farming improves animal welfare and health.

Hlásný (10, 11) predicted an increase in milk Mg content (also milk Ca and Na concentrations) up to the former level due to general decrease in artificial NPK soil fertilization under conditions in the Czech Republic (CR) resulting in relevant changes in mineral composition of fodder crops. In this way he identified the higher milk Mg content as a potential indicator of higher ecology level in agriculture. This hypothesis was derived from the well known antagonistic relationships between K soil fertilization (if higher), consecutive Mg (also Ca and Na) level (lower) in cultural plants (roughage) and cow feed rations and possible transfer of Mg ions into milk.

The present study focused on evaluation of potential impact of organic farming on raw cow-milk quality, composition and properties as compared to the current conventional milk production.

MATERIALS AND METHODS

The altitude of investigated farms was 361.5 ± 121.2 m for O herds and 257 m for C herd. Bulk milk samples (BMSs; each from eight dairy cows) were obtained regularly every two weeks in winter and spring seasons of one year from one conventional (C) Holstein (H) herd (150 cows; n = 36 BMSs, 8 cows in one BMS) and two times in winter and summer seasons of one year from four organic dairy herds (O; n = 16 whole BMSs). The number of cows (H) in the four investigated O herds ranged between 25 and 400. The cows were fed in a way typical of O herds under the CR conditions (alfalfa, clover and grass silage, hay, concentrate and mineral mixtures in the form of total mixed rations (TMR) according to relevant milk yield (MY) and standard demands). The O herds were kept in free stables and grazed during summer as it is stipulated by law on organic farming in the CR. The C herd was kept in free stable and fed TMR throughout the year (maize, alfalfa, clover and grass silage, hay, concentrate and mineral mixtures according to relevant milk yield (MY) and standard demands). All cows were milked twice a day. Other farm characteristic about MYs and herds were recorded as well.

Milk analyses were performed regularly by an accredited testing laboratory in Rapotin (n. 1340, EN ISO 17025, Accreditation certificate No. 124/2004) according to standard operation manuals currently in force. The following abbreviations and units were used for the investigated milk indicators: TS = total solids (%); AS = alcohol stability (ml, consumption of 96 % ethanol for protein coagulation in 5 ml of milk); TA = titration acidity according to Soxhlet-Henkel (ml 0.25 mol.l^{-1} NaOH solution for the titration of 100 ml of milk); pH = actual milk acidity; RCT = rennet coagulation time (second); CQ = subjective estimation of curd cake quality determined by examination and palpation and graded from 1 (excellent) to 4 (poor); CF = cheese curd firmness = depth of penetration of the corpuscle falling into curd cake in a standard way, the value is the opposite to firmness (mm); WV = whey volume, obtained during the process of enzymatic cheesemaking from the curd cake (ml); FAM-T = fermentation ability of milk, i. e. the yoghurt test (incubation with thermophilic yoghurt culture YC-180-40-FLEX = *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *L. d.* subsp. *bulgaricus* at 43 °C for 4 hours) with microbial culture (by the titration of yoghurt acidity in ml of 0.25 mol.l^{-1} NaOH.100 ml^{-1}); FAM-pH (actual acidity of yoghurt, pH); FAM-LAB (noble lactic acid bacteria in CFU.ml^{-1}; CFUs were counted on plates with GTK M (Milcom Tábor) agar with glucose monohydrate, triptone-peptone, dehydrated yeast extract and skim milk powder after conventional incubation at 30 °C for 72 hours); FAM-L (lactobacilli in CFU.ml^{-1}); FAM-S (streptococci in CFU.ml^{-1}); FAM-RSL (ratio streptococci/lactobacilli), all the previous indicators for FAM were measured after the yoghurt test fermentation; residues of inhibitory substances (+/–; by microbiological Delvo-test, mostly antibiotic drugs); Ca, P, Na, Mg, K and I, Mn, Fe, Cu and Zn as milk macro- and microelements were expressed in mg.kg^{-1} (with the exception of I = μg.l^{-1}).

TS was determined by means of MilkoScan 130 B (Foss Electric, Denmark) with regular calibration according to relevant reference method results. Active acidity was measured by a pH-meter CyberScan 510 (Eutech Instruments) at 20 °C. The instrument was regularly calibrated by standard buffer solutions (pH 4.0 and 7.0 Hamilton Duracal Buffer, Switzerland) for measurement of each milk sample set. Chemical elements were determined by atomic absorption spectrophotometer SOLAAR S4 plus GFS97 (Graphite Furnace). Milk iodine (I) concentration was determined photometrically at 420 nm using alkaline mineralization (KOH), brucine (modified Sandell-Kolthoff reaction) and an apparatus Spekol 11 (Carl Zeiss, Jena, Germany).

Statistical processing of data included determination of basic statistical parameters and testing of differences. C milk results were used as a reference for comparison with O milk. To ensure more accurate statistical evaluation, the milk quality indicators with abnormal frequency distribution of values were transformed logarithmically.

RESULTS AND DISCUSSION

The MY of the C herd was 8 900 kg and average MY of O herds was 7037 ± 422 kg per lactation. The C herd milk yield was higher by 26.5 %. The MY was higher by 17 % in the C herds in the Czech Republic (17). All Delvo-test results were negative, i. e. investigation of milk technological properties was carried out on milk samples free of residues of inhibitory substances (mostly antibiotics) which is important for validation of results of fermentation tests.

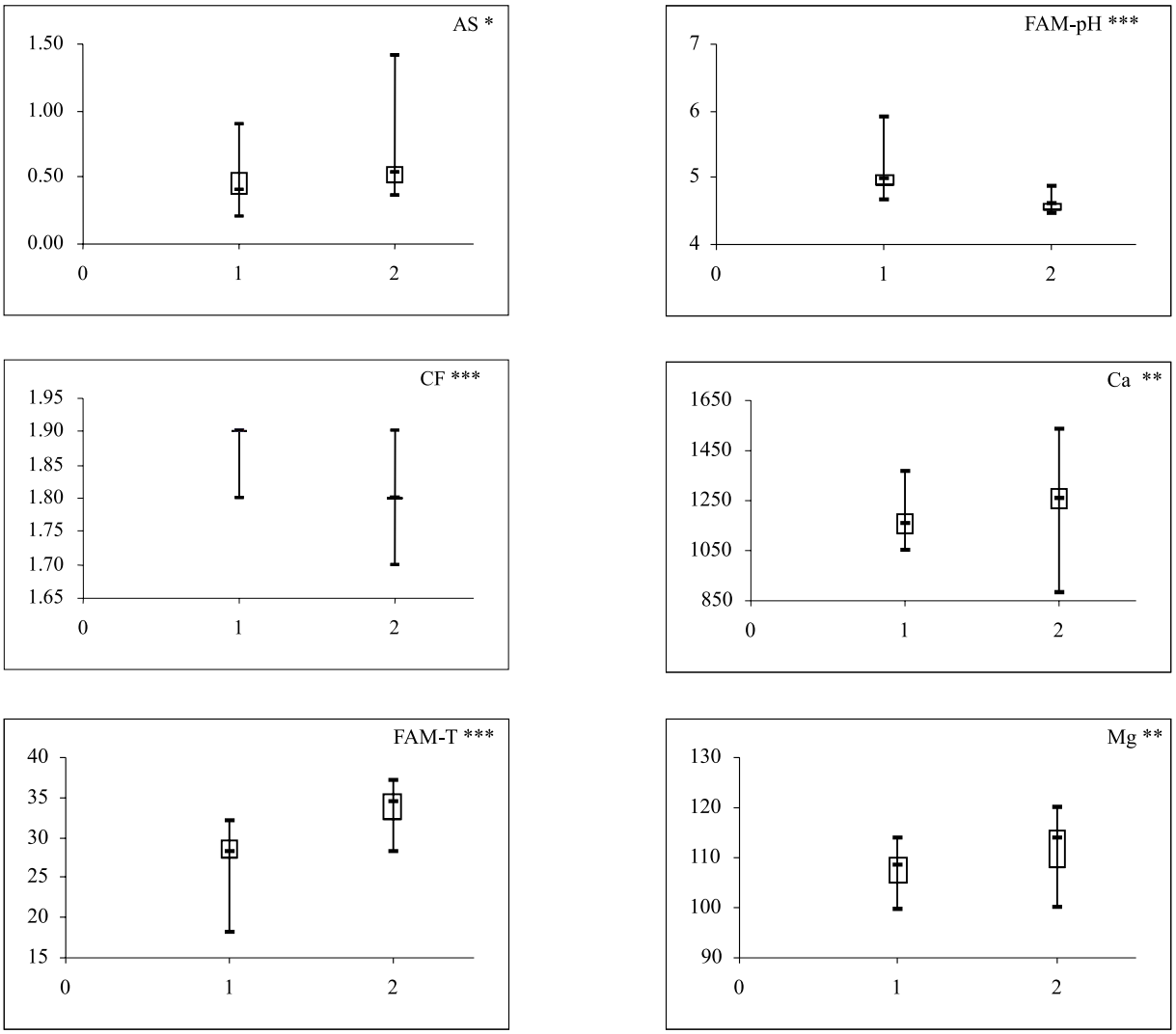
Table 1 shows that TS, WV, FAM-pH, FAM-S, FAM-LAB, I and Cu were significantly higher in C milk. On the other hand AS (Fig. 1), TA, CF, FAM-T, Ca, P, Mg, K and Fe were significantly higher in O milk. There were no significant differences in pH, RCT, CQ, FAM-L, FAM-RSL, Na, Mn and Zn between C and O milk. Overall, the differences mentioned were slightly more advantageous for O milk from both technological and nutritional point of view, particularly because of AS (0.46 < 0.58 ml, C vs. O), CF (1.88 > 1.81 mm), FAM-T (27.3 < 33.8 ml of 0.25 mol.l^{-1} NaOH.100ml^{-1}), FAM-pH (5.1 > 4.6), Ca (1172 < 1257 mg.kg^{-1}), P (950 < 1004 mg.kg^{-1}) and Mg (107.4 < 112.0 mg.kg^{-1}) results. The difference in iodine (Tab. 1) could be ascribed to regular use of iodine disinfectant for the treatment of teats on C farms to prevent mastitis. Such treatment is less frequent in O herds.

The results (Tab. 1 and Fig. 1) also showed that with regard to FAM, O milk could produce slightly better environment for yoghurt fermentation than C milk because of acidity results (FAM-pH (4.60 < 5.08; P < 0.001) and FAM-T (33.76 > 27.27 ml; P < 0.001)) despite the fact that counts of noble bacteria (FAM-L, FAM-S and FAM-LAB) were a little lower. It could be related to higher metabolic activity under the mentioned conditions. However, also the basic initial TA was higher in O milk, so the obtained results should not be overestimated. Nevertheless, in terms of basic quality, both sources of milk were good.

Table 1. Differences in cow milk mineral composition and properties between conventional (C) herd and organic (O) herds

MI	Unit	C			O			sign.
		x	sd	vx	x	sd	vx	
TS	%	12.83	0.472	3.7	12.49	0.293	2.3	*
AS	ml	0.46	0.169	36.7	0.58	0.240	41.4	*
TA	ml	7.17	0.521	7.3	8.71	0.476	5.5	***
pH		6.70	0.052	0.8	6.68	0.028	0.4	Ns
RCT	sec.	177	33.739	19.1	163	37.725	23.1	Ns
CQ	class	2.81	0.786	28.0	2.63	0.806	30.6	Ns
CF	mm	1.88	0.038	2.0	1.81	0.057	3.1	***
WV	ml	32.78	1.758	5.4	30.38	4.870	16.0	*
FAM-T	ml	27.27	4.000	14.7	33.76	2.303	6.8	***
FAM-pH		5.08	0.331	6.5	4.60	0.118	2.6	***
FAM-L	$CFU.ml^{-1}$	43702778	24120785	55.2	32437500	15248907	47.0	Ns
Log		7.5524	0.3139		7.4674	0.2022		Ns
FAM-S	$CFU.ml^{-1}$	1345277778	961417068	71.5	718125000	235633013	32.8	*
Log		9.0303	0.3052		8.8289	0.1689		*
FAM-LAB	$CFU.ml^{-1}$	1388980556	979634387	70.5	750562500	242431837	32.3	*
Log		9.046	0.3032		8.8492	0.165		*
FAM-RSL		33.0771	13.828	41.8	25.8927	14.510	56.0	Ns
Ca	$mg.kg^{-1}$	1172.01	81.813	7.0	1257.25	138.502	11.0	**
P	$mg.kg^{-1}$	950.06	55.142	5.8	1004.44	74.138	7.4	**
Na	$mg.kg^{-1}$	452.97	30.013	6.6	467.06	75.834	16.2	Ns
Mg	$mg.kg^{-1}$	107.41	3.470	3.2	112	5.955	5.3	**
K	$mg.kg^{-1}$	1563.45	52.669	3.4	1682.56	58.516	3.5	***
I	$\mu g.l^{-1}$	462.84	103.916	22.5	174.31	116.274	66.7	***
Mn	$mg.kg^{-1}$	0.02	0.005	25.0	0.02	0.007	35.0	Ns
Fe	$mg.kg^{-1}$	0.15	0.070	46.7	0.27	0.206	76.3	**
Cu	$mg.kg^{-1}$	0.08	0.023	28.8	0.06	0.008	13.3	**
Zn	$mg.kg^{-1}$	4.20	0.616	14.7	3.86	0.583	15.1	Ns

MI – milk indicator; x – arithmetical mean; sd = standard deviation; vx = variation coefficient (in %); statistical significance: *, **, ***, ns = $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, $P > 0.05$, resp.; TS = total solids; AS = alcohol stability; TA = titration acidity; pH = milk acidity; RCT = rennet coagulation time; CQ = curds cake quality; CF = curd firmness; WV = whey volume; FAM-T = fermentation ability of milk by titration; FAM-pH (at determined acidity); FAM-LAB (noble lactic acid bacteria); FAM-L (lactobacilli count); FAM-S (streptococci count); FAM-RSL (ratio streptococci/lactobacilli)



Box graph: median (the central short horizontal line); top edge of 1st and 3rd quartile (the tetragon); variation range, maximum – minimum (the vertical line); AS = alcohol stability; CF = curd firmness; FAM-T = fermentation ability of milk by titration; FAM-pH (at determined acidity))

Fig. 1. Example of data distribution in sets of AS, CF, FAM-T, FAM-pH, Ca and Mg in conventional (1) herd and organic (2) herds

The results mentioned above (increase in Ca and Mg) are in agreement with our previous results (7, 8, 9) and more or less (Mg increase) with the results reported by Janů *et al.* (12). Hlásný (10, 11) mentioned higher Mg content in milk in the period with lower use of mineral NPK fertilization in the fifties of the past century. Between 1980 and 1989 the level of NPK fertilization was relatively high in the CR (1986–1989, 230.4 kg of mineral nutrients per hectare without natural organic fertilizers) and was comparable or a little lower compared to the present level in many developed countries. After 1989 the mineral NPK fertilization in CR was reduced dramatically (by 68.3 %) due to economic reasons (1991–2000, 73.1 kg). Also the current application of NPK artificial fertilizers on O farms (0 kg according to relevant law on organic farming) is completely different as compared to C farms (2005–2006, 95.6 kg). The results obtained showed that the system of rearing of dairy cows can influence significantly milk mineral composition and properties and that the above mentioned hypothesis (10, 11) about possible mineral composition change (increase in milk Mg and Ca) stands on a real basis.

Another explanation of increased content of Ca could be related to the fact that organic dairy cows spend more time on pasture exposed to solar radiation as compared to C herds in cowsheds. Thus organic cows may utilize Ca from feedstuffs more effectively than dairy cows in conventional herds with respect to physiological presumption of better calciferol production.

CONCLUSIONS

The results obtained showed that organic milk could be a slightly better source of calcium, phosphorus and magnesium in human nutrition at comparable milk yield of dairy cows and low NPK soil fertilization, but also inferior source of iodine as compared to conventional milk. Organic milk can also produce slightly better environment for yoghurt fermentation. In general, the changes detected in organic milk were more advantageous for human nutrition and dairy technology. Nevertheless, there is still lack of results which prevents us to draw final conclusions and therefore the results presented should not be overestimated. Both sources produced milk of good quality. More research is needed to asses unambiguously organic milk benefits.

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ANIMAL HAIR FORENSIC ANALYSIS

Takáčová, D., Buleca Jr., J., Nagyová, A.

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

takacova@uvm.sk

ABSTRACT

Forensic veterinary medicine is a science based on specialized ways and methods of ascertaining, investigating and evaluating the evidence of veterinary character which can be used by bodies involved in criminal proceedings, by courts in civil legal proceedings or by bodies of specialized state administration. Forensic veterinary medicine is based on detection of traces and ascertaining of information encoded in material traces as a result of interactions of various influences on scrutinized objects in concrete environment and time. The potential trace can also be an animal hair which can help to clear up the case.

Key words: analysis; animal; hair; methods

INTRODUCTION

According to the Code of criminal procedure, everything that can contribute to reasonable explication of a case provided it was obtained by legal manner is considered a reliable evidence. Real evidence are various things, such as parts of cadavers, feedstuffs, saliva, animal hair, etc. The veterinary expertise of such real evidence is done by specialised laboratories with participation of various experts. Procured evidence is applicable in contravention of the law as well, particularly in connection with animal cruelty and animal protection thereupon it is jurisdiction subject matter of state veterinary administration.

Veterinary forensic medicine plays a vital role in the enforcement of wildlife laws and laws pertaining to domestic animals (e.g. acts of cruelty). The examination of hair for individual identification is less eligible in the forensic veterinary medicine than in the human forensic medicine. The hair examination process involves many different steps, the first of which is to determine whether the hair originates from an animal or a human being. The purpose of examination is to ascertain which individual or individuals could have come into contact with an object (e.g. claim compensation in the case of animal destroying: an analysis has proved that inflicted injuries were made by fox breaching the place and not by a dog; or poaching cases).

ANIMAL HAIR IDENTIFICATION METHODS

Macroscopic forensic analysis

There are two distinct parts of a hair, hair shaft and hair bulb set in the skin. Hairs on different areas of the body of an animal can vary widely in colour and length. The animal hair can be confounded with fibres; there is a resemblance among human hair and anthropoid apes hairs, horse short hairs and goat hairs (1). Regular placing of animal hair is natural for horses and cattle. Dog hairs are ordered into bundle. Sheep and goat hairs are ordered in characteristic groups. Hog bristles have a special order.

Microscopic forensic analysis

Animal hairs can be identified by light microscopy. The easiest way to differentiate between hairs and fibres is examination under a light microscope. For preparation of slides, dry mount and wet mount are implemented. The dry mount may be useful before the wet mounting to study the exterior

texture and the overall colour of the hair. The wet mount is essential in hair analysis because of the refractive index of Canada balsam (close to that of the keratin in the hair). Wet mount provides information about interior structure of the hair (inclusions and granules).

A collection of a suitable known animal hair standard is necessary before a meaningful comparison can be conducted. Hairs should be collected from each area of an animal, because they can vary widely.

A strand of hair has 3 layers: cuticle, cortex and medulla. Cuticle consists of overlapping scales of extinct keratin cells and its function is to protect the cortex. Cortex is bellow cuticle, it is comprised of spindle-shaped cells that give the hair their texture and house pigment that gives hair its distinct colour. Medulla is in the middle of the hair, during the growth period coursing the whole hair up to the cortex. It consists of one or more cell rows of cubical or stratified shape. After degeneration of cells they are filled with air. Depending on hair the hollow medulla is continuous or fragmented.

Forensic science investigators determine the medullar index of hair which is the diameter of the medulla relative to the diameter of the hair, expressed as a fraction (6).

$$\text{Medullar index} = \frac{\text{Diameter of medulla}}{\text{Diameter of hair}}$$

Humans have a medullar index of less than 1/3, and the medullar index of animals is 1/2 or greater. Animals may have hairs with different scale of medulla and cortex on various areas of the body. Differences could be observed between summer and winter coats and between species and breeds of animals. Contrary to bristles, animal moustache has thicker cortex and narrow medulla.

Although certain hairs can be attributed to certain species, it is not possible to identify hairs as belonging to a specific animal to the exclusion of other similar animals. An example of this occurs when dog hairs can be associated with a particular breed but cannot be identified as hair of specific dog within that breed (2).

GENETIC ANALYSIS

DNA analysis

Identity test is one of the possible application of DNA analysis with the result of individual identification of the originator of the biological traces and can conduct the investigation to the exclusion of litigable trace (red herring). Kalbe *et al.* (4) confirmed, that DNA can be obtained also from animal hair. Animal hair fibres are constructed from cellular material. In the living cells of animals DNA carries the hereditary information. Nuclei contained within immature hair follicles can be directly visualised using the technique of in situ DNA hybridisation (3). It is well known, that human hair shafts contain minute amounts of genomic DNA and detectable mtDNA. Due to the low quantities of DNA and the bad quality of hair roots or the loss of roots, forensic scientists have focused on the application of more efficient DNA extraction methods on hair remnants found e.g. at crime scene. The extraction of DNA from animal hairs very often failed when root cells were absent. According to Kollárová (5), 600 cells from the hair bulb are sufficient for analysis. Nowadays there are available methods on the basis of which it is possible to perform genetic analysis of animal hair and to identify animal species (3). Pfeiffer *et al.* (7) described an experimental study on forensic DNA-typing of dog hair by means of a Ca^{2+} improved DNA extraction method, quantification and amplification.

OTHER INVESTIGATIONS

Hair analysis allows one to identify hair damage, e.g. snapped hair (sturdy pulling causes visible fissures in hair surface, medulla is affected rarely). Burning or crushing will cause the shaft to curl and bubble, burned hair has tendency to coil after loss of water and hairs are frail and split, the influence of blow and bullet wounds are also very easy to identify. Electric current may tear the hair bulb. By means of hair analysis it is also possible to determine arsenic and thallium poisoning.

Determination of human origin By Marcelle Lambert and Balthazard, Human and Animal Hair, Paris, Steinheil, 1910 (8)

HUMANS	ANIMALS
Medullary Canal	
Finely granulated network of airducts	Airduct network consists of moderately voluminous vesicles
Medullary cells invisible, without preliminary dissociation	Medullary cells are quite obvious. The value of medullary index is above 0.5
The value of the medullary index is less than 0.3	In the underhairs (duvet), the medulla is aligned in rungs or moniliform
The underhairs (duvet) have no medulla	(i.e. resembling a string of beads)
Cortical Substance	
Forms thick mantle	Constitutes a rather thin hollow cylinder
Pigment consists of very fine homogenous granulations	Pigment is of irregular granulations, always larger than granules in human pigment
Cuticle	
Fine scales, slightly raised and overlapping strongly	Thick scales, raised and less strongly overlapped than human cuticle scales

CONCLUSION

Forensic scientists in the veterinary field have to draw conclusions based on what the evidence shows. Answering the questions related to issues such as cruelty to animals, malpractice, identification of live and dead species as well as their derivatives are of interest to help the judicial system.

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INFLUENCE OF CADMIUM AND CHROMIUM ON THE ACTIVITY OF CHYMOTRYPSIN AND TRYPSIN IN DROPPINGS OF JAPANESE QUAILS

**Korének, M., Koréneková, B., Skalická, M.
Naď, P., Sokol, J., Húska, M.**

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

korenekova@uvm.sk

ABSTRACT

The aim of the study was to observe the effect of cadmium (Cd) and chromium (Cr) on the activity of chymotrypsin (ACHT) and trypsin (AT) in droppings of Japanese quails. The birds (24) were divided into 4 groups, 6 subjects each. The animals in group 1 (G1) received 0.12 mg Cd.day^{-1} per quail in drinking water, whereas those in group 2 (G2) were given 0.12 mg Cr.day^{-1}. The quails in group 3 (G3) were supplied a combination of 0.12 mg Cd.day^{-1} and 0.12 mg Cr.day^{-1}. The quails in group 4 were not given any supplements, acting as controls. ACHT and AT were determined after 35 and 58 days of supplementation. The addition of Cd in drinking water resulted in inhibition of ACHT and AT ($P < 0.001$) in group G1 in comparison to the control. Chromium showed a positive effect on the increase in ACHT ($P < 0.001$) and AT ($P < 0.05$) in G2 supplemented with Cr. A significantly higher ($P < 0.001$) ACHT and AT was observed in group G3 supplemented with Cd and Cr compared to group G1 supplemented with Cd. Our results indicated that long-term supplementation of Cr can affect positively the level of ACHT and AT in droppings of Japanese quails.

Key words: cadmium; chromium; chymotrypsin; Japanese quail; trypsin

INTRODUCTION

Japanese quail (*Coturnix japonica*) belongs to genus Coturnix, order Galliformes, which include chickens, turkeys, pheasants, quails, peafowl, grouse, partridges, guinea fowl and related birds. Japanese quail mature in about 6 weeks and are usually in full egg production by 50 days of age. With proper care, hens should lay 200 eggs in their first year of lay. Life expectancy is about 2 years. According to Baumgartner and Hetényi (2) Japanese quails reach slaughter weight by 4 weeks after hatching. Quails have been used for investigation of physiological processes in the body of fowl and appeared suitable for observation of interactions between essential chemical elements and xenobiotics under in vivo conditions (11). Because they have short hatching cycle, rapid growth and reproduction, quails are suitable as experimental animals (7, 10).

Trivalent chromium (Cr) is an essential mineral required by animals in trace amounts. Chromium enhanced the action of insulin and appeared important in carbohydrate, fat and protein metabolism and storage in the body (12). The addition of high-chromium yeast decreased serum glucose, total cholesterol and triglycerides in diabetic rats (26). Chromium is widely used as a supplement. Chromium supplements are available as chromium chloride, chromium nicotinate, chromium picolinate, high-chromium yeast and chromium citrate (6).

Cadmium (Cd) is an environmental pollutant that has serious toxicity in humans and animals. Cd is accumulated mainly in the kidneys and liver of animals (18). The birds reflect the level of environmental contamination which should be monitored (19). The exposure to Cd, even by a single dosage, can hamper quail reproduction for a very short time, mainly by decreasing egg production and thinning the eggshell (20). Decrease in body weight in Japanese quails due to Cd exposure at sublethal levels was reported by Sant'Ana et al. (25). However, manifestation of these effects in exposed organisms is the result either of the unique action of Cd or the

consequence of synergism/antagonism with other elements. Cadmium is bound to the sulfhydryl (-SH) groups of proteins. It can affect various metabolic processes, especially energy metabolism, membrane transport and protein synthesis (9).

Chymotrypsin and trypsin, important members of the family of serine proteases, are proteolytic enzymes acting in the digestive system. They are synthesized in the pancreas by protein biosynthesis. Determination of chymotrypsin and trypsin activity in faeces is relevant to pancreatitis, lung diseases and some other pathological changes (1). Protease inhibitors have the potential for regulation of proteolytic activities in specific pathways (4). Serine protease inhibitors are the best-known and most characterized inhibitors. They are classified into 18 different families, based on amino acid sequences, structural similarities and mechanism of reaction with their respective enzymes (17). Cadmium inhibits activity of many enzymes, such as catalase, mitochondrial ATP-ase, alkaline phosphatase, some proteases, dehydrogenases and chymotrypsin in poultry droppings (16).

The aim of the present study was to observe the activity of chymotrypsin and trypsin in droppings of Japanese quails after long-term administration of cadmium and chromium.

Table 1. Composition of commercial feed supplied to Japanese quails

Composition	Value ($g.kg^{-1}$)	Minerals	Value ($mg.kg^{-1}$)	Vitamins	Value
Crude protein	153	Zinc	60	A	8 000 $IU.kg^{-1}$
Energy	11.5 $MJ.kg^{-1}$	Calcium	45	D3	1 600 $IU.kg^{-1}$
Ash	160	Phosphorus	5.0	E	10 $mg.kg^{-1}$
Fibre	60	Sodium	2.5	B12	4.0 $mg.kg^{-1}$
Lysine	7.0	Manganese	40		10 $mg.kg^{-1}$
Methionine Cystine	6.0	Iron	40		
Methionine	3.5	Copper	4.0		
Linoleic Acid	15				

MATERIAL AND METHODS

Twenty four 40-days-old Japanese quails, weighing about 170 g each, were included in the experiment. All experiments were performed observing the ethical requirements for animal handling approved by the University of Veterinary Medicine, Košice, the Slovak Republic. The experimental conditions complied with ethical standards, animal welfare and proper care of animals. Japanese quails were used as model animals because they are close to wild birds and are a better indicator of environmental exposure than rodents that are kept strictly under laboratory conditions.

The quails were kept in cages under microclimate conditions optimal for their growth. They were fed complete mixed feed for poultry HYD 10 (manufactured by Agrofarm Product s.r.o., Slanská Huta, the Slovak Republic), as a full-value feed, throughout the experiment (Table 1). The feed mixture was provided ad libitum. Composition of the feed is given in Table 1. It complied with the Regulation of government of the Slovak Republic No. 440/2006. The mean feed consumption was about 40 g $feed.day^{-1}$ per quail.

The cadmium content in feed (0.007 $mg.kg^{-1}$) and water (0.001 $mg.l^{-1}$) used in this study were below the maximum permissible limits for Cd in feeds in the Slovak Republic (0.5 mg. kg^{-1} at 12 % moisture) and drinking water (0.003 $mg.l^{-1}$) complied with the Regulations of the Slovak government No. 347 and 438/2006. Thus the birds were not exposed to Cd through their diet or drinking water.

Experimental design

Soluble salts $CrCl_3.6H_2O$ and $CdCl_2.H_2O$ (extra pure, Merck, Germany) were used without further purification to prepare stock solutions in 1 litre of distilled water from which the selected doses, contained in 1 ml, were given to birds in their drinking water.

The quails were divided to 4 groups, 6 subjects in each. The first experimental group was administered solution of $CdCl_2.H_2O$ at a dose of 0.12 mg $Cd.day^{-1}$, the second group solution of $CrCl_3.6H_2O$ at a dose of 0.12 mg $Cr.day^{-1}$ and the third group received both elements (Cd + Cr), Cd at a dose of 0.12 mg $Cd.day^{-1}$ and Cr at a dose of 0.12 mg $Cr.day^{-1}$. The fourth group was supplied tap water without any supplements and served as a control.

The analysis of specific activity of chymotrypsin and trypsin

The specific activity ($U.g^{-1}$) of chymotrypsin (ACHT) and trypsin (AT) in droppings of Japanese quails was determined by the methods described by Rosival et al. (18). The measurements were carried out on day 1 of the experiment and after 35 and 58 days of supplementation of the investigated elements (Cd, Cr), at the time of maximum production of eggs (11–14 weeks of age).

The chymotrypsin activity was determined after hydrolysis of chromogenic substrates Succinyl-$(Gly)_2$-Phe-p-nitroanilin and the activity of trypsin after hydrolysis of L-TAPA (Tosyl-Arg-p-nitroanilin). Chymotrypsin (trypsin) was extracted from 1g of fresh mixed droppings, using 10 ml of 0.5 $mol.l^{-1}$ NaCl with 0.1 mol^{-1} $CaCl_2$.

The suspension obtained was centrifuged for 5 min at 5000 x g and the supernatant was used as an enzyme source. An extract aliquot (0.1 ml) was added to 0.1 ml of substrate

dissolved in buffer (1.8 ml) by which the reaction was initiated. The rapidity of chymotrypsin (trypsin) catalysed hydrolysis of Succ-$(Gly)_2$-Phe-pNA or trypsin catalysed hydrolysis of L-TAPA (Tosyl-Arg-pNa) was established kinetically at 405 nm on Specol 200 (Karl Zeiss Jena, Germany). The sensitivity of the method used for determination of chymotrypsin and trypsin was 0.5–1.0 $\mu g.ml^{-1}$ at the starting concentration of the substrate, and 1 $mmol.l^{-1}$ in reaction media under optimal reaction conditions (pH 7.8–8.0, temperature 25 °C). The results obtained were analysed statistically by Student t-test (Microsoft Excel 7.0), setting the significance levels at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The data are presented as means ± standard deviations.

RESULTS AND DISCUSSION

The results of our study showed an increase in the activity of chymotrypsin and trypsin at the end of the experiment in comparison with the beginning (Tabs. 2 and 3). The activities of chymotrypsin and trypsin were related to the age of quails.

The long-term administration of Cd to Japanese quails supplemented only with Cd (G1) resulted in significant ($P < 0.001$) inhibition of the activity of chymotrypsin (132.1 $U.g^{-1}$) and trypsin (113.1 $U.g^{-1}$) in droppings of these quails in comparison to the controls (195.9; 125.2 $U.g^{-1}$). A similar inhibitory effect of Cd on ACHT was confirmed in droppings of laying hens after 6 months exposure to 0.3 $mg.kg^{-1}$ $CdCl_2.H_2O$ by Koréneк *et al.* (13) and on trypsin after Cd supplementation to broilers by Kosa *et al.* (14, 15).

Heavy metals can alter markedly the metabolism and function of essential elements by competing for binding position and/or ligands in any biological system. Deficiency of essential elements results in dysfunction, but their excess may also be harmful. However, at higher doses, Cd blocks metabolism of these elements and competes with them for some intracellular bonds (27).

The administration of Cr affected positively ($P \leq 0.001$) the level of both ACHT (478.0 $U.g^{-1}$) and AT (116.4 $U.g^{-1}$) in droppings of Japanese quails in the group supplemented only with Cr (G2) in comparison to controls (G4 – 195.9; 125.2 $U.g^{-1}$) and the Cd-supplemented group (G1 – 132.1; 113.1 $U.g^{-1}$).

The pancreas has two major functions: production of insulin and production of digestive enzymes. The very important role of insulin is the attachment to free amino acids for delivery to the pancreas. Chromium 3+, or glucose tolerance factor (GTF), is the specific mineral that allows insulin to bind to these amino acids. This is a very important fact regarding chromium supplementation (5).

Our results showed that ACHT (270.5 $U.g^{-1}$) and AT (210.7 $U.g^{-1}$) was significantly higher ($P \leq 0.001$) in the Cd-Cr-supplemented group (G3) after 58 days of the experiment in comparison to the controls (G4 – 195.9; 125.2 $U.g^{-1}$) and Cd-supplemented group (G1 – 132.1; 113.1 $U.g^{-1}$). The addition of Cr can lead to competitive activation of chymotrypsin and trypsin. The protective effect of Cr against Cd was reflected in the middle and at the end of the experiment.

The effect of essential elements, including Cr, on reduction of heavy metals toxicity was described by Chowdhury and Chandra (8). Beňová *et al.* (3) found, that low doses of ionising radiation improved adaptive responses of some species *(Artemia franciscana)*. Examination of groups exposed to Cd and Cr, either separately or in combination, showed better viability of irradiated birds. At higher doses of radiation a protective effect of Cr was assumed.

The major effect of high levels of Cd appears to be the interference with absorption of essential minerals. Excess intake of essential minerals can decrease or eliminate some of these effects of Cd and, at the same time, reduce Cd level in the kidney, a target organ for Cd accumulation and functional damage.

Environmental toxicants such as Cd cannot be completely avoided. It is important, therefore, to define low intake levels of essential nutrients at which toxicity of Cd is exacerbated and high intake levels of essential nutrients at which toxicity of Cd is minimized.

CONCLUSION

The results of the present study indicate an antagonistic relationship between Cr and Cd regarding the activity of chymotrypsin and trypsin. Cadmium inhibits significantly the activities of proteases which play critical role in animal digestion. For this reason the inhibition of these enzymes by cadmium may affect a variety of important physiological functions. An appropriate Cr supply reduces Cd retention in the organism of quails, Cd content in the gut and enhances activity of chymotrypsin and trypsin in quail droppings. The supplementation of Cr as an essential micronutrient in animals could perform an important protective function, mainly in industrially polluted areas.

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Table 2. Effect of Cd, Cr and Cd-Cr supplementation on the activity ($U.g^{-1}$) of chymotrypsin (ACHT) in quail's droppings

Activity of Chymotrypsin	Mean	Day 1 Sd	Max.	Mean	Day 35 Sd	Max.	Mean	Day 58 Sd	Max.
Cd	120.5***	6.1	127.7	135.3***	7.8	144.3	132.1***	7.7	141.6
Cr	174.5***	5.4	181.2	232.8***	6.6	242.2	478.0***	6.3	486.2
Cd-Cr	150.8	6.4	158.6	213.9***	8.7	223.7	270.5***	9.8	281.6
Control	152.8	6.2	160.3	160.4	6.1	168.7	195.9	5.6	203.7

Statistical significance: *– $P < 0.05$, **– $P < 0.02$, ***– $P < 0.001$

Table 3. Effect of Cd, Cr and Cd-Cr supplementation on the activity ($U.g^{-1}$) of trypsin in quail's droppings

Activity of Trypsin	Mean	Day 1 Sd	Max.	Mean	Day 35 Sd	Max.	Mean	Day 58 Sd	Max.
Cd	98.3**	7.4	106.5	102.8***	6.5	111.7	113.1***	5.2	119.2
Cr	115.3**	6.8	124.3	125.8*	7.1	135.9	116.4*	7.5	126.0
Cd-Cr	112.9*	5.5	119.8	141.5***	6.1	149.4	210.7***	9.7	222.8
Control	101.3	7.6	111.2	116.4	6.6	125.4	125.2	5.2	131.9

Statistical significance: *– $P < 0.05$, **– $P < 0.01$, ***– $P < 0.001$

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MECHANICAL PROPERTIES OF BONES

Baranová, D.[1], Pešek, Ľ.[2], Šály, J.[1]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice
[2]Technical University, Letná 9, 040 01 Košice
The Slovak Republic

baranovad@uvm.sk

ABSTRACT

Examination of mechanical properties of broiler bones is interdisciplinary in essence and involves physiological processes in bones and whole organism of chickens. It has been recognized that different composition of diet affects considerably the growth, mineral composition and structure of bones. This in turn affects their mechanical properties and may produce conditions for development of various osteopathies. The study of mechanical properties of bones allows one to explain new relationships and mechanisms of development of relevant diseases. When investigating mechanical properties of bones and deriving relations for their calculation we used terminology common in the field of technical materials and adjusted it to veterinary medicine research needs.

Key words: broiler; mechanical properties of bones; poultry

INTRODUCTION

Selection in development of commercial chicken meat hybrids focused on increase in growth rate, feed efficiency and meatiness. Rowland (15) observed that increasing growth rate and slaughter yield resulted in increased incidence of abnormalities of extremities, particularly tibia and other loaded components of the skeleton. The most frequent abnormalities of legs observed in rapidly growing birds included perosis, rachitis, osteoporosis and tibial dyschondroplasia (3).

Changes in mechanical properties of bones developing at their disorders can further worsen their condition and contribute to lameness. For example, decreased elasticity of bones may result in damage to growth plate and subsequent morphological changes and locomotion disorders (4). Rowland (15) reported that abnormalities of broiler extremities caused important economical losses due to decreased weight gain and increased mortality.

WAYS OF EVALUATION OF MECHANICAL PROPERTIES OF BONES

According to Capps (3) the strength of poultry bones can be determined by various testing techniques involving both compact and cancellous portion of bones.

Merkley and Miller (12), Lott *et al.* (11) Rowland *et al.* (16) presented in their papers the strength of bones in kilogrammes of force needed to break the various extremity bones regardless of their size. Such comparisons were frequently made between birds of varying size and age. Because there is a positive correlation between the size of bones and the body weight, Anderson *et al.* (1) and Huff *et al.* (10) proposed adjustment of calculation of stress according to the size of bones.

Huff *et al.* (10) assumed that tibiotarsus is a compact cylinder and considered only external diameter of bones. They also measured the modulus of elasticity, reporting the degree of rigidity or stiffness, and again assumed that bone is a compact cylinder.

According to Huff *et al.* (10), the bone bending strength(s) at break can be calculated as follows:

$$S = \frac{8.F.L}{\pi.D^3}$$

*where F is the bone breaking force, L – effective bone length (70 mm), and D – diameter of the bone at centre; * – stands for multiplication*

However, bone is not a solid cylinder and therefore comparisons between bones with changing internal composition without changes in external diameter can lead to wrong conclusions when only the external diameter is used in calculations. Crenshaw *et al.* (5) observed that dietary calcium and phosphorus produced no changes in the external diameter of pig bones but there were differences in the internal diameter.

Patterson *et al.* (14) had not considered the bone a solid cylinder and presented calculation of bending stress at bone brake based on both the external and internal bone diameter.

$$S = \frac{F.L.C}{4.0.0491.(B.D^3 - b.d^3)}$$

where C is half-diameter of the bone in the plane parallel to the braking force, B and D – external bone diameters, b and d – internal bone diameters, B and b – bone diameters in the plane vertical to the breaking action and D and d – bone diameters parallel to the breaking force.

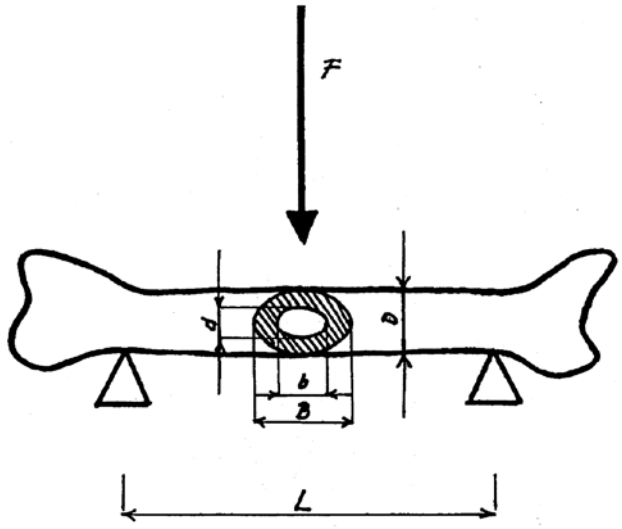

Fig. 1. Illustration of individual bone parameters, direction of breaking force F and effective bone length L (orig.)

Stress is the measure of bone strength which takes into consideration differences in the size and shape of materials when tested according to Patterson and Cook (13).

Capps (3) determined the strength of the total structure of poultry bone considering both compact and cancellous bone and using the three-point bending test. Harner and Wilson (8, 9) validated 3 techniques for evaluation of mechanical properties of bones. They compared the three-point bending, shear and torsion. The three-point bending method was usable only when the bones were straight, had a symmetrical cross-section and their length to diameter ratio was greater than 10. The shear test was successful with straight bones having a symmetrical cross-section. Results of bending stress studies based on linear regression model indicated that the strength of bones may be predetermined by body weight. The authors observed an influence of body weight on the final bending strength and final bending stress of tibiotarsus.

Morphology of bones changes so as to produce an optimum structure for relevant stresses in relation to body weight and centre of gravity. Changes in the ratio of muscle to bone growth rates result in changes in poultry bone strength (2, 6).

Investigations in dogs showed that the cancellous bone is the subject of the highest remodelling activity induced by the high ratio of its surface to volume (9). It has been assumed that the cancellous bone of poultry is subject to more rapid changes at changing load related to growth of muscles. This process is affected by protein and amino acid nutrition but also by heavy metals. The importance of mechanical properties of cancellous bones in the subchondral region has not yet been adequately explained in poultry. There is also a lack of knowledge about changes in bone strength in relation to their disorders (3).

Capps (4) observed 4 mechanical properties of cancellous bones: strength (compression strength – σ_u), defined by maximum load in the scheme "stress-deformation"; deformation (δ) measured at the moment of brake; modulus of elasticity (E) calculated as a function of strength, deformation and non-deformed thickness of a bone slice. The modulus of elasticity is a material-dependent property and not a function of shape, symmetry, or bone size.

$$E = \frac{P.L}{A.\delta}$$

P – *strength at braking*
L – *original thickness of a non-deformed slice*
A – *cross-section (area)*
δ – *deformation at fracture*

Bulk modulus (R) provides information needed for evaluation of the degree of structure deformation at failure (fracture). It is calculated as follows:

$$R = \frac{\sigma_u.\Delta}{2.L}$$

σ_u – *strength*
Δ – *deformation*
L – *original thickness of a non-deformed slice*

Crenshaw *et al.* (5) reported on a method used to compare the strength of pig bones differing in size and shape. This method for calculation of bone strength involves the following: external and internal diameter of bones, parallel and perpendicular applied force and moment of inertia related to the real shape of bone.

In addition to the already mentioned mechanical properties, there are other mechanical parameters that are used to evaluate differences between properties of various materials and substances of other than biological essence. These parameters allow one to evaluate changes in structure of materials which in turn indicate their origin, for example toughness, which is the work needed to induce material failure (17).

MATERIAL AND METHODS

Of different poultry bones tibiotarsus is a bone suitable for examination of mechanical properties. The tibia were dissected from fresh carcasses and stripped of soft tissues. They were stored in plastic bags at –20 °C and warmed up to 20 °C before the testing. Static tests of mechanical properties of bones were carried out on a universal tensile testing machine FP 100/1 using a three-point bending test at ambient temperature 20 °C. The range of loading force was 1 kN for large bones and 400 kN for small bones. The suitable distance of supports for small bones is 36 mm and for large ones 60 mm and is selected so that it is as big as possible but, at the same time, the bone bears on the support at location x. Bones are placed on supports in such a way so that the plane of the smaller dimension of bone is parallel with the loading force.

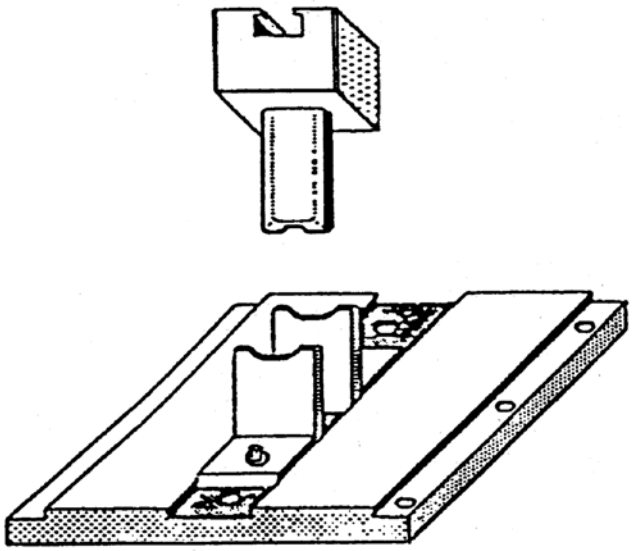

Fig. 2. Detailed view of the machine FP 100/1 for tensile testing according to Patterson *et al.* (14)

Deflection of bones is scanned and registered with 10-fold magnification. The rate of loading, expressed as the crosshead speed and therefore the rate of application of the loading force, is 6.1 mm.min^{-1} (9.8 s.mm^{-1}), and in some machines 6.9 mm.min^{-1} (8.7 s.mm^{-1}), but 6 mm.min^{-1} (10 s.mm^{-1}) is considered a sufficient accuracy of reporting, as used in the study by Fukal *et al.* (7).

Patterson *et al.* (14) carried out similar tests and used a loading rate of 5.08 mm.min^{-1}. This difference in the loading rate used can be considered insignificant regarding the comparison of results. The authors used rounded supports (Fig. 2); such an adjustment enables better positioning of bones on supports during testing and decreases the local pressure at the bone-support contact. This also prevents undesirable turning of the bone during testing and decreases damage to the specimen at the bone-support contact before breaking. Our tests were carried out only with straight supports. Parameters of bones (Tab. 3) were measured with 0.01 mm accuracy using a technical slide calliper.

Parameters of mechanical properties of bones

Loading force-deformation expressed by bone deflection is used for evaluation of the respective parameters. The initial portion of the force-deformation diagram is always non-linear. At first the deformation (deflection) increases rapidly but the corresponding force grows slowly. This is due to settling of the bone on supports at the bone-support contact site. Then the non-linearity of the relationship gradually decreases and eventually changes to linear when the stress is proportional to deflection up to the limit of elasticity.

The stress below the elasticity limit causes only reversible (elastic) deformation of bones. F_1 is the force up to which the *force-deflection curve* is linear. This is a force corresponding to the *limit of elasticity* (R_E) of the bone, i. e. the stress up to which the bone is deformed elastically, reversibly, and after unloading returns to its original shape and dimensions. This force is used to determine the relevant *stress-limit of elasticity.*

From the beginning of loading up to the limit of elasticity there is a direct relationship between the bending stress σ and deformation ε of the most stressed fibres on the bone surface: $\sigma = E . \varepsilon$. With additional loading and increasing the applied force the bone deformation becomes partly elastic, reversible, and partly permanent (plastic), irreversible.

After unloading at this stage, the bone remains permanently deformed although its total deflection somewhat decreases (by

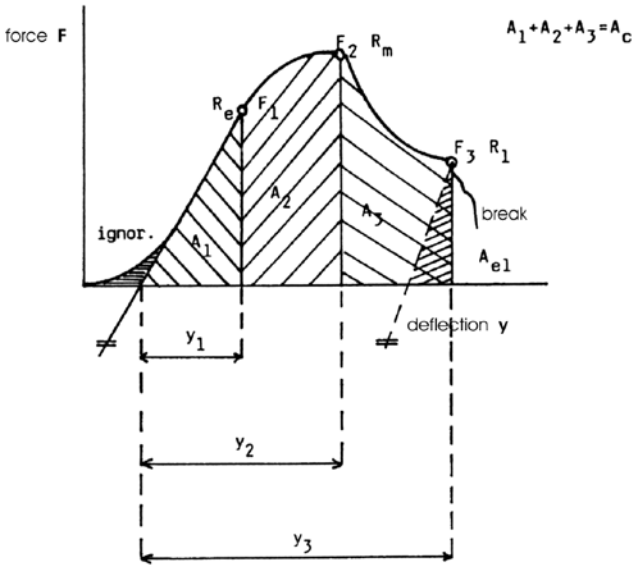

Fig. 3. The bone force-deflection curve with indication of respective parameters (orig.)

elastic deformation). The force F_2 is the highest force that the bone can sustain. After reaching the level F_2, the highest bending stress is attained on the surface of the flexed bone, the so-called *bending strength* R_m. Further loading can result in a gradual decrease in the loading force to F_3. After reaching the F_3 level a sudden failure occurs indicated by a sudden decrease in the force and braking of the bone to two parts often accompanied by an acoustic effect.

The breakage is rapid and is called brittle fracture. The force F_3 at break is related to *fracture stress* R_f. The bone can break (force F_3) even without decrease of the force from the level F_2 (absence of force decrease beyond the maximum). In this case $F_2 = F_3$. Some bones do not break even after "great" deflection. In such a case we cannot determine neither force F_3 nor deflection Y_3 and the fracture stress is equal to the bending strength.

According to the character of the loading diagram we recognise the following typical cases:

Type K – brittle fracture. Braking of bones occurs at maximum force. After reaching the maximum there is no decrease in force, $F_2 = F_3$, bending strength = fracture stress. Both the total and plastic deformation (deflection Y) is small.

Type N – unbroken bone. After reaching the maximum force (F_2) there is a decline in force but the bone will not break. Neither fracture stress R_1 nor bone toughness can be determined.

Type T – transient or plastic (tough) deformation. After reaching the maximum force F_2 the loading force decreases down to the moment when, at force F_3, the bone is fractured. The fracture is accompanied by a sudden abrupt decrease in force and breaking the bone to two pieces, frequently associated with an acoustic effect. This is a rapid, brittle fracture. The decrease in force from the level F_2 to F_3 differs for different bones. If $F_2 = F_3$, the fracture T changes to fracture K and $F_2/F_3 = 1$. The considerations which apply to forces F_2, F_3, apply obviously also to the stresses: bending strength, fracture stress R_1. The lower the ratio F_2/F_3 (< 1) the higher the deformation after reaching the maximum (Y_3) and also the toughness.

Type TK – combination of T and K. In some cases, reaching of maximum force F_2 and subsequent decrease to force F_3 does not result in breaking of bone to two pieces but in incomplete fracture. This can occur also at F_2 without decrease to F_3. Similar to the previous type, incomplete fracture is also accompanied by a sudden abrupt decrease in loading force. This decrease is lesser than that observed with type T and some force still persists and gradually decreases resulting eventually in complete fracture. In this case it is reasonable to evaluate the moment of first "failure" of the bone, i.e. the force corresponding to the first sudden decrease in force should be regarded as force F_3.

Diagrams of types K and N are the two ultimate extremes and the remaining ones are considered transient types.

The *Area A* below the *loading force – bone deflecton* curve is the work done by the force F at deflection of bone by Y. The work (energy) needed to deform the bone up to the failure is considered bone *toughness*. As the bone breaks, a portion of energy is released – elastic energy. Its magnitude corresponds to the area of a triangle. This elastic energy (not consumed by the sample – the energy returned at fracture) is not included in the energy for calculation of bone toughness.

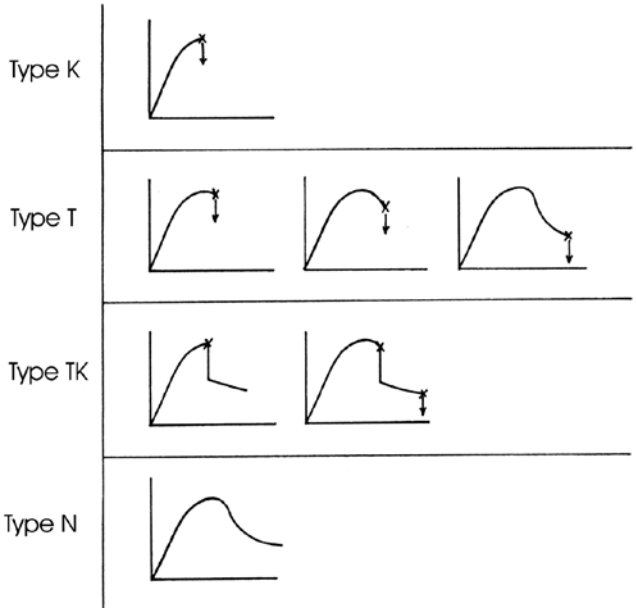

Fig. 4. Types of force-deformation diagrams (orig.)

A substantial expended work (large force, big deflection) means that the bone is tough while small expended work (small force and/or small deflection) indicates that its toughness is low and the bone is brittle. In order to compare the toughness of bones of different size (different diameter) it appears appropriate to recalculate the work expended on deformation up to failure per bone cross-section area of bone at the point of fracture.

Toughness – total work below the *force – deflection* curve – elastic energy/area of bone cross-section = $(A_c - A_{el})$/area of bone cross-section. This means that the work needed to deform the bone up to its failure may be considered its toughness. Neither toughness nor fracture stress can be determined for unbroken bones.

Total energy (work) = plastic energy + elastic energy.

Modulus of elasticity E (Table 1) expresses relationship between deformation and stress in the elastic zone (linear relationship between loading force and deflection) and is characterised by the slope of this straight line. Modulus of elasticity is a measure of the degree of rigidity (stiffness) of the bone – higher value of E means that the bone is more rigid and less yielding than another bone with lower E. Higher E also means that grater force is needed to produce the same deformation then in bone with lower E. Or, an identical force will produce higher deformation in bone with lower E than in that with higher E. We refer to elastic deformation.

Moment of inertia I of a stressed cross-section (mm^4 – Table 1) is a geometrical characteristics of a cross-section which allows one to calculate the minimum bending stress in the most stressed cross-section of the bone.

Area of bone cross-section. The bone is a hollow structure with external and internal cross-section perimeters acquiring approximately elliptic shapes. The bearing cross-section of bone is the area between the outer and inner ellipses (diameters D, B and d, b, respectively).

Table 1. Formulas for calculation of relevant parameters

(1)	**Moment of inertia**	**I** [mm^4] = $(B.D^3 - b.d^3).\pi / 64$
(2)	**Area of bone cross-section at the point of fracture**	**Area** [mm^2] = $(D.B - d.b).\pi / 4$
(3)	**Limit of elasticity** s = distance between supports = 36 mm for small bones = 60 mm for large bones	**Re** MPa] = F1.s.D / (8.I)
(4)	**Modulus of elasticity** Constant of proportionality between stress σ and deformation ε.	**E** [MPa] = $F1.s^3 / (48.I.Y1)$
(5)	**Bending strength**	$\mathbf{R_m}$ [MPa] = F2.s.D / (8.I)
(6)	**Fracture stress**	$\mathbf{R_1}$ [MPa] = F3.s.D / (8.I)
(7)	**Toughness**	**Huz** [N / mm = 10^{-3} J / mm^2] = $(A_c - A_{el})$ / area $\mathbf{A_{el}} = F3^2.Y1 / (2.F1)$

When evaluating the mechanical properties of bones the usual procedure is that after obtaining the primary results one checks the results for those bones for which extremely low or extremely high values were obtained. In some cases it is justified to correct them or eliminate them from the set of data. Neither toughness nor fracture stress can be determined for unbroken bones. Because of that some samples lack some parameters and thus individual sets of data consist sometimes of different number of values that are used for statistical processing and analysis of variance. This ensures elimination of cases potentially associated with erroneous measurement, testing or evaluation. In some cases it may happen that due to resting of bones on supports at loading, the force-deflection curve is not quite linear in the elastic region and therefore it is unsuitable for determination of the elasticity modulus E.

Table 2. Parameters of bones

D [mm] = external bone diameter in the plane parallel to the loading force (smaller outer diameter)
B [mm] = external bone diameter in the plane perpendicular to the loading force (bigger outer diameter)
d [mm] = internal bone diameter in the plane parallel with the loading force (smaller inner diameter)
b [mm] = internal bone diameter in the plane perpendicular to the loading force (bigger inner diameter)

Table 3. Parameters relevant to the diagram of bone deflection

F1 = force F_1 at the limit of elasticity [N]. In the force – deflection diagram the force F1 is the force up to which the relationship between force and deflection is linear
F2 = maximum loading force F_2 [N].The absolutely highest force that the bone can sustain during loading. It is used to determine the bending strength
F3 = force F_3 in the moment of bone breaking [N]
Y1 = bone deflection Y_1 corresponding to force F_1 [mm]
Y2 = bone deflection Y_2 corresponding to force F_2 [mm]
Y3 = bone deflection Y_3 corresponding to force F_3 [mm]
A1 = area A_1 [mm^2] below the *force-deflection* curve, starting from the beginning of loading up to force F_1.
The *force-deflection* relationships recorded during the test serve for determination of the area below the loading curve. Area A1 is calculated as a triangle A1 = F1*Y1/2. The area A1 does not include the initial work resulting from the bone bearing on its supports and impression produced by supports (the initial crosshatched area).
A2 = area A_2 [mm^2] below the *force-deflection* curve, between the forces F_1 and F_2. Area A2 is determined by planimetry as a trapezoid area
A3 = area A_3 [mm^2] below the *force-deflection* curve between the forces F_2 a F_3. Area A3 is determined by planimetry as a trapezoid area. When the bone fractured at the maximum force F_2, area A3 was not determined
A1 [J] = area below the *force-deflection* curve, starting from the beginning of loading up toforce F_1
A2 [J] = area below the *force-deflection* curve, between the forces F_1 and F_2
A3 [J] = area below the *force-deflection* curve, between the forces F_2 and F_3
Ac [J] = A1 + A2 + A3 – **total work** (energy) **expended** on deformation and failure
Ael [J] = **elastic energy** released at fracture

According to the preset extent of measurement of force and deflection (deformation) we recalculated the areas in mm^2, obtained from the force-deflection diagram, to the real values in work (energy) units.

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HARVESTING OF ADULT RAT BONE MARROW DERIVED STEM CELLS EXPRESSING NESTIN

Šulla, I.[1], Balik, V.[2], Capková, J.[3], Šarišský, M.[4]

[1]Department of Anatomy, Histology and Physiology, University of Veterinary Medicine Komenského 73, 041 81 Košice
[2]Department of Neurosurgery, P. J. Šafárik University Faculty of Medicine, AD Centre, Košice
[3]Department of Anaesthesiology and Intensive Medicine, P. J. Šafárik University Faculty of Medicine
[4]Department of Pharmacology, P. J. Šafárik University, Faculty of Medicine, Tr. SNP 1, 040 11 Košice
The Slovak Republic

sulla@uvm.sk

ABSTRACT

The aim of the present study was to develop a reproducible and reliable method of harvesting of bone marrow stem cells (BMSCs) for their further cultivation leading to generation of neural precursors. Six adult male Sprague-Dawly (S-D) rats under inhalation narcosis, induced by halothane and maintained by a mixture of 1–2% of halothane with N_2O and medical oxygen (1 : 1) administered by a special mask, were subjected to double-trephination of both thigh bones and the bone marrow (BM) was flushed out by saline with heparin and aspirated to a syringe with 2 ml of Dulbecco's modified eagle medium (DMEM) with heparin. The harvested cells were numbered, cultured and finally analysed by flow cytometry for expression of CD45, CD90 and nestin. All rats survived experiments uneventfully. The surgical wounds healed without complications. Yields of BMSCs harvested from individual rats ranged from 1.5 to $4.0.10^6$ and their sequential expansion in appropriate culture media with foetal bovine serum (FBS) resulted in obtaining a sufficient amount of nestin-positive bone marrow derived stem cells (BMDSCs). The study proved that the proposed technique is a suitable method for harvesting BMDSCs, their further cultivation and generation of nestin-expressing BMDSCs. Long-time survival of donors in an excellent condition enables continuation of the experiments by autotransplantation of BMDSCs.

Key words: bone marrow cells; harvesting technique; nestin expression; rat

INTRODUCTION

Multiple experimental studies as well as clinical observations have provided a definitive evidence that the central nervous system (CNS) of adult mammals has only a limited capacity for neurogenesis and axonal re-growth (2, 12, 16). However, the ability of peripheral nerves to heal and re-establish functional connections with appropriate targets following an injury shows that a process of regeneration is not completely foreign to neural tissues (12).

Conceptually it represents a recapitulation of normal development of the CNS, when all types of neuronal and glial elements derive from a single class of progenitor cells, called neural stem cells – NSCs (1, 4, 15, 18–20). The ability of NSCs to self renew and their potential for controlled differentiation into neurons, astrocytes or oligodendrocytes makes neural progenitors very attractive for the treatment of many neurological disorders, including traumatic or ischemic paraplegia, brain injury, stroke and neurodegenerative diseases (1, 3, 7, 8, 13).

Several tissues have been considered as a possible source of NSCs for transplantation and replacement therapies, among them embryonic stem cells and foetal brains (1–5, 11, 14, 19). However, their clinical use is limited by ethical and technical problems, as it requires not only high numbers of foetal cells, but immunosuppression is recommended as well (5). Alternatively, several reports suggested that mesenchymal stem cells isolated from the bone marrow of a recipient are able to dif-

ferentiate to neural precursors, suitable for autotransplantation (1, 4, 15, 18, 20).

The aims of our study were to evaluate some problems related to cultivation and isolation of NSCs from bone marrow of adult rats and long-term survival of donors in the best possible condition for further autotransplantation experiments with replacement of the autologous bone marrow derived stem cells expressing nestin.

MATERIAL AND METHODS

The experimental protocols were elaborated in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 and approved by the State Veterinary and Food Administration of the Slovak Republic in Bratislava (decision No. SK P 10552/03-220), as well as the Ethical Commission of the P. J. Šafárik University, Faculty of Medicine in Košice. All manipulations with experimental animals were performed under general anaesthesia.

Six adult male Sprague-Dawley (S-D) rats, weighing 480–690 g, were used in the study. The general anaesthesia was induced by inhalation of a mixture of 4 % halothane (*Halothanum thymolo 0.01 % stabilisatum* – "Narcotan", LÉČIVA, Prague, The Czech Republic) with medical oxygen in a closed plexiglass box. After 2–3 min, when the surgical phase of narcosis was reached, the experimental animals were removed from the box, placed on a heated operating table in lateral position and the anaesthesia was further maintained by the mixture of 1–2 % of halothane with N_2O, and oxygen (50 : 50) administered via a special mask designed for rats (Fig. 1). Lateral areas of both thighs were shaved, then the exposed one was disinfected with iodine solution (*Povidonum iodatum* – "Betadine", Egis Pharmaceuticals, Budapest, Hungary). The disinfected skin and femoral fascia were incised to separate layers and the ventrolateral surface of the femoral bone was approached between *musculus rectus femoris* and *musculus vastus lateralis*.

After the removal of periosteum, two burr-holes (one at the proximal and second at the distal end of the femoral shaft) were drilled by a tooth-drill (Fig. 2). The bone marrow was flushed out by 2 ml of saline with 0.1 ml of heparin (*Solutio heparini natrici sterilisata et titrata 5000 u. i.* – "Heparin", Zentiva, Prague, The Czech Republic), instilled into one of burr-holes and aspirated with an injection needle to a syringe with 2 ml of a collection medium (DMEM with heparin – "Invitrogen", Gibco, United Kingdom). Following aspiration of bone marrow, the femoral fascia and skin were sutured by an atraumatic sewing material (6/0 "Mersilk", Ethicon, Edinbourgh, Scotland), the experimental animal was put on the operated side and the bone marrow was aspirated from its contralateral femor using the same technique.

Before finishing the surgical procedure, 150 mg of ampicillin (*Ampicillinum ut sal natrico* – "Ampicillin 0.5", Biotika, Slovenská Ľupča, The Slovak Republic) in 2 ml of saline and 2 mg of tramadol (*Tramadoli hydrochloridum* – "Tramadol AL 100", Aliud Pharma GmbH Co KG, Leichingen, Germany) was administered intramuscularly; each medicament to a different place. The rats were transferred to separate disinfected compartments where they were offered drinking water and food granules *ad libitum*. Blood samples (0.5 ml) aspirated by a thin needle from the right heart ventricle of randomly chosen two rats just prior finishing the general anaesthesia were used as sham controls. The blood samples were taken to a syringe containing 0.2 ml of heparin solution.

Immediately following the aspiration of BM from both thigh-bones, the material was dissected to small pieces, homogenized and filtered through a 70 μm filter to remove bone fragments, then diluted 1 : 1 with Hank's balanced salt solution (HBSS, Gibco, United Kingdom) and mononuclear cells were isolated by Ficoll-Urographin density centrifugation (Sigma, Schering, Germany) at 1 600 rpm for 30 minutes. After centrifugation, mononuclear cells were collected from the interface. The isolated cells were washed two times in Dulbecco's modified Eagle medium (DMEM), suspended at 10^6 cells.ml^{-1} in five different types of culture media (see Tab. 1 for details) routinely used for the generation of stem cells, and transferred to 6-well culture dishes (Sarstedt, Germany).

The cells were cultured for two weeks, half volume of the corresponding culture medium being replaced with a fresh medium every third day. Following fourteen days of culturing the cells were stained with a combination of FITC/PE/PE-Cy5, i. e. monoclonal antibodies (IgG_1) against rat CD 90, nestin, and CD 45. Then the expression of CD 90, nestin and CD 45 was analyzed by three-colour flow cytometry.

RESULTS

General inhalation anaesthesia by halothane with N_2O and medical oxygen proved to comply with the requirements of the study. The induction phase of narcosis was short, its course smooth, awakening of experimental animals without undue excitation. All six rats survived the surgical procedures uneventfully. None of them had any complication related to bilateral double-trepanation of thigh-bones, aspiration of bone marrow or puncture of the right heart ventricle (two rats). Only minutes after finishing administration of anaesthetics, the rats began to move, gained consciousness and their agility was completely restored shortly afterwards. They drank water, devoured food granules, and moved freely around their cages. Surgical wounds healed *per primam intentionem*, i. e. quickly and without complications (Fig. 3).

Microscopic analyses of cells isolated from the fresh material harvested from both thigh bones revealed that the total number of isolated bone marrow mononuclear cells was 1.5–4.0 million per sample. After 14 days of *in vitro* cultivation, the proportion of CD 90/nestin co-expressing stem cells increased from less than 1.0 % to 19.2–24.9 % in individual samples (Figs. 4 and 5).

Cultivation of adult rat bone marrow derived cells in medium No. 1 (Culture Medium 1 – for its exact composition see Table 1) led to the generation of a mixture of floating and adherent cells of variable size, predominantly CD45 ±, CD90 + and nestin –. In contrast, cells expanded in medium No. 5 (Culture Medium 5 – its

Fig. 1. Surgical procedure in one of the experimental animals under general anaesthesia Anaesthetics were administered by a special mask designed for rats

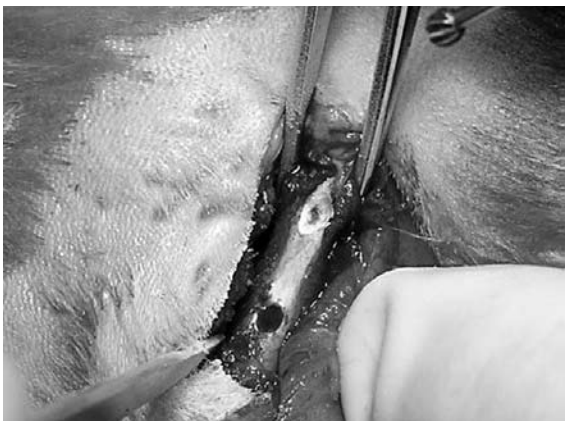

Fig. 2. Drilling of a second burr-hole at the proximal end of the left thigh bone shaft in a rat

Fig. 3. Situation two months following aspiration of bone marrow. The right femoral region of the experimental animal from Fig. 2

composition is shown in Table 1) were large, adherent, with fibrillary morphology (Fig. 4), without any pattern of CD 45 expression, but CD 90 +++ and nestin +++.

On the other hand, medium No. 2 (Culture Medium 2 – composition is in Table 1) was not able to maintain viability of BMDCs. All mononuclear cells died and disintegrated shortly after suspension in these two culture media.

DISCUSSION

Recent studies on animals have strongly suggested that transplantation of neural stem/progenitor cells could be a promising therapeutic option for patients with ischemic or traumatic damage of CNS, neurodegenrative processes of the brain and spinal cord, as well as many other pathological conditions (1, 2, 4, 15, 18, 20). That is why in the recent years the study of stem/progenitor cells has attracted such a widespread interest in the scientific community round the world. On the other hand, many cancer types including gliomas, resemble undifferentiated cells in their gene expression and phenotypic characteristics.

It is a common, although unproven idea, that astrocytomas arise from astrocyte precursors, oligodendrogliomas from oligodendrocyte precursors and mixed gliomas arise from progenitors of both, astrocytes and oligodendrocytes (17). This means that any possibility of malignant transformation of implanted or autonomous (present in normal adult subjects in subventricular zone of the brain) NS/PCs deserves serious evaluation (10, 17).

Of the several kinds of cell candidates – including umbilical cord blood progenitors, adult neural stem cells (NSCs), embryonic stem cells (ESCs), embryonic neural cells (ENCs) – the bone marrow or adipose tissue derived stem cells (BMSCs, ATSCs) are by far the most accessible and their use is not associated with ethical problems (1, 3, 14, 19). BMSCs have a potential to differentiate into glial cells, Schwann cells or neurons, and they can produce many important growth factors and cytokines (1, 2, 11, 15, 18, 20).

Having appropriate equipment at our disposal, necessary experience with tissue culture and being able to gather a team of enthusiastic specialists in different fields of neuroscience and medicine, we decided to evaluate some aspects of a hypothetical idea that modified neural stem/progenitor cells could initiate a neoplastic process in the CNS (10, 17). To avoid any possible adverse reactions, we decided to perform autotransplantation of NS/PCs isolated from own bone marrow of the potential candidates for replacement therapy (5).

Sometimes cats, dogs, or primates are subjects of experimental studies (7, 19). Due to emotional reasons we rejected this option. That is why adult male Sprague-Dawley rats were chosen as the experimental animals most appropriate for our purposes. This decision was influenced by several facts, namely that rats are much bigger than mice, S-D rats are more robust than Wistar rats, so they should be able to tolerate better the stress associated with complex and repeated surgical procedures and the volume of harvested bone marrow, gained by a

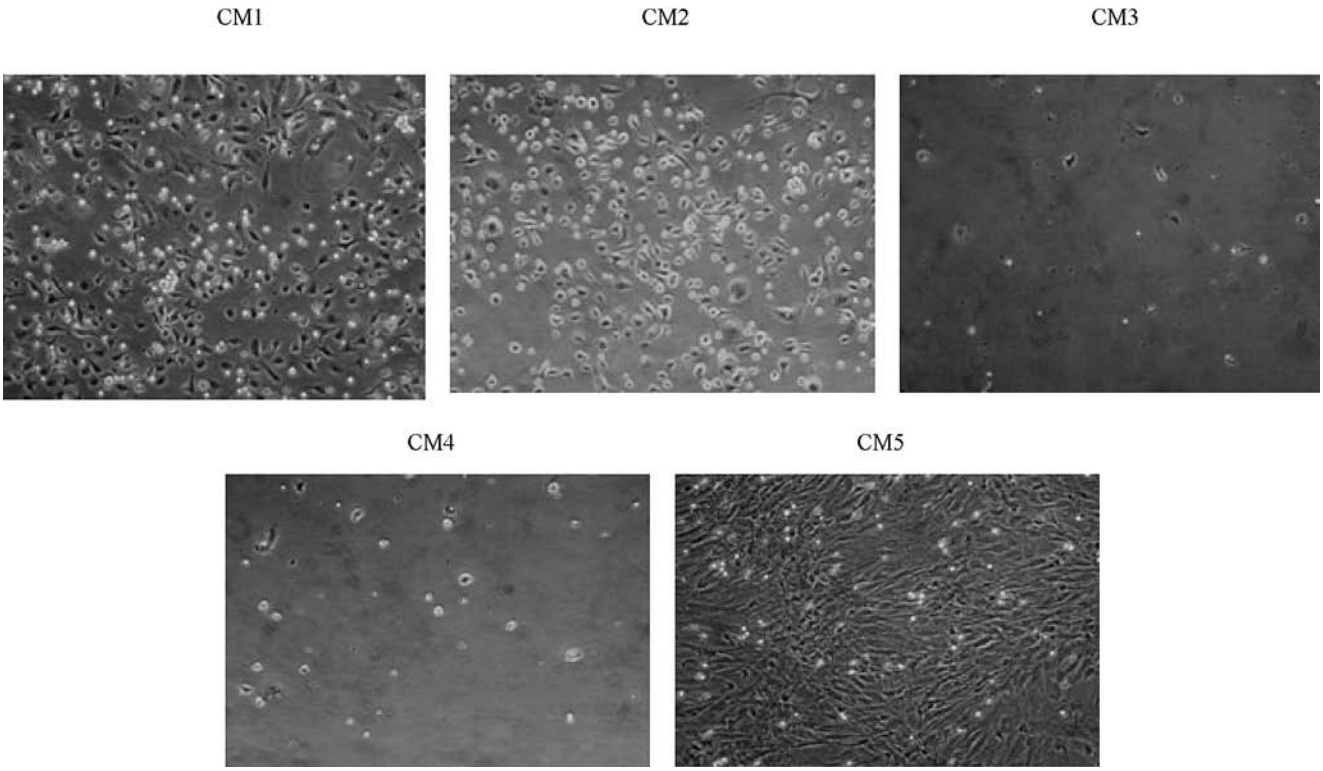

Fig. 4. Light microscopic analysis of rat bone marrow cells cultured for 14 days in different culture media (CM1-CM5)

simple aspiration from trephined thigh bones, should be sufficient for BMSCs isolation without undue danger to the life or health of experimental animals (18).

The constant progress of pharmacology and development of new anaesthesiological drugs has significantly influenced the veterinary surgery in our country during the last few years. Different combinations of *diazepam*, *phenycyclidinum*, *cyclohexaminum*, etc., have been introduced to the everyday veterinary clinical practice (9). We considered utilization of above mentioned drugs in the planning phase of our experiments very seriously due to positive experience with this type of general anaesthesia by other authors and a fact that endotracheal intubation of rats is rather difficult (1, 9). However, the possibility to use halothane or N_2O with medical oxygen administered by a special mask on the one hand, problems with intravenous application and a little higher risk of possible failure of intraperitoneal anaesthesia on the other hand, together with suggestions of an experienced anaesthesiologist (J. C.) who has been a member of our team, finally prevailed.

At last we decided to use in our experiments an inhalation anaesthesia induced by halothane and further maintained with a mixture of halothane with laughing gas and medical oxygen. The results proved that above mentioned type of general anaesthesia was suitable for S-D rats and completely complied with the requirements of our experiments.

Various techniques of stem/progenitor cells harvesting have been advocated during recent several years (1, 5, 15, 18, 20). Some of them require utilisation of the whole foetal or embryonal brain, some removal of both femora, their longitudinal splitting and excochleation of bone marrow or resection of epiphyseal plates and flushing out the bone marrow by a syringe inserted into the shaft, which means the sacrifice of donors (5, 10, 19). We considered such methods unethical, moreover, they would exclude any possible autotransplantation of isolated BMSCs. These disadvantages of stem/progenitor cells harvesting inspired us to develop and test a modified surgical procedure. The described operative technique proved easy and straightforward. In our opinion it could be recommended as a method of choice for harvesting rat bone marrow derived stem cells.

Since nestin is predominantly expressed by neural stem/progenitor cells (NS/PCs), expression of this marker is considered to be the first step in progression of BMDSCs to the neural lineage (15). Under differentiating conditions BMDSCs display a distinct neuronal shape, acquire neuron-like functions and their progeny are competent to differentiate along the neuronal pathway (15). Following 14 day *in vitro* cultivation of BMDSCs in culture medium No. 5, the proportions of CD 45 negative (marker expressed in all haematopoietic lineages at all stages of development) and CD 90 (cell surface marker expressed mainly by mesenchymal stem cells (MSCs))

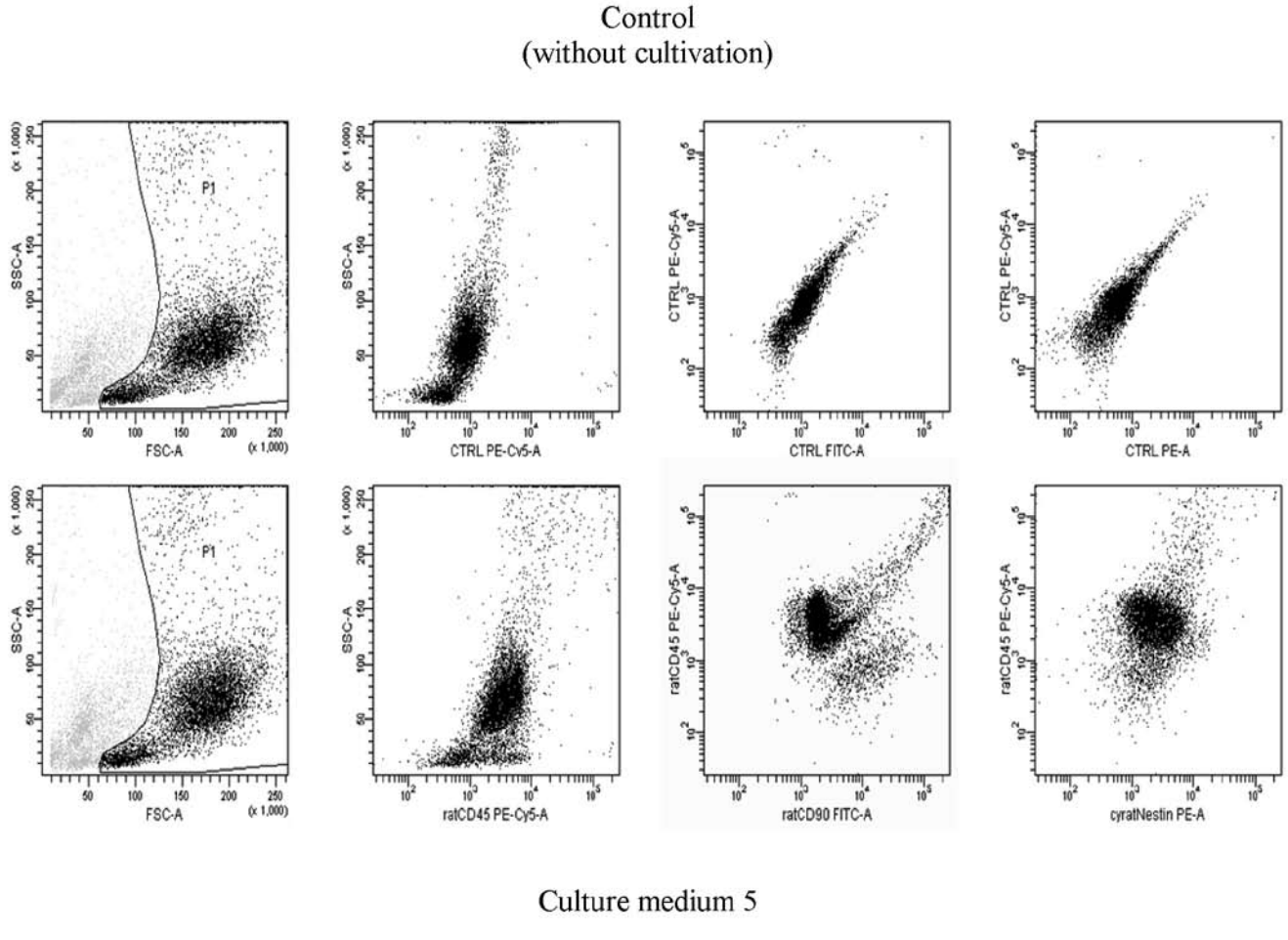

Fig. 5. Flow cytometry analysis of rat bone marrow cells cultured for 14 days in 3 different culture media (Control, CM5). Cells generated under different culturing conditions display distinct patterns of CD90 and nestin expression

and nestin-expressing cells raised from less than 1 % to 19.2–24.9 %.

The results were promising, however, further investigations *in vitro* and *in vivo* are necessary, especially in order to detect the potential of these cells to differentiate into mature cell types of the CNS and to determine whether they possess functional/electrophysiological characteristics of neurons.

Although some experimental studies in animals or pre-clinical human studies demonstrated the effectiveness and safety of MSCs therapy, there are still many questions to be answered regarding the mechanisms of engraftment, homing, intercellular interactions, immunological profiles, in vivo differentiation as well as long term safety.

CONCLUSIONS

The present study showed that a modified technique of bone marrow stromal cells harvesting, culturing and isolation of nestin-expressing NS/PCs was an easy and straightforward procedure causing no unnecessary traumatisation of experimental animals. The Alpha-MEM-based medium supplemented with embryonic stem cells qualified FBS and leukemia inhibitory factor proved to be the most appropriate environment for the rapid *in vitro* expansion of bone marrow derived stem cells with a capacity for further development along the neural lineage.

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Table 1. The composition of culture media (CM) used in the study

Components	CM1	CM2	CM3	CM4	CM5
Basal medium	StemSpan SFEM	StemSpan SFEM	NeuroCult NS-A BM (Rat)	DMEM/F-12	Alpha-MEM
Supplement	StemSpan CC100 (1%)	StemSpan CC100 (1%)	NeuroCult NS-A PS (Rat) (10%)	N-2 Plus Supplement (1%)	ES-FBS (10%)
rh EGF	–	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($20\ ng.ml^{-1}$)	–
rh FGF-b	–	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($20\ ng.ml^{-1}$)	–
rh LIF	–	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)
rat ESGRO	–	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)	+ ($10\ ng.ml^{-1}$)
Heparin	–	–	+ (0.0002%)	–	–
L-Glutamin	–	–	–	+ (2 mM)	+ (2 mM)
PNC/STM	+	+	+	+	+
	+ ($100\ IU.ml^{-1}$/ $100\ \mu g.ml^{-1}$)	+ ($100\ IU.ml^{-1}$/ $100\ \mu g.ml^{-1}$)	+ ($100\ IU.ml^{-1}$/ $100\ \mu g.ml^{-1}$)	+ ($100\ IU.ml^{-1}$/ $100\ \mu g.ml^{-1}$)	+ ($100\ IU.ml^{-1}$/ $100\ \mu g.ml^{-1}$)

The reagents were purchased from Chemicon, Invitrogen, R&D Systems and Stem Cell Technologies (all USA)
StemSpan CC100 contains Flt-3, SCF, IL-3 and IL-6 PNC/STM, penicillin/streptomycin; BM, basal medium; ES-FBS, embryonic stem cells-tested FBS; PS, proliferation supplement; rat ESGRO, recombinant rat LIF

Legend:
rh EGF – recombinant human Epidermal growth factor
rh FGF-b – recombinant human basic Fibroblast growth factor
rh LIF – recombinant human Leukemia inhibitory factor
ES-FBS – embryonic stem cell-qualified foetal bovine serum
alpha-MEM – minimum essential medium
FITC/PE/PE-Cy5 – alternative names for mouse IgG_1
CD45 – is a protein tyrosine phosphatase located in haematopoietic cells, CD90 – in T-lymphocytes
nestin – is a type VI – intermediate filament (IF)-associated protein expressed mostly in nerve cells,
CNS neural stem cells as well as a few non-neural cell types in the embryo- nestin contributes to the cytoskeleton
HBSS – Hank's balanced salt solution
Number and viability of cells was estimated by trypan blue staining.

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DISTRIBUTION OF S-100 PROTEIN IN MANDIBULAR SALIVARY GLAND OF THE SHEEP

Marettová, E., Legáth, J.

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

marettova@uvm.sk

ABSTRACT

The distribution of S-100 protein in the sheep mandibular salivary glands was studied using indirect immunohistochemistry. The distribution of S-100 protein in the exretory ducts system of compound glands followed a characteristic pattern. The gland ducts of all types displayed positive immunoreactivity for S-100 protein. Positive reaction was observed in intercalated ducts, intermediate reaction in the intralobular and interlobular ducts. In the interlobular ducts few S-100 stronger positive cells were interspersed among the moderately stained cells. Neither serous nor mucous secretory cells presented positive reaction to S-100 protein. The myoepithelial cells were negative to S-100 protein. Endothelial cells of blood vessels and nerve fibres displayed strong positive reaction.

Key words: mandibular salivary gland,; S-100 protein; sheep

INTRODUCTION

S-100 protein has been identified in exocrine glands of several mammalian species and a specific role in secretion has been suggested for this protein. Salivary glands have been studied widely in mammals. Hashimoto *et al.* (7, 8) studied S-100 protein in the rat submandibular salivary gland, Makino *et al.* (15), Lee *et al.* (14) and Okura *et al.* (19) studied S-100 protein in human salivary gland tissues and cultured submandibular gland epithelial cells. While Lee *et al.* (14) observed S-100 protein positive cells in some acinar basal cells in the late developmental stage, this protein apparead to be negative in the adult stages. On the other hand, Okura *et al.* (19) found ductal cells positive to S-100 protein in normal salivary gland tissue. Lauboeck and Egerbacher (13) localized S-100 protein and its subunites in a variety of bovine exocrine glands. In this species S-100 protein was identified in the majority of serous secretory cells of mixed salivary glands, although secretory acini in some serous glands remained unreactive for this antigen. Mucous cells were constantly negative.

Staining for S-100 protein has been widely used in histological studies and in diagnostic pathology in order to identify neuroectodermal derivatives. In normal human submandibular and sublingual gland tissues, ducts were moderately positive for S-100 as were ductal cells in non-neoplastic salivary gland associated with pleomorphic adenoma (17, 19).

The purpose of the present study was to investigate the immunoreaction of the secretory cells and the cells of the salivary ducts against S-100 protein in physiological state. Exposure to sawdust and chemicals used in the industry, pesticides, and industrial solvents may increase the risk of salivary gland cancer.

MATERIAL AND METHODS

Seven clinically healthy adult ewes of both sexes weighing between 35–43 kg, aged 2–4 years, were used in this investigation. The samples of mandibular gland were dissected out from sheep and goats in a slaughterhouse. The tissue samples were fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections of thickness 5 µm were deparaffinized with xylene and dehydrated in ethanol using a decreasing gradient.

The sections were pretreated with 3 % H_2O_2 in methanol to block endogenous peroxidase activity and preincubated with 2 % goat serum to mask unspecific binding sites.

The sections were incubated with the first antibody and washed in a phosphate-balanced salt solution (PBS). Afterwards, the sections were incubated with biotinylated secondary antibody for 45 min, washed in PBS and finely incubated with avidin-biotin-peroxidase complex according to Hsu *et al.* (10) (ABC kits, Vector Laboratories, USA). After that the sections were washed with PBS and the reaction product was obtained by incubation for 10 minutes at room temperature using a mixture of an equal volume of 0.02 % hydrogen peroxide and 0.1 % 3,3'-diaminobenzidine tetrahydrochloride made in Tris buffer. To obtain negative controls, the first antibody was substituted by PBS or by normal rabbit- serum.

RESULTS

Positive reaction to S-100 protein in the sheep mandibular salivary gland was limited to the gland duct system while both serous and mucous secretory cells gave negative reaction. The degree of reactivity varied in the intercalated, intralobular, interlobular and main excretory ducts. While the intercalated ducts stained strongly with S-100 protein, the intralobular ones displayed an intermediate reaction. In the excretory interlobular ducts a weak to moderate positivity for S-100 protein was observed (Figs. 1, 2). transition in immunoreactivity between the intercalated and intralobular ducts was also observed. Positive reaction was observed in the cytoplasm while cell nuclei were negative in all ducts.

Myoepithelial cells were consistently negative while positive reaction was observed in nerve fibres. Nerve fibres were located next to the ducts, mainly in the interstitial tissue septa, on the perifery of blood vessels (Fig. 3), and fine nerve fibres were observed also among the secretory acini. Besides, small-size nerve branches and ganglionic cells in the interstitium localized among the glandular lobules were strongly positive (Fig. 4).

DISCUSSION

Calcium is an important second messenger for fluid secretion and exociyosis of secretory cells (20, 21). Merritt and Rubin (16) found that increasing intracellular concentration and secretory processes are closely related in pancreas and in large salivary glands. Several authors suggested a transduction of calcium signals by calcium-binding proteins, such as S-100 protein (3, 4, 5, 9).

Contradictory reports were published on the location of S-100 protein in salivary glands of various animal species and findings related to the mandibular gland were species specific. In the bovine mandibular gland (13), of all salivary ducts only the intercalated ducts were strongly positive. In sheep mandibular glands positive reaction was observed in all ducts, being the strongest in the intercalated ducts mediate in striated ducts and less intensive in interlobular ducts.

Differences were also described in other types of ducts of salivary glands. Molin *et al.* (17) found different reaction for S-100 protein in the individual salivary ducts of different rat salivary glands. In the mandibular gland, immunopositive cells were localized to intercalated, striated, and excretory ducts, as we observed in sheep. In the parotid salivary gland, the intercalated ducts were negative and differed in this respects from those of the mandibular and sublingual glands. There is no expanation for this discrepancy in expression of S-100 protein in major salivary glands. We assume that it could be caused by species differences. No differences among animal species were found in the small Ebner´s salivary gland. The reaction for S-100 protein in the Ebner's salivary gland in other animals was positive, similar to serous cells of other salivary glands.

Lauboeck and Egerbacher (13) identified S-100 protein in most serous acinar cells of mixed glands, while some secretory units failed to react to S-100 antigen in certain serous glands. Mucous cells were generally found to be negative but mucoid cells in the lacrimal and Harderian gland stained for S-100 protein. Molin *et al.* (17) studied S-100 protein in three major rat salivary glands and were not able to demonstrate immunoractivity in acini in the rat submandibular, sublingual and parotis glands. As for the secretory cells of the ovine salivary gland, no positive reaction to S-100 protein was found in serous and mucous secretory cells. Lee *et al.* (14) observed S-100 protein in secretory cells of salivary glands. Nakazato *et al.* (18) supported these findings and confirmed that mucous cells do not react for S-100. In sheep mandibular glands both serous and mucous secretory cells were constantly negative for S-100 protein.

There are also differences in the reaction to S-100 protein in the myoepithelial cells. Some studies reported that myoepithelial cells stained positively stained with antibody to S-100 protein. Makino *et al.* (15) expresed positive reaction to S-100 protein in myoepithelial cells of human parotid glands. Also Haimoto *et al.* (5) and Kahn *et al.* (12) described S-100 protein positivity in myoepithelial cells in normal human tissues of salivary gland tumour. For this reason 100-protein was considered to be one of the markers employed in the identification of myoepithelial cells (1, 6). Okura *et al.* (19) observed ductal cells in normal human salivary gland tissues positive for S-100 protein and found that myoepithelial cells were uniformly negative. Myoepithelial cells were negative for S-100 protein also in bovine submandibular salivary glands (13). Dardick *et al.* (2) who did not find S-100 protein in the myoepithelial cells assumed that S-100 protein staining of the rich network of unmyelinated nerves in the interstitial tissue, obvious ultrastructurally, was misinterpreted as myopeithelium. Thus, it is apparent that S-100 protein positivity in normal salivary gland tissues is exhibited by ductal cells rather than myoepithelial cells.

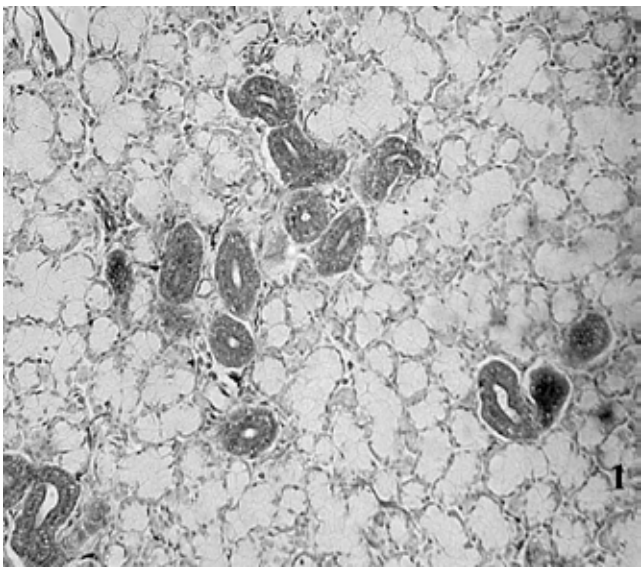

Fig. 1. Intralobulary ducts positive for S-100 protein. Magn. × 240

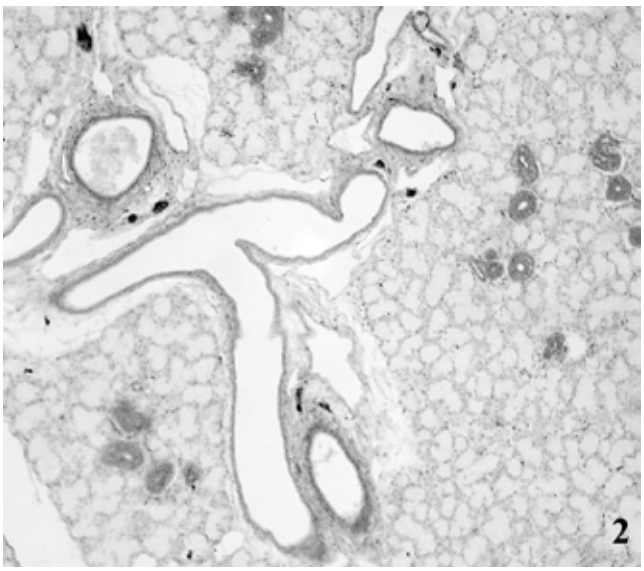

Fig. 2. Interlobulary ducts (in the centre) and intralobulary and intercalated ducts (on the periphery) positive for S-100 protein. Magn. × 120

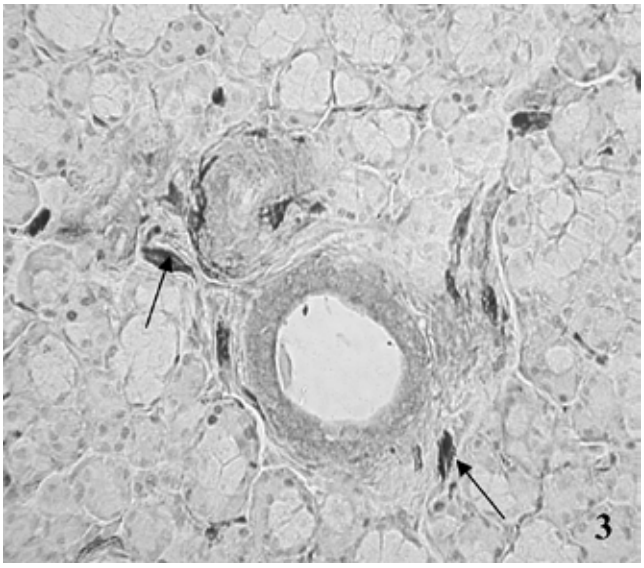

Fig. 3. Intralobulary duct positive for S-100 protein in high magnification. Next to the duct numerous positive nerve fibres and blood vessel are seen (arrow). Magn. × 530

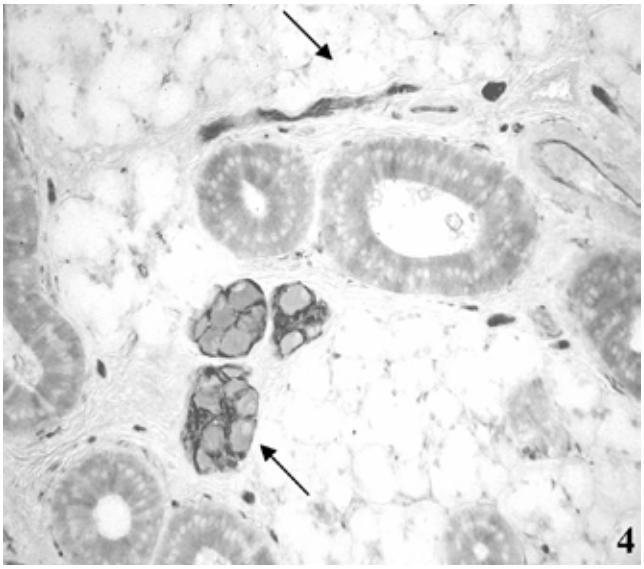

Fig. 4. Ganglionic cells and nerve fibres (arrow) positive for S-100 protein are next to interlobular duct. Magn. × 450

In conclusion, sheep mandibular glands displayed a uniformly distributed immunoreactivity for S-100 protein in all ducts. The discrepancies described in the literature may reflect differences in the metabolism, namely proteins in the serous cells. In addition to the salivary proteins, the serous cells are involved in active secretion of watery fluid containing a variety of inorganic ions in a physiologic state as well as after toxic atack.

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INNERVATION OF THE LINGUAL PAPILLAE IN THE SHEEP AND CATTLE

Marettová, E., Legáth, J.

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

marettova@uvm.sk

ABSTRACT

The distribution of nerve fibres was investigated in tongue papillae of the cattle and sheep with regard to the localization of protein S-100. Nerve fibres are concentrated in the connective tissue of mucosa from where they enter the connective tissue of lingual papillae. Positive nerve fibres for S-100 protein were found to enter the filiform papillae in reduced number and were concentrated at the top of the papillae. Dense accumulation of nerve fibres was found in the fungiform, lenticular and vallate papillae where nerve fibres were distributed centrally in the core of connective tissue of the papillae. Only a few fine nerve fibres were seen at the base of the taste buds in contact with the gustatory cells of taste buds. No nerve fibres were seen to penetrate into the taste buds though these displayed mild positive S-100 protein reactivity. Inside the lining epithelium of lingual papillae nerve fibres were not observed.

Key words: tongue; papillae; innervation; immunohistochemistry; sheep; cattle

INTRODUCTION

In the animals the various lingual papillae are present primarily on the tongue where they are generally divided into mechanical and sensory types (5). Their gross anatomy and structure was studied using histological, histochemical and electron microscopical methods (2, 3, 8, 11, 15, 16, 17, 19, 22, 23). By them the presence of ganglionic structures containing numerous cellular bodies and bundles of fibres, myelinic and amyelinic fibres and nerve corpuscles were found in various organs (12, 13). In the papillary stroma along stromal myelinic fibres the presence of the S-l00 protein was demonstrated (4).

The taste buds, which are composed mainly of clusters of sensory cells, are located in the stratified squamous epithelium of lingual papillae. Mack *et al.* (11) studied number and distribution of the taste buds in the pig which is the best model for human as both are omnivorous (12). The aim of the present study was to localize nerve structures in the sheep and cattle lingual pappillae with special reference to taste buds using the immunohistochemical method.

MATERIAL AND METHODS

Five adult sheep of both sexes weighing between 35–43 kg, 2–4 years old, and five clinically healthy cattle were used in the investigation. The samples of mandibular glands were dissected out of cattle and sheep in a slaughterhouse. The tissue samples were fixed in 10 % buffered formalin and embedded in paraffin. The paraffin sections of thickness 5 µm were deparaffinized with xylene and dehydrated in decreasing ethanol gradient. The sections were pre-treated with 3 % H_2O_2 in methanol to block endogenous peroxidase activity and pre-incubated with 2 % goat serum to mask unspecific binding sites. They were incubated with the first antibody (polyclonal S-100 protein, Sigma) and washed in phosphate-balanced salt solution (PBS). Afterwards, the sections were incubated with biotinylated secondary antibody for 45 min. washed in PBS, and finely incubated with avidin-biotin-peroxidase complex according to Hsu *et al.* (6) (ABC kits, Vector Laboratories, USA). After that the sections

were washed with PBS and finally reaction product formation was achieved by incubating for 10 minutes at room temperature using a mixture of an equal volume of 0.02 % hydrogen peroxide and 0.1 % 3,3'-diaminobenzidine tetrahydrochloride made in Tris buffer. For negative controls, the first antibody was substituted by PBS or normal rabbit serum.

RESULTS

The thick bundles of nerves run among the muscle bands of the tongue from which smaller nerve bundles reach the connective tissue of the mucosa layer. Single nerve fibres and small bands enter into primary and secondary connective tissue papillae and reach the basal membrane of the basal surface of the lining epithelium (Figs. 1, 2). No nerve fibres were found to enter into the epithelium of filiform papillae. In fungiform papillae numerous nerve fibres positive for S-100 protein were seen, concentrated inside the top of primary papillae (Fig. 3). The most dense accumulation of the S-100 protein nerve fibres in both species studied were seen in the vallate papillae. Numerous nerve fibres from the connective tissue of the mucosa layer entered into the vallate papillae and ran directly to the apical surface of the papillae (Fig. 3). Despite the presence of numerous nerve fibres in the corresponding lamina propria of fungifom and vallatae papillae of the tongue (Fig. 4) only a few fine nerve fibres were seen at the base of the sensory cells of the taste buds. No nerve fibres were seen to enter

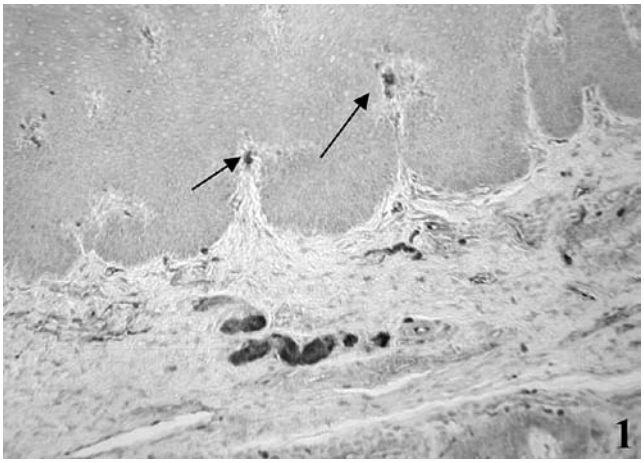

Fig. 1. Section of the cattle tongue in the filiform papillae. Small bands of nerve fibres are seen in the connective tissue. Fine nerve fibres reach the top of the connective tissue (arrows). Magn. × 200

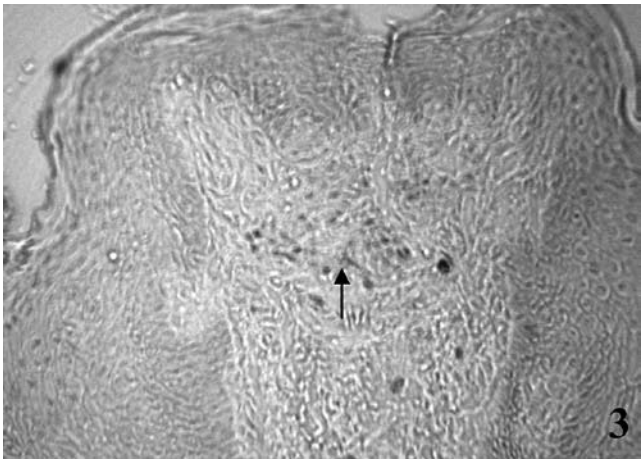

Fig. 3. Section of the sheep fungiform papilla. Nerve fibres are found centrally in reduced number in the papillae (arrow). Magn. × 200

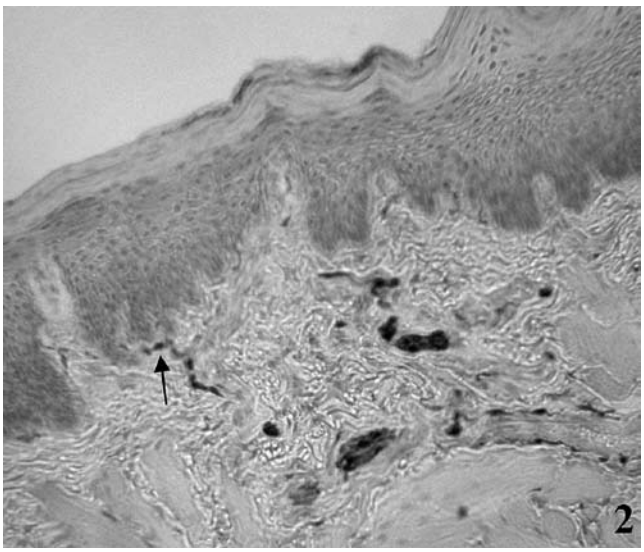

Fig. 2. Section of the sheep tongue in the interpapillary area. From the small bands of nerve fibres in the connective tissue branches of nerve fibres run at the basal surface of the epithelium (arrow). Magn. × 200

Fig. 4. Section of the cattle vallata papilla. Many S-100 protein positive nerve fibres are found in the base of the papilla. Fine nerve fibres run directly to the apical surface of the papilla. Magn. × 10

into taste buds and to have visible contact with gustatory cells. It is worth to mention that whole taste buds displayed positive reaction to S-100 protein. Outside the lingual papillae, nerve fibres of various thickness and positive for S-100 protein were distributed horizontally in the connective tissue of the mucosa.

DISCUSSION

In the tongue of the cattle and sheep numerous protein S-100-containing structures were found. The nerve fibres were found at several locations, i.e. in the glandular tissue, connective tissue, and around blood vessels. The density and distribution of the nerve fibres in these locations varied. Less abundant nerve fibres were found in the filiform and fungiform papillae. The occurrence of nerve fibres in the filiform papillae was studied electronmicroscopically by Böck (2, 3). The orientation of the nerve fibres in the observed species resembled that in sheep and cattle, and ran towards the tip of the papilla in parallel to the capillary loops. The entrance of the nerve fibres into the epithelial cells, described by Böck (3) and Sato *et al.* (18), could not be confirmed with S-100 protein.

Inside the vallate papillae the nerve fibres have been most numerous and thus were found in various animals species. Richly innervated vallate papillae were described by Vittoria *et al.* (20) in buffalo and in human tongue by Astbäck (1). According to Budetta *et al.* (4) the papillary stroma of the buffalo lingual papillae contains myelinic and amyelinated nerve fibres. We observed high density of nerve fibres in the vallate papillae in cattle and sheep. The presence of terminal nerve corpuscles, reported by the authors, could not be confirmed in the species studied after immunostaining with S-100 protein.

Studies made on the lingual papillae in various animal species (9, 10) detected presence of nerve fibres inside taste buds. These studies concluded that the taste buds in the lingual papillae are innervated by sensory nerve fibres which make contact with certain types of gustatory cells. Our observations made in the sheep and cattle tongue correspond with findings obtained by Yoshie *et al.* (21) who described neuron-specific proteins in the taste bud in guinea pigs. The authors mentioned did not find any nerve fibres positive for S-100 protein in none of the cells of the taste bud, but exclusively in the subepithelial elements of the connective tissue. Within the epithelium of the vallate papillae no peptidergic fibres were found in the mentioned study. On the other hand, Kusakabe *et al.* (10) investigated human circumvallate papillae and observed immunoreactive peptidergic nerve fibres densely distributed in the connective tissue core of the circumvallate papillae and some fibres associated with the taste buds.

Though previous immunohistochemical studies have revealed that immunoreactivity for neurofilament protein is not contained in every kind of neuron (7), later publications reported that sensory neurons were intensely immunoreactive to anti- neurofilament protein sera. Sato *et al.* (18) noted a dense distribution of neurofilament protein and S-100 protein in the human taste buds of the fungiform and vallate papillae. Our observations of sheep and cattle tongue showed a fine nerve fibres densely distributed in the connective tissue core of the vallate papillae, but no fibres were seen inside the taste buds after S-100 protein immunostaining.

The sensory lingual papillae are rich in sensory nerves because of their dual innervation with general somatic and gustatory nerves. These findings extend the knowledge about both the general and neurochemical messenger-based innervation of fungiform papillae which can serve as a solid basis for functional investigations of normal, experimental and clinical materials.

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PRESENCE OF AMINO ACIDS IN SPECIFIC TISSUES OF THE TWO HYBRIDS OF COMMON CARP *(Cyprinus carpio,* L.*)*

Buchtová, H.[1], Svobodová, Z.[1,2], Velíšek, J.[2]

[1]University of Veterinary and Pharmaceutical Sciences Brno
[2]University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodňany
The Czech Republic

buchtovah@vfu.cy

ABSTRACT

The aim of the study was to compare the amino acid composition in relation to edible parts in two groups of carp, i.e. hybrid of Hungarian and Northern mirror carp (M2 × M72) and scaly hybrid of Přerov carp and Ropsha carp (PŠ × ROP) in harvest size (K_3). The authors have demonstrated the existence of frequent significant differences ($P < 0.05$ and $P < 0.01$, respectively) in the quantitative composition of amino acids in the hybrids (M2 × M72, PŠ × ROP) in dependence on specific tissues. Only leucine (Leu) and methionine (Met) (M2 × M72) and threonine (Thr) (PŠ × ROP) were the same in all carp parts investigated. Fish muscle protein was rich in lysine (Lys), leucine (Leu), asparagine (Asp) and glutamine (Glu). In comparison with other edible parts, muscle tissues of both of the hybrid contained more histidine (His) ($P < 0.01$). Of all the edible parts investigated, the highest essential amino acid (EAA_{sum}) concentrations (M2 × M72: $P < 0.05$, PŠ × ROP: $P < 0.01$) were found in the testes, EAA_{sum} were also higher in hard roe of PŠ × ROP scaly hybrid ($P < 0.01$). The hard roes of female carp (PŠ × ROP) contained, besides the non-essential glutamine (Glu), mainly valine (Val), while hard roes of female carp (M2 × M72) contained, besides glutamine (Glu), mainly glycine (Gly). The hepatopancreas of the hybrids (M2 × M72, PŠ × ROP) contained significantly ($P < 0.05$ and $P < 0.01$, respectively) more valine (Val), phenylalanine (Phe), arginine (Arg), serine (Ser) and glycine (Gly) but less lysine (Lys) and histidine (His) than the muscle tissues.

Key words: carp; chemical composition; fillet; gonad; hepatopancreas; protein

INTRODUCTION

Results on concentration results of individual amino acids in different tissues of edible parts in carp may be important for the use of new biotechnologies in commercial processing of fish. Specific chemical composition (concentration of proteins, particularly concentrations of Lys and Glu) of muscle tissues is relevant to biotechnology (production of mechanically separated fish meat, use of transglutaminase enzyme for its processing), which are currently being experimentally tested for the processing of carp in the Czech Republic prior to their introduction to freshwater fish processing technologies (2, 3, 4). In some parts of the world (U.S.A., Japan, Germany), these technologies are commonly used for processing of salt-water fish, mainly of the family *Gadidae*, and are the subject of research (5, 6, 7).

This article is a follow-up to the paper by Buchtová *et al.* (1), in which the authors discussed the differences in the percentage composition of amino acids in edible parts (fillet, hard and soft roe, hepatopankreas) between the hybrids tested. The experiment made it possible to determine the differences in amino acid composition between the fillet and the remaining edible parts separately for the M2 × M72 hybrid and for the promising PŠ × ROP hybrid. In this case we may assume the existence of numerous significant differences in the amino acid spectrum, which testify to the existence of physiologically determined representation of individual amino acids according to the type of the tissue analysed. The M2 × M72 mirror hybrid carp is intensively reared in the Czech Republic for commercial purposes. The PŠ × ROP scaly hybrid was chosen

as a promising breed for aquaculture purposes on the basis of the above-mentioned comprehensive research.

The aim of the study was to compare the amino acid composition in relation to edible parts (fillet, gonads, hepatopancreas) in two groups of carp, i.e. mirror hybrid of Hungarian and Northern mirror carp (M2 × M72) and scaly hybrid of Přerov scaly carp and Ropsha scaly carp (PŠ × ROP) in harvest size (K_3).

MATERIALS AND METHODS

Performance test of scaly phenotypes of the common carp were started in 2001 by the fish farming company Rybníkářství Pohořelice. In the experiments, Přerov scaly carp (PŠ) were used at the maternal position. The PŠ was crossed with males of other carp breeds (top crossing) namely the Přerov scaly carp (PŠ) for the production of a pure line, Ropsha scaly carp (ROP) and Northern mirror carp (M72). The mirror carp hybrid of the Hungarian and the Northern mirror carp (M2 × M72) were used as controls. The establishment of test populations and the test protocol were designed according to Linhart *et al.* (8).

In the first test year, the fry of each experimental group were stocked separately into two ponds (totally 6 ponds of 0.06 ha) together with its control group (M2 × M72) of different scaliness. The stocking density was 20 000 fish per 0.06 ha. At advanced fry stage (after 6 weeks), the carp were fished out, checked for weight and the stocking density was reduced to 1,600 fish/0.06 ha. Because natural diet was abundant, the fry were only fed limited amounts of sieved wheat meal and, exceptionally, carp feed mixture pellets (KP1). The next growth and survival check was made at the end of the vegetation period before the carp were transferred to special wintering tanks (2001).

From 2002 onward, the fish were group marked and kept in mixed stocks in three ponds, namely the Antonín (2 700 carp/1.27 ha), Bohumír (3 400 carp/1.60 ha) and Jaroslav ponds (2 400 carp/1.13 ha). Two-year old grass carps were introduced in all ponds (150 grass carp per pond) to control macrophytes. For additional feeding of the fish tested, uncrushed whole wheat was used. Checks of fish weight and survival checks were always made before and after the wintering period.

The performance assessment of dressing out parameters was made at the end of the 2003 vegetation period for K_3 from all three fishponds (258 carp in total). A total of 15, 14 and 35 carp of each group were randomly selected from fishponds Antonín, Bohumír and Jaroslav, respectively.

The fish were processed in a standard manner in the Mušov Freshwater fish slaughter house of the Rybníkářství Pohořelice Comp. (9). The dressing out was performed manually. The fish were scaled, gutted and filleted. Gonads were separated from viscera. The head was cut from the skeleton by circular cut in front of the pectoral fin girdle so that the fin girdle remained at the body. Fillets were made with skin separating the flesh from spine and rib bones.

Laboratory tests of quantitative and qualitative composition of amino acids were made in 40 carp: 10 of each line (5 females + 5 males) from the pond Jaroslav. Fish from that pond scored best results in efficiency tests (10). The determination of quantitative and qualitative composition of amino acids was performed by HPLC.

To prepare samples for amino analysis, 0.5 g from each mixed sample with 0.0001 g accuracy (PRECISA 240 A, France) were used (homogenization: Moulinex ILLICO Y92, Ireland). The samples were prepared by acid hydrolysis ($HCl = 6\ mol.l^{-1}$) for 24 hours at 110 °C. The amino acid assay was performed on a AAA T 339 automatic amino acid analyzer (Mikrotechna Praha, Czech Republic). For their separation, sodium – citrate elution buffers in a chromatographic column with catex (OSTION LG ANB, Czech Republic) were used. After colour reaction with ninhydrin, separated amino acids were detected by a flow photometer. AMIK software 3.0. (Czech Republic) was used to calculate retention times and areas of individual amino acid peaks, and to process the data. Reagents necessary for preparation of samples, buffers and AAA operation were supplied by the amino analyzer manufacturer. Solution of standard amino acid mixtures, also supplied by the AAA manufacturer, were used as external amino acid standards.

Basic statistical values (means, S.D.) of the parameters investigated were processed in Excel 97. Statistical significance was evaluated using the multifactorial analysis of variance (ANOVA, Statgraphics 6.0).

RESULTS

All the groups of carp M2 × M72 ($P < 0.05$) and PŠ × ROP ($P < 0.01$) and all the edible parts studied, the highest part of essential amino acid (EAA_{sum}: Thr, Val, Ile, Leu, Phe, Lys, His, Arg, Met) and the lowest part of non-essential amino acid ($NEAA_{sum}$: Asp, Ser, Glu, Pro, Gly, Ala, Tyr) were found in soft roe. Compared with fillets and the hepatopancreas, EAA_{sum} were also higher in hard roe of PŠ × ROP scaly hybrid ($P < 0.01$). The significantly lowest EAA_{sum} and the highest $NEAA_{sum}$ were found in female gonads of M2 × M72 mirror carp controls ($P < 0.05$). EAA_{sum} and $NEAA_{sum}$ in muscle tissues and the hepatopancreas of PŠ × ROP hybrids were also practically identical (Fig. 1).

From the qualitative point of view, the same spectrum of amino acids was found in analysed parts (fillets, gonads and hepatopancreas) of all groups of carp. However, frequent differences were found in amino acids among different edible parts. Only Leu and Met (M2 × M72) and Thr (PŠ × ROP) were the same in all carp parts investigated (Figs. 2 and 4).

Significant differences ($P < 0.05$ and $P < 0.01$) were observed in the composition of amino acids identical for the carp groups investigated (M2 × M72, PŠ × ROP) (Figs. 2–5).

In comparison with other edible parts, muscle tissues contained

- significantly more Phe, Lys, His, Asp a Glu but less Ser and Gly than hard roe
- significantly more Phe, His, Asp and Glu but less

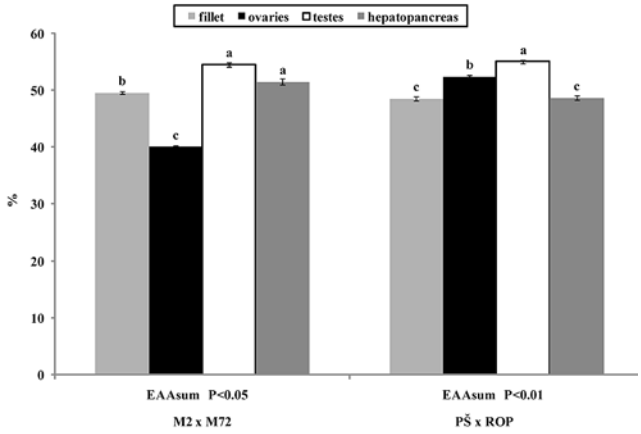

Fig. 1. Percentage of essential amino acid contents (EAA_{sum}: Thr, Val, Ile, Leu, Phe, Lys, His, Arg, Met) (in % of total amino acids investigated) in edible parts of mirror (M2 × M72) and scaly (PŠ × ROP) crossbreds of the common carp (Cyprinus carpio, Linnaeus 1758).

Groups with different alphabetic superscript differ significantly at the given level of probability.
M2 – Hungarian mirror carp, M72 – Northern mirror carp, PŠ – Přerov scaly carp, ROP – Ropsha scaly carp

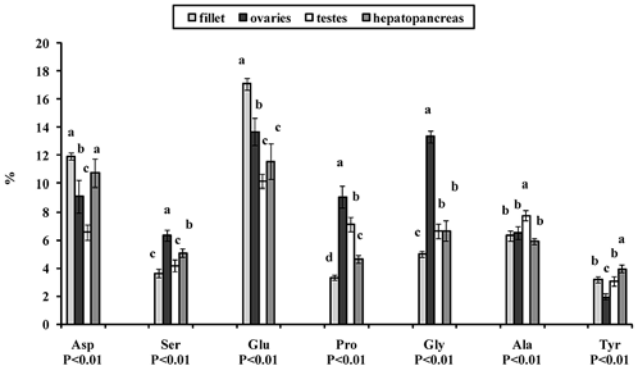

Fig. 3. Percentage of non-essential amino acid contents (in % of total amino acids investigated) in edible parts of the group (M2 × M72) of the common carp (Cyprinus carpio, Linnaeus 1758).

Groups with different alphabetic superscript differ significantly at the given level of probability.
M2 – Hungarian mirror carp, M72 – Northern mirror carp

Lys, Arg, Gly and Ala than soft roe

• significantly more Lys and His but less Val, Phe, Arg, Ser and Gly than the hepatopancreas.

Compared to soft roe, hard roe contained significantly more Ser, Glu and Gly but less Lys and Arg.

Other significant differences ($P < 0.05$ and $P < 0.01$) in amino acids were not the same in all the carp groups investigated (M2 × M72, PŠ × ROP).

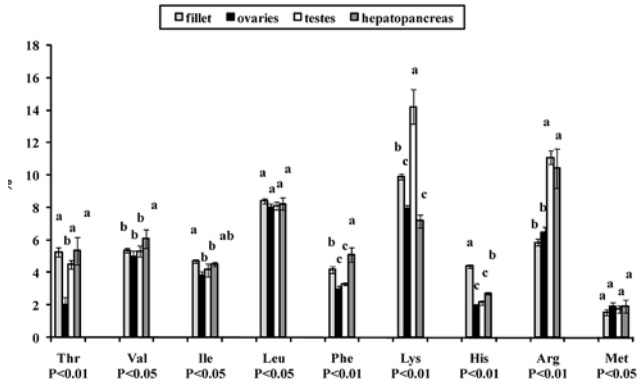

Fig. 2. Percentage of essential amino acid contents (in % of total amino acids investigated) in edible parts of the group (M2 × M72) of the common carp (Cyprinus carpio, Linnaeus 1758).

Groups with different alphabetic superscript differ significantly at the given level of probability.
M2 – Hungarian mirror carp, M72 – Northern mirror carp

M2 × M72 mirror carp hybrid (Figs. 2 and 3).

Muscle contained significantly more Thr, Ile and Tyr but less Pro than hard roe; more Ile but less Pro than soft roe, and more Glu but less Pro than the hepatopancreas. Compared with soft roe, hard roe contained significantly more Asp and Pro but less Thr, Ala and Tyr.

PŠ × ROP scaly carp hybrid (Figs. 4 and 5).

Muscle contained significantly more Met and Pro but less Val, Ile, Arg and Ala than hard roe; more Met but less Ile and Pro than soft roe, and more Pro than the hepatopancreas. Compared with soft roe, hard roe contained significantly more Val, Leu and Phe but less Ile and Pro.

DISCUSSION

Meat of Czech Carp (registered in Trade Mark Registry of the Czech Republic on 22 October 2001) is highly appreciated for its specific aroma, delicate taste, short cooking times and digestibility. Muscle is the main part of fish body used for human nutrition. The muscle tissues and edible organs from both groups of carp (M2 × M72, PŠ × ROP) in this experiment investigated contained highly nutritious proteins (11). Figs. 2–5 showed that each fish muscle protein was rich in two essential (Lys, Leu) and two non-essential (Asp, Glu) amino acids. In comparison with other edible parts, muscle tissues both hybrid contained significantly more His ($P < 0.01$). The importance of these amino acids, as essential precursors for the synthesis of proteins and other molecules with enormous biological importance, was reported by Wu et al. (12). Glu is the most abundant free amino acid in

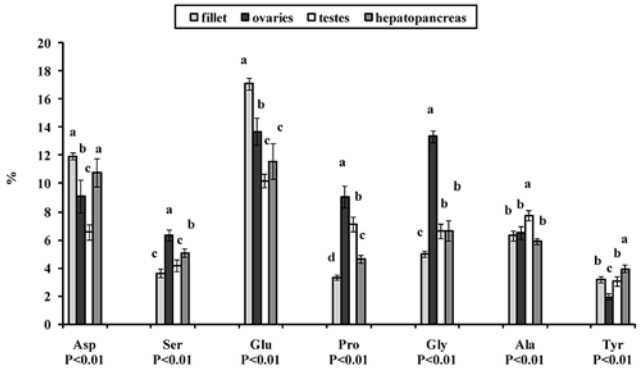

Fig. 4. Percentage of essential amino acid contents (in % of total amino acids investigated) in edible parts of the group (PŠ × ROP) of the common carp (Cyprinus carpio, Linnaeus 1758).

Groups with different alphabetic superscript differ significantly at the given level of probability. PŠ – Přerov scaly carp, ROP – Ropsha scaly carp

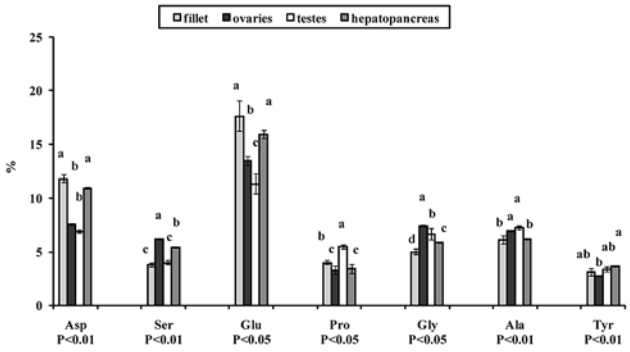

Fig. 5. Percentage of non-essential amino acid contents (in % of total amino acids investigated) in edible parts of the group (PŠ × ROP) of the common carp (Cyprinus carpio, Linnaeus 1758).

Groups with different alphabetic superscript differ significantly at the given level of probability. PŠ – Přerov scaly carp, ROP – Ropsha scaly carp

the body, comprising nearly 60 % of the free intracellular amino acids in skeletal muscle. As a donor of nitrogen in the synthesis of purines and pyrimidines Glu is essential for the proliferation of cells (13). These references dealing with marine fish were used because of lack of relevant data on freshwater fish in available papers.

The authors have demonstrated the existence of significant differences ($P < 0.05$ and $P < 0.01$, respectively) in the quantitative composition of amino acids in the hybrid types (M2 × M72, PŠ × ROP) of the common carp in dependence on specific tissues. The proteins differed in their composition of individual amino acids, which depends on the type of the tissue analysed. Only Leu and Met (M2 × M72) and Thr concentrations (PŠ × ROP) were the same in all carp parts (fillet, gonads, hepatopancreas) investigated (Figs. 2 and 4). Kim and Lall (14) assumed that the composition of amino acids in muscles of a specific fish species was relatively stable. Few papers have reported the effect of dietary protein levels on the body amino acid composition in fish (15). Schwarz and Kirchgessner (16) reported that dietary protein level had no obvious on the body amino acid pattern in common carp. On the other hand, considerable variability in the composition of amino acids, particularly in blood plasma of fish, was reported in relation to differences in the diet (13).

Of all the edible parts investigated, the highest EAA_{sum} concentrations (M2 × M72: $P < 0.05$, PŠ × ROP: $P < 0.01$) were found in the testes, EAA_{sum} were also higher in hard roe of PŠ × ROP scaly hybrid ($P < 0.01$) (Fig. 1). Higher amounts of proteins in hard roe of largemouth bass (*Perciformes*, *Centrarchidae*) than in muscle tissue have also been reported by Portz and Cyrino (17).

Some authors believe that quantitative and qualitative composition of specific amino acids in carp gonads may vary in dependence on the sexual cycle (18). The reason for differences frequently found in the content of amino acids among fillet, hard and soft roes and hepatopancreas probably lies in different stages of their gonadogenesis, which are related to proven differences in growth capabilities between the groups of carp studied (M2 × M72, PŠ × ROP), and the differences in the weight of gonads and the gonadosomatic index (GSI) (10). Detailed data on the morphology, composition and fertility of carp hard roe were published by Linhart et al. (19).

In the Czech Republic, carp gonads are harvested during their commercial processing in freshwater fish processing plants. Gonads are then used to make fish soup. Hard roe of carp in the Czech Republic is also used for the production of Golden Caviar (20). The hepatopancreas of rod-caught carp is also consumed mainly in anglers' households. In comparison with other edible parts, hepatopancreas of both hybrids (M2 × M72, PŠ × ROP) contained significantly more Val, Phe, Arg, Ser and Gly but less Lys and His than the muscle tissues (Figs. 2–5). Data on the amino acid composition of the hepatopancreas of the carp available to date are scarce.

CONCLUSION

In conclusion, results of the present study manifest the existence of frequent significant differences ($P < 0.05$ and $P < 0.01$, respectively) in quantitative composition of amino acids in the hybrids (M2 × M72, PŠ × ROP) in dependence on specific tissues (fillet, gonads, hepatopancreas). Only Leu and Met (M2 × M72) and Thr (PŠ × ROP) were the same in all carp parts investigated. Fish muscle protein was rich in Lys, Leu, Asp and Glu.

The hard roes of female carp (PŠ × ROP) contained, besides the non-essential Glu, mainly Val, while hard roes of female carp (M2 × M72) contained, besides Glu, mainly Gly.

From the point of view of the specific composition of Lys and Glu, muscle tissues are very suitable for mechanical separation of fish meat and for introduction of modern biotechnologies, such as the use of transglutaminase enzyme, which are currently tested in the Czech Republic in carp. The use of biotechnologies in hard roes, where we found highly significantly ($P < 0.01$) lower Lys concentrations compared with muscle tissues, will require further experimental verification.

ACKNOWLEDGEMENT

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TUMOUR DISEASES IN DOGS

Capík, I.[1], Ševčík, A.[1], Ševčíková Z.[2]

[1]Clinic of Small Animals
[2]Insitute of pathological anatomy
University of veterinary medicine, Komenského 73, Košice
The Slovak Republic

capik@uvm.sk

ABSTRACT

Diagnosis and treatment of neoplastic diseases show increased occurrence in small animal practice. This clinical study provides a summary and analysis of occurrence of tumours in dogs treated at the Clinic of surgery, orthopaedics and roentgenology in two 3-year periods with 10-year interval between them. The objective of the study was to identify the most frequently affected tissues, histogenetic proportion of tumours and their mutual ratio-relationship over the period of ten years. Our results indicated a relatively higher risk of occurrence of neoplastic diseases in Boxers, Dachshunds, Schnauzers and Poodles, in descending order. Of the most frequently affected tissues the skin and its adnex rated as first (52.3% and 48.2%, resp.) in both observation periods. The second most frequently affected organ was the mammary gland which accounted for 26.6–27.9% of all treated tumours. The following primarily affected organs were oro-nasal cavities (7.8% and 8.9%), male reproductive organs (testes – 5.4 and 4.9%) and female reproductive organs (ovaries, uterus, vagina – 4.7 and 2.4%). An increased number of surgically treated tumours was associated with the fifth to twelfth year of the life of dogs. We observed that the total number of malignant neoplasms increased in the second observation period by 27.3% on average.

Key words: dog; histopathology; incidence; neoplasm; predisposition

INTRODUCTION

Tumour diseases result from the action of various external and internal factors (3, 4). The most frequently mentioned factors responsible for initiation and development of the neoplastic process include ultraviolet radiation, oncogenic viruses and chemical carcinogens. The internal influences that may support development of tumours include dietetic and hormonal influences, genetic predisposition and age. In many cases it is not possible to identify the cause due to synergistic effect of both external and internal initiating factors.

The ultraviolet portion of solar radiation has been described as a common cause of skin carcinomas. They are observed more frequently in cats than in dogs (5). In addition to the influence of ultraviolet radiation, skin carcinomas are induced also by traumatic and viral influences or frequent exposure to X-rays. The most frequent skin neoplasms are carcinoma keratoides cutis (cancroid, spinaliom, carcinoma spinocellulare, tilelike cellular carcinoma) and from among the benign tumours adenomas, located particularly in head, neck and perianal areas (7, 8). Predisposition to adenomas has been described in Cockers Spaniels and Poodles (1, 2, 9).

The influence of hormones has been associated with adenomas of perianal glands (6). In males, particularly older ones, the incidence was described as 10 times higher compared to females. Occasionally this type of tumours has been diagnosed also in neutered dogs. Breed predisposition (Cocker Spaniel, Bulldog, Spitz-dog, Beagle) has been described also with this tumour with 2–5 times higher frequency than in other breeds (10). Adenocarcinomas of perianal glands are rare. These neoplasms occur more frequently in females but do not account for more than 1% of perianal tumours. Adenocarcinomas that are not removed surgically may metastasise relatively quickly into pelvic lymphatic nodes (11).

The oncogenic viruses (RNA, DNA) implicated most frequently in inducing neoplastic processes are feline leukemia

virus, papovaviruses, adenoviruses and herpesviruses (3). The majority of papillomas in young cattle, horses and dogs are of viral origin (papovaviruses) and may vanish spontaneously after some period. However, they may also be transformed to tilelike cellular carcinoma (10). Recently the importance of skin carcinomas has been increasing, particularly in larger agglomerations. We can speak about civilisation disease caused mostly by excessive irritation and load on the organisms due to chemical pollution of the atmosphere.

The influence of food as one of the internal factors on the development of tumours has been recognised since long time ago (11). Fat is the component of food that acts as an internal factor contributing to the development of many tumours. It is one of the most suspicious food components supporting growth of mammary gland tumours. Excess body weight is also a risk factor that may result in initiation of tumours.

Human oncology obtained evidence indicating relationship between various chemicals and increased risk of urinary bladder carcinomas. Similar risk involving urinary bladder tumours was identified also in dogs exposed frequently to contact insecticides for the purpose of controlling external parasites (flees, ticks).

MATERIAL AND METHODS

The present study provides a summary and analysis of occurrence of tumours in dogs treated at the Clinic of surgery, orthopaedics and roentgenology in two 3-year periods with 10-year interval between them.

During the first 3-year period of our investigations conducted at the Clinic for small animals we examined altogether 5 567 dogs and during the second 3-year period 4 648 dogs.

The evaluation involved only the dogs with history related to neoplastic diseases which were either treated surgically in order to remove tumours or were euthanized because of the extent of tumour growth and unfavourable prognosis. Therefore it does not provide absolute review of incidence of tumour diseases in population of dogs in the respective area.

The following information was recorded for each patient: breed, gender, age, tissue affected by the neoplastic process and the result of histopathological examination.

By recording the proportion of affected breeds we tried to reveal the developmental trend and potential predisposition of breeds to tumours.

At the same time we investigated the relationship between the number of affected dogs of certain breeds and the frequency of surgical therapy in the respective breeds. During investigation the animals of respective breeds were not divided to subgroups (e. g. the breed Schnauzer included small, medium and large Schnauzer dogs).

The tumours were divided according to their location in tissues with the aim to evaluate the occurrence of tumours in various tissues and organs.

The objective of the study was to identify the most frequently affected tissues, histogenetic proportion of tumours and their mutual ratio-relationship over the period of ten years.

RESULTS

The number of dogs treated within the two periods of observation differed. The number of dogs treated during the first period was almost by 1 000 dogs higher (5 567) than that in the second period (4 648) (Fig. 1).

During both observation periods the number of treated breeds increased. While in the first year of the first period we examined dogs of 58 breeds plus mongrels as one separate group, in the third year of the same period we treated dogs of 75 breeds plus mongrels.

In the first 3-year period as much as 87.2 % of treated dogs belonged to 14 breeds including the mongrels. The percentage proportion of dogs of each of these 14 breeds reached minimally 1 %. The remaining 61 breeds were represented by less than 1 % of the total number of treated dogs (altogether 12.8 %).

In the second 3-year period we examined dogs of 87 breeds in the first year and 97 breeds in the third year. Again, higher than 1 % proportion was reached only with 24 breeds including the mongrels the proportion of which reached 81.9 % and the majority of breeds (i. e. 73 breeds) accounted for less than 1 % (altogether 18.1 %) of the total number of dogs treated in the respective period (Fig. 2).

In the first period of observation out of total number of treated dogs (n = 5 567) 126 were subjected to surgical therapy which accounts for 2.3 % of all treated dogs.

In the second observation period 247 of the total number of 4 648 examined dogs were surgically treated, i. e. 5.3 % proportion (Fig. 3).

Relationship between incidence of tumours and the age of dogs is illustrated in Fig. 4. This figure shows that the prevalence of neoplastic diseases started to increase markedly in the fifth year of dog life. With the increasing age, particularly after reaching twelve years of age, the incidence of tumours decreased gradually.

Observation of primary localisation of neoplastic growth showed that the tumours involved most frequently the skin and adnex and the surgical therapy of mammary gland tumours rated as second. No significant differences in prevalence of tumours in the respective tissues were observed between the two periods of examination. In the first period we recorded a somewhat wider range of tissues affected by neoplastic growth (Fig. 5).

The relationship between percentage proportion of breeds affected by neoplastic growth in the two observation periods is shown in Fig. 6. In both observation periods the percentage proportion of surgically treated tumours in mongrels, Poodles, Boxers and Dachshunds was higher than the percentage proportion of treated dogs of the respective breeds.

Higher percentage proportion of surgically treated tumours in relation to percentage proportion of treated dogs of the respective breed during only one investigated period was observed for German Shepherd and Cocker Spaniel in the period of 1995–97 and Schnauzer, Springer Spaniel and Rottweiler in the period of 2005–07.

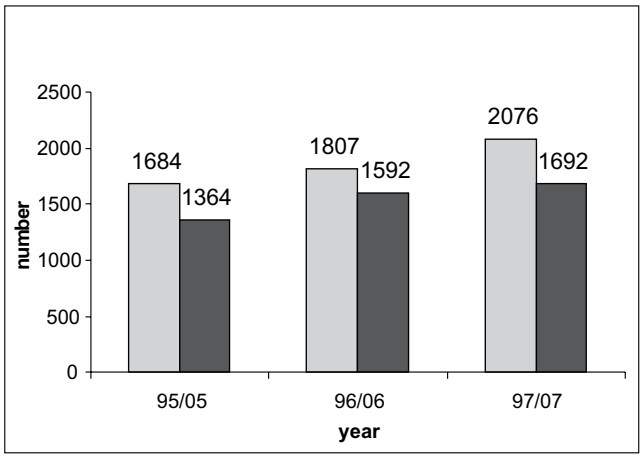


Fig. 1. Comparison of the number of examined dogs after 10-year interval

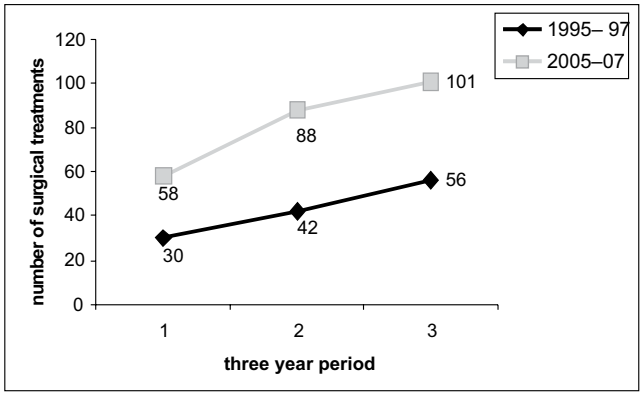


Fig. 3. Number of surgically treated tumours in the investigated periods

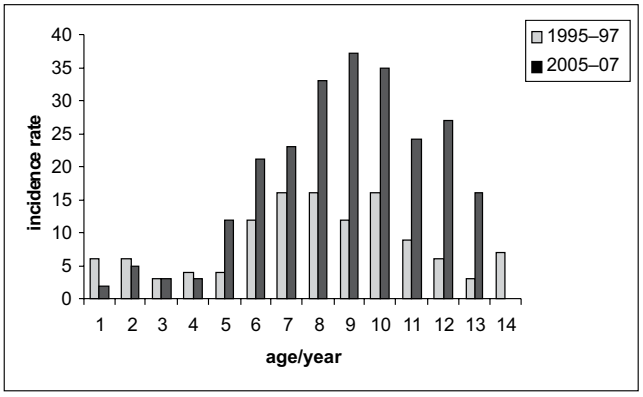


Fig. 4. Relationship between age and prevalence of surgical treatment of tumours

The most frequently affected tissues were skin and its adnex which accounted for approx. 50 % of all surgically treated neoplasms. The mammary gland rated as second most frequently affected organ showing approx. 27 % prevalence of tumours.

The third place in the number of surgical interventions

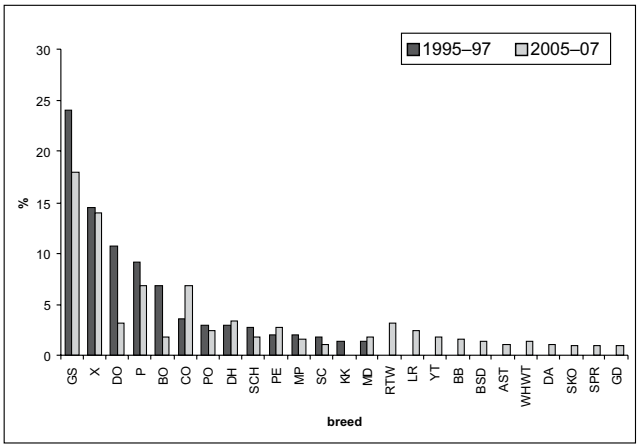


Fig. 2. Percentage proportion of breeds in the examined periods that accounted more than 1 % of all treated dogs

GS – German Shepherd, X – mongrel, DO – Doberman, P – Poodle, BO – Boxer, CO – Cocker Spaniel, PO – Pomerian, DH – Dachshund, SCH – Schnauzer, PE – Pekingese, MP – Miniature Pincher, SC – Slovak Cuvac, KK – Kavalier King Charles Spaniel, MD – Maltese Dog, RTW – Rottweiler, LR – Labrador Retriever, YT – Yorkshire Terrier, BB – Bavarian Bloodhound, BSD – Bern Sheepdog, AST – American Staffordshire Terrier, WHWT – West Heighland White Terrier, DA – Dalmatian, SKO – Slovakian Hound, SPR – Springer Spaniel GD – Great Dane

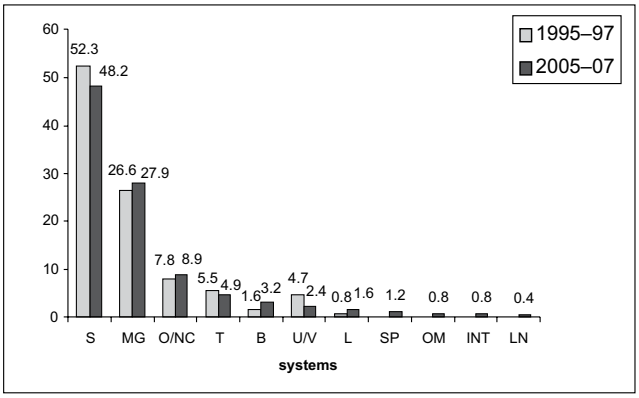
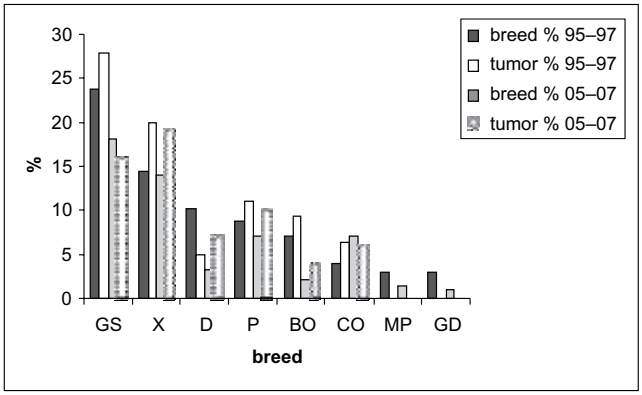
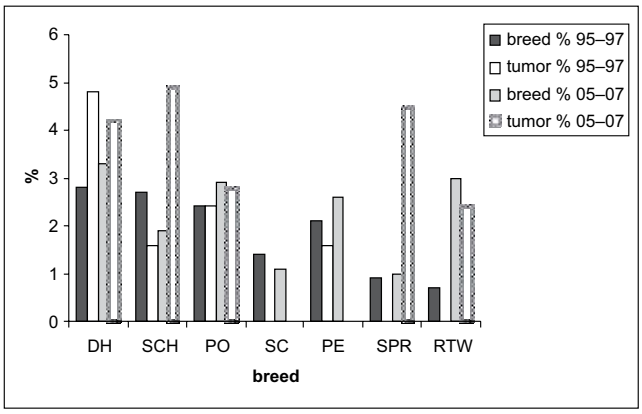


Fig. 5. Percentage proportion of tumours in individual organ systems in 10-years interval

Legend: S – skin and adnex, MG – mammary gland, O/NC – oral and nasal cavity, T – testes, B – bones, U/V – uterus and vagina, L – liver, SP – spleen, OM – omentum, INT – intestines, LN – lymph nodes

was occupied by tumours located in oral and nasal cavities. Their proportion in the first and second examination periods reached 7.8 % and 8.9 %, respectively. We observed an increase in the number of surgical interventions in the nasal cavity (rhinotomy, maxilectomy). While in the years 1995–97 only two nasal cavity tumours were treated surgically, in the period of 2005–07 the number of surgically treated tumours of this type increased to eight which represented 400 % increase.





Figs. 6a, b. Relationship between percentage proportion of breeds in the periods of 1995–97/2005–07 and prevalence of neoplastic diseases in the respective breeds

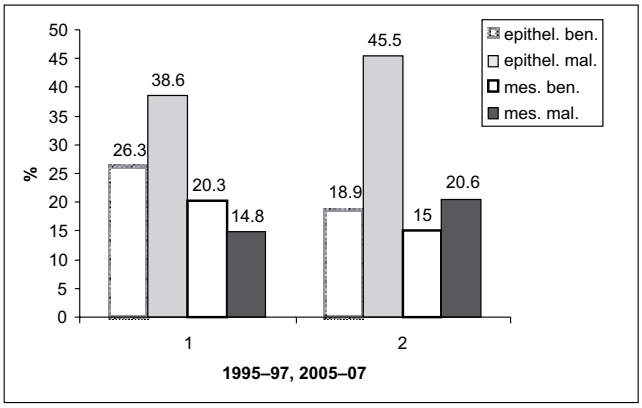


Fig. 7. Proportion of skin tumours

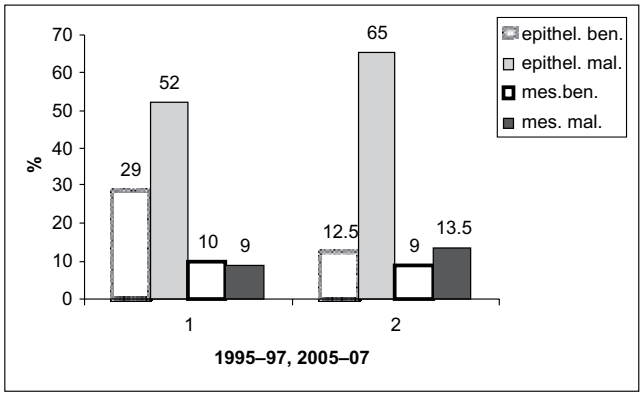


Fig. 8. Proportion of mammary gland tumours

Results of histological examination of most frequently observed skin and mammary gland tumours are shown in Figs. 7 and 8.

DISCUSSION

Neoplastic diseases constitute a group of diseases frequently diagnosed in veterinary practice. In the recent years we observed an increase in the occurrence of these diseases. Also our observations of prevalence of tumours in dogs in two 3-year periods (years 1995–97 and 2005–07) confirmed this trend. Comparison of the number of surgical interventions showed a 130 % increase in surgical therapy of tumours in the period of 2005–07 in comparison with the period of 1995–97. We observed an increase in the number of surgically treated cases despite smaller number of dogs examined in the period of 2005–07. This decrease in the total number of treated dogs can be explained by establishment of new private veterinary ambulances and the logical redistribution of patients, resulting in partial decrease in the total number of patients.

The relationship between the age of dogs and prevalence of neoplastic diseases indicated an increase in prevalence between years five and twelve of age. In the two observed periods we found differences in the character of treatment related to therapy of tumours. While no distinct differences in frequency of surgical therapy of 5 to twelve years old dogs was observed in the period of 1995–97, in the second observation period (2005–2007) the frequency of surgical interventions used for the treatment of tumours increased for the dogs eight to ten years old.

One of the factors resulting in a decreased number of dogs treated for tumours after the year twelve of age can be the decrease in the number of dogs which live longer due to some other diseases or natural mortality. We have in mind particularly large breeds of dogs which generally have shorter life than smaller breeds.

Of the most frequently affected tissues the skin and its adnex rated as first (52.3 % and 48.2 %, resp.) in both observation periods.

The second most frequently affected organ was the mammary gland which accounted for 26.6–27.9 % of all treated tumours. The following primarily affected organs were oro-nasal cavities, male reproductive organs (testes) and female reproductive organs (ovaries, uterus, vagina).

Investigations of the potential relationship between breeds and neoplastic processes indicated relatively

higher susceptibility of some breeds to these diseases. We assumed that breeds with highest proportional representation are guarantors of future preservation or even increase in the number of respective breeds. However, our results also showed some "fashionable" trends in dog keeping which were reflected in a marked decrease in the number of Dobermans and Boxers and increase in the number of Rottweilers. However, for the majority of observed breeds we did not detect substantial changes between the two observation periods in proportion of the most frequently kept breeds which allowed us to draw some relevant conclusions.

Our results indicated a relatively higher risk of occurrence of neoplastic diseases in Boxers, Dachshunds, Schnauzers and Poodles, in descending order. The risk for mongrels was about the same as for Poodles. However the group of mongrels consisted of dogs derived from both large and small breeds and therefore this group could not be considered homogenous regarding the relevant category of evaluation and could not be compared with pedigree dogs.

When evaluating the neoplastic diseases we used the ratio of benign and malignant growth as the last parameter. Regarding tumours of skin and mammary gland, we observed an increase in malignant skin and mammary gland tumours by 23.5 % and 28.7 % resp., when comparing the situation in 2005–07 with that in the period of 1995–97.

Similar results were obtained also for other affected systems. A considerable increase in the number of malignant tumours was detected in the oral and nasal cavities. While the majority of oro-nasal tumours in the period of 1995–97 were oral epulides (87 %), in the period of 2005–07 we recorded not only 120 % increase in the total number of oro-nasal tumours but also significant increase in the number of nasal tumours. With regard to the susceptibility of breeds to development of nasal tumours our observations supported the assumption of relatively higher susceptibility of Cocker Spaniel breed to this type of tumours.

CONCLUSION

Our results allowed us to draw the following conclusions:

- The number of dog tumours increased over the evaluated period.
- With the increasing number of tumour diseases a wider range of tissues was affected although skin and mammary gland tumours were present in the highest proportions (78.9 % – 1995–97, 76.1 % – 2005–07).
- An increased number of surgically treated tumours was associated with the fifth to twelfth year of the life of dogs.
- No distinct changes in primary localisation of tumours were observed between the evaluated periods.
- Our results indicated that some dog breeds are more susceptible to development of tumour diseases.
- We observed that the total number of malignant neoplasms increased in the second observation period by 27.3% on average.

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WELANIMAL PROJECT (LLP-LdV-TOI-2007-TR-002) A NEW APPROACH ON DIFFERENT ASPECTS OF WELFARE, ENVIRONMENT AND FOOD INTERACTIONS IN CENTRAL AND SOUTHESTERN EUROPE WITH THE USE OF ICT

Bozkurt Z.[1], Endre S.[2], Sossidou E.[3], Katalin G.[4] Mishev P.[5], Ludovic C.[6], Venglovosky J.[7]

[1]Afyon Kocatepe University
Turkey
[2]Szent István University Faculty for Agricultural and Environmental Sciences
Hungary
[3]National Agricultural Research Foundation
Greece
[4]University of West-Hungary Faculty of Agricultural and Food Sciences
Hungary
[5]University of National and World Economy
Bulgaria
[6]Banat University of Agricultural Sciences and Veterinary Medicine
Romania
[7]University of Veterinary Medicine in Kosice
The Slovak Republic

Zhra.bozkurt@gmail.com

ABSTRACT

In recent years, the effects of welfare needs of animals, environment and "from farm to fork" food quality have been demonstrated scientifically.

The project (WELFOOD) product and results of training tools related to the animal welfare, environment and food quality interactions will be enriched considering cultural, socio-economic and religious aspects in order to determine a common work definition for all sectorial workers having different moral and social values on the subject of animal welfare and food safety. To produce training tools that are used in communication technologies will be determined.

The target group of the project are students, teachers, sectoral entrerprise workers and their family members (elderly or disabled members, wife/husband and children) and project partners are Central and southeastern Europe countries (Turkey, Greece, Bulgaria, Romania, Hungary and Slovakia).

INTRODUCTION

Despite the continuous debate on whether animals bear conscious experience, the recent scientific studies by ethologists have demonstrated that the animal's perception of world is not simple and crude. The possibility of thinking and feeling abilities of non-humans cause to criticise our responsibilities toward animals and more significantly to raise the feeling of guilt for animal-derived food and anxiety on food quality and safety.

INNOVATIVE RESULTS OR THE CONTENT THE PROJECT IS BASED UPON

In WELFOOD three main training curricula and products have been developed: *Animal welfare, Environmental influence on animal* and *their welfare* and *Food quality and safety.*

In this project, training curriculum for animal welfare needs, animal and environmentally friendly product techniques were prepared as a starting point of consumers' structure. However, the current project will cover and be adapted to the central and southeastern European region. Therefore, the area of vocational training associated with animal welfare and food quality and safety in WELFOOD project in Europe will be extended to eastern Europe.

Products of WELFOOD project will be translated into national languages. It will take into account regional and social differences and characteristics. Coordinator and one of the partners of the WELFOOD project will be partners of the new project and will contribute as internal evaluator and advisor for development and usage of project' products, and will guide the use of the products.

In 6 countries in central and southeast Europe regional differences will be evaluated and training products of WELFOOD project will be adapted according to regional conditions. Social, economic, cultural and religious approaches on animal welfare concepts of European community will be handled and welfare understanding and definition will be more globalized. More extensive sampling will be done for current discomforts on animal welfare and prevention of cruelty to animals kept on European farms. Conventional national vocational training curricula will be improved by a multidisciplinary approach and European dimension will be brought in. In the region, a training curriculum which will produced for the use on both national and European scale.

EXPECTED IMPACT OF THE PROJECT

The expected short-term impact of the project is envisaged in training of target groups within their cultural perspective in the topic of animal welfare and food safety with the use of courses and printed products, encouragement of the use of ICT techniques and provision of knowledge, skill and quality (provision of certificate). In the long term the members of target groups should qualify as hidden workforce (sectoral workers' family and partners, children, elderly etc who work without payment) and reinforce the workforce market. Orientation of work force on new technology will support production of high-quality and healthy food, and establish workers' knowledge on animal welfare.

METODOLOGY OF THE WELANIMAL PROJECT

1. Current situation (knowledge) analysis of local conditions and comparison with the WELFOOD project's products and adaptation in the partnership region.

A "WELANIMAL evaluation platform" will be assembled by encouraging all organisations, establishments and institutions to participate in work with the target groups and sectors at the possible widest level. Therefore, as the current knowledge and skill of target groups in each partner country towards animal welfare, environmental impacts on and of animals and food safety and safety and training requirements will be adequately and completely determined, sectoral requirements will be met by the Project products where possible.

Each partner within his country will analyse the knowledge and skill of workers on farms and in food sectors in food production chains about interactions between animal welfare, food quality and safety, evaluate the laws and regulations that affect directly or indirectly animal welfare in the fields of vocational training curriculum, agriculture, food and transportation.

2. Personal initiatives to improve animal welfare and food safety and development of training methods.

Within the project, in the central and southeast European region the potential effects of socio-cultural, economic and religious factors on animal welfare definition and sensitivity of workers in animal husbandry and food industry and on animal product consumers will be determined by the method of questionnaire. The data obtained within this project will be compared with WELFOOD project products. Then, the WELFOOD vocational training curriculum in the light of effects and related results of regional cultural, economic and religious differences will be revised.

It is planned that a new common European work definition for the graduates of animal welfare and food safety training and facilities, except for the internet, towards worker's family and children in animal farm and their reflections to the training modules will be developed. ICT methods of project training will be adapted and the results will be translated into national languages and English.

3. Development of Methodology, training modules and educational materials and tools.

The European common work description on mutual basis will be provided as the education material and detailed ICT tools related to topics in English and Turkish languages will be prepared and used in the training curriculum. To develop an animal welfare and food quality guide for academicians, veterinarians and agricultural engineers, to produce a summary CD-ROM on the Project includes the methodology and teaching materials, related cartoons as an education materials regarding the program for people who have no access to the web and the course program. Radio and TV programs covering all the related societies will be considered. Enough feedback on education modules and materials from the target groups will be provided and the project outputs and effects will be determined on national and international basis.

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