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ANTIOXIDANT PROPERTIES OF SELECTED ETHANOL PLANT EXTRACTS

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ABSTRACT

The growing interest in the substitution of synthetic antioxidants by natural antioxidants in the cosmetic and food industry has focused on the research of plant sources. The antioxidative effects of four commercial ethanolic extracts were studied utilizing two different *in vitro* assays. Pure extracts (0.2%) of agrimony (*Agrimonia eupatoria* L.), lemon balm (*Melissa officinalis* L.), sage (*Salvia officinalis* L.), and red grape (*Vitis vinifera*) were used. For the detection of potential synergic effects of antioxidant components, the antioxidant activities of red grape in combination with other extracts were also observed. The ability of scavenging free radicals was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) reduction spectrophotometric assay, and the inhibition of lipid oxidation were tested by the modified TBARS (thiobarbituric acid reactive substances) assay. The total phenolic contents of extracts were measured by the Folin-Ciocalteu method and the total flavonoids content was determined according to the aluminum chloride colorimetric method. Extracts of agrimony, lemon balm, red grape and their combinations revealed strong DPPH scavenging activity (65.90–91.34%) and the total phenolic compounds varied between 21.8 and 70.9 μg.ml^{-1}. The extract from sage showed the lowest DPPH scavenging ability (65.88%) and total phenolic content (21.8 μg.ml^{-1}). The content of flavonoids varied from 13.5 μg.ml^{-1} in sage, to 27.1 μg.ml^{-1} in lemon balm extracts. In the TBARS assay, the maximum inhibition of lipid oxidation was reached in the extract of lemon balm (77.74%) and red grape (65.8%), whereas the lipid inhibition in the extract of sage was only 16.47%. The correlation between the antioxidant activity and the total phenolic contents of extracts, indicated that phenolic compounds were a major contributor of antioxidant activity of these plant extracts. The synergic effect of extract combinations was not found. All tested combinations showed lower antioxidant activity in comparison to individual extracts.

Key words: antioxidant activity; phenols; plant extract; total phenolic content

INTRODUCTION

Many species of herbs have been recognized to have medicinal properties and beneficial impact on health, e.g. antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic potential (1). Antioxidant activity is one of the most important because it helps the organism to fight against oxidative stress, defined as an imbalance between oxidants, potentially leading to damage. In modern medicine the balance between antioxidation and oxidation is believed to be a critical factor for maintaining a healthy biological system (3). A general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds (polyphenols, carotenoids), due to their well known health benefits.

Synthetic phenolic antioxidants, such as BHT (buthylated hydroxytoluene), BHA (buthylated hydroxyanisole), and TBHQ (tert-butylhydroxyquinone), effectively inhibit lipid oxidation. However, concern from consumers regarding such additives has stimulated investigations into the benefits of natural antioxidants as substitutes for synthetic antioxidants (4). Crude extracts of herbs rich in phenols are of increasing interest in the food industry, because they retard oxidative degradation of lipids and thereby improve the qual-

ity and nutritional value of food (26). Epidemiological studies have shown that the consumption of foods and beverages rich in phenols can reduce the risk of heart disease (14).

Many plants have been identified as having potential antioxidant activities. Bioactive phenols, especially flavonoids are very interesting as antioxidants because of their natural origin and the ability to act as efficient free radical scavengers (15, 17). A direct relationship between antioxidant activity and the phenolic content of plant extracts has been reported (7, 10), but depends on; maturity, cultivars, horticultural practices, geographic origin, growing season, postharvest storage conditions and processing procedures (8, 11).

Red grape skin, a by-product obtained after wine production, is a cheap material for the extraction of antioxidant compounds (23). Grape skin is a source of anthocyanidins, anthocyanins, and natural pigments with antioxidant properties acting through the inhibition of lipoperoxidation, and which also have antimutagenic activities (20). Herbs from the *Lamiaceae* family are well-known for their antioxidant properties and in recent years their extracts have been used as antioxidants in the food industry. Phenolic acids, flavonoids and terpenoids isolated from agrimony belong to the main antioxidant constituents. The main antioxidant effect of sage has been reported in relation to the presence of phenolic diterpenes (such as carnosic acid and carnosol) and, especially, rosmarinic acid (2, 21).

Two main types of assays can be performed to measure the antioxidant ability of natural substances: (a) assays for radical scavenging ability and (b) assays measuring the ability to inhibit lipid oxidation. As all methods are based on different chemical and physical principles of oxidation monitoring, the antioxidant activities may vary according to the assay used (22), particularly in matrices rich in oxidisable lipids (lard, vegetable oils, egg yolk; 19). In addition, the comparisons of the results from different experiments are often complicated because of the different chemical composition of the extracts, which are also dependent on several factors.

The aim of our study was to determine the antioxidant activity and content of the total phenols and flavonoids in commercially prepared ethanolic extracts of; sage (*Salvia officinalis* L.), agrimony (*Agrimonia eupatoria* L.), lemon balm (*Melissa officinalis* L.), and red grape (*Vitis vinifera* L.), and their combinations.

MATERIALS AND METHODS

Samples of commercial dry plant extracts (extraction was performed with 40 and 50 % ethanol) used in our experiment were purchased from Calendula a. s. (Nová Ľubovňa, Slovensko). All chemicals and reagents were of analytical grade and if not stated otherwise, purchased from Sigma-Aldrich (Germany) and from Merck (Germany). The dry extracts were diluted with ethanol at 0.2 % concentration (0.2 g in 100 ml of ethanol) and were prepared for subsequent measurements.

Determination of antioxidant activity using stable DPPH radical

Free radical scavenging ability using a DPPH radical (2,2-diphenyl-1-picrylhydrazyl) according Heilerova *et al.* (5) was performed. The radical stock solution was prepared fresh daily. DPPH solution in methanol (0.025 g.l^{-1}) in a volume of 3.9 ml was pipetted into a 1 cm cuvette and the absorbance value A_0 was read against a blank at 515 nm using a spectrophotometer Helios γ v 7.1, Thermospectronic (Great Britain). The plant extracts in a concentration of 0.2 % were added to the cuvette containing the DPPH solution (3.9 ml) in the amount of 0.1 ml. The absorbance was measured in 1 minute intervals during 7 minutes (A_7). The measurements were performed in four replications.

The percentage inhibition of the DPPH radical by the samples was calculated according to the formula:

$$\% \text{ inhibition} = \{A_0 - A_t / A_0\} \times 100$$

where A_0 is the absorbance of the control at t = 0 min;
A_t is the absorbance of the antioxidant at t = 7 min.

TBARS assay

A thiobarbituric acid reactive substances (TBARS) assay without lipid peroxidation inducer according Miguel *et al.* (19) was used to measure the potential antioxidant capacity of the extracts. Egg yolk homogenates were used as a lipid-rich media. The absorbance of the samples prepared according the procedure was measured at 532 nm (Helios γ v 7.1, Thermospectronic, Great Britain). All samples were prepared in four replicates. The results were expressed as the antioxidant index (AI %) and calculated using the formula:

$$AI\ \% = (1 - t/c) \times 100$$

where c – the absorbance value of the fully oxidizes control
t – the absorbance of the tested sample

Determination of total phenolic content

The total phenol content was determined spectrophotometrically using Folin-Ciocalteu reagent (25) and 1 ml of 0.2 % plant extract (diluted with water 1 : 10). Samples (1 ml, 4 replicates) were introduced into test tubes, 5 ml of Folin-Ciocalteu's reagent (diluted 1 : 10 with water) and 4 ml Na_2CO_3 (75 g.g^{-1}). The absorbance was measured at 765 nm after incubation at room temperature (21 °C) for 30 minutes (spectrophotometer Helios γ v 7.1, Thermospectronic). The total phenolic content was expressed as microgrammes of gallic acid equivalents per 1 ml of 0.2 % of ethanol extract. Analysis was performed in four replications.

Determination of total flavonoids

The total flavonoids were measured by a colorimetric assay developed by Kim *et al.* (12). A 1 ml aliquot of an appropriately diluted sample or a standard solution of quercetin (0.5 mg.ml^{-1}) was added into a 10 ml volumetric flask containing 4 ml of H_2O. Immediately, 0.3 ml 5 % $NaNO_2$ was added to the flask. After 5 minutes, 0.3 ml 10 % $AlCl_3$ and at 6 minutes 2 ml 1 M NaOH were added to the mixture. The samples were diluted by adding immediately 2.4 ml of H_2O and were mixed thoroughly. Absorbance of the samples was determined at 510 nm (spectrophotometer Helios γ v 7.1, Thermospectronic) versus a water blank. The flavonoids were expressed as quercetin equivalents (μg.ml^{-1}).

Statistical analysis

All of the data were analysed statistically using GraphPad Prism Software, Version 4.00 (2003). One-way analysis of variance (ANOVA) with the post hoc Tukey's multiple comparison test was

used to evaluate the statistical significance of differences among the groups of extracts and $P < 0.05$ was considered as the statistically significant difference. All data were presented as mean values and standard deviation (mean ± SD).

RESULTS

The results of the antioxidant capacity of the extracts and their combinations are shown in Table 1. The antioxidant capacity with the use of the DPPH radical ranged from 65.90 to 91.34 %. It decreased in the following order: red grape > agrimony > lemon balm > agrimony + red grape > lemon balm + red grape > sage + red grape > sage. There were significant differences between the percentage inhibitions of the DPPH radical in individual extracts ($P < 0.05$). Synergic effects of combinations of red grape extract with other extracts were not detected. All combinations showed the lower DPPH scavenging capacity in comparison to their individual extracts. The only combination with higher scavenging activity was red grape in combination with sage ($P < 0.05$), but it was a significantly lower value than the extract of red grape ($P < 0.05$).

The results of TBARS after ethanol extract additions are reported in Table 1. In comparison to the DPPH method, the values were lower in all extracts and combinations and ranged from 16.47 % in sage to 77.74 % in lemon balm. The highest ability to inhibit the lipid peroxidation was detected in lemon balm and red grape extract. The significant decreasing of antioxidant activity in comparison to the results obtained by the DPPH method was recorded in agrimony. The antioxidant activity of sage was the lowest and the synergic effect of extract combination was also not confirmed.

Table 1. Antioxidant capacity of extracts expressed in percentage inhibition

Extracts	Antioxidant capacity %	
	DPPH	TBARS
Agrimony	$90.85^{a} \pm 0.31$	$40.16^{b} \pm 5.59$
Sage	$65.90^{e} \pm 0.71$	$16.47^{c} \pm 4.94$
Lemon balm	$85.43^{b} \pm 1.16$	$77.74^{a} \pm 2.52$
Red grape	$91.34^{a} \pm 0.72$	$65.88^{a} \pm 5.34$
Sage + Red grape	$73.26^{d} \pm 2.84$	$43.80^{b} \pm 3.01$
Lemon balm + Red grape	$74.74^{d} \pm 0.38$	$48.66^{c} \pm 14.27$
Agrimony + Red grape	$79.84^{c} \pm 1.18$	$50.01^{b} \pm 23.80$

$^{a, b, c, d, e}$ – Results with different superscript in the column differ significantly ($P < 0.05$)

The results of the spectrophotometric measurements of the total phenols and flavonoids are recorded in Table 2. The amount of the total phenols varied from 21.8 to 70.9 μg. ml^{-1} of the tested extracts ($P < 0.05$). The higher content of the total phenols was recorded in red grape extracts and it decreased in the order: red grape > agrimony + red grape > agrimony > lemon balm + red grape > lemon balm > sage + red grape > sage. The flavonoids content ranged from 13.5 (sage) to 27.1 $\mu g.ml^{-1}$ (lemon balm). The highest content of flavonoids was detected in the lemon balm and red grape extracts. The lowest content of flavonoids was recorded in sage extracts, similarly to the results from the total phenols determination.

Table 2. The total phenolic content ($\mu g.ml^{-1}$) and related amount of flavonoids ($\mu g.ml^{-1}$) in extracts and their combinations

Extracts	Total phenols (GAE)	Flavonoids (Q)
Agrimony	$65.2^{b} \pm 0.6$	$18.5^{c} \pm 0.4$
Sage	$21.8^{d} \pm 0.3$	$13.5^{d} \pm 0.2$
Lemon balm	$40.2^{c} \pm 0.8$	$27.1^{a} \pm 0.4$
Red grape	$70.9^{a} \pm 0.9$	$22.0^{b} \pm 0.5$
Sage + red grape	$39.2^{c} \pm 0.3$	$19.7^{c} \pm 0.4$
Lemon balm + red grape	$62.5^{b} \pm 1.2$	$23.2^{b} \pm 0.3$
Agrimony + red grape	$65.6^{b} \pm 0.2$	$19.4^{c} \pm 0.2$

GAE – gallic acid equivalents; Q – quercetin

$^{a, b, c, d}$ – Results with different superscript in the column differ significantly ($P < 0.05$)

DISCUSSION

The ethanol extracts of all herbs at 0.2 % concentration used in this study demonstrated free radical scavenging activity and the ability to inhibit the lipid oxidation processes. However, the efficiency of each species differed depending upon the particular assay methodology, reflecting the complexity of the mechanisms involved in the total antioxidant capacity (18). Furthermore, different methods of preparation of the extracts and the location and harvesting could influence their antioxidant capacity (13). The most significant example is agrimony, it showed high DPPH scavenging activity, but its ability to inhibit lipid oxidation, as determined by the TBARS method, was the second lowest. The extracts of lemon balm and red grape were, in both performed methods, detected as extracts with high antioxidant activity. According to Katalinic *et al.* (9), lemon balm can be considered "the antioxidant queen" between 70 selected medicinal plants

used in experiments. The high antioxidant activity for lemon balm and sage was also confirmed in a study according to Chrpova *et al.* (6). However, sage extract in our study showed the lowest antioxidant activity by both of the determinations. It could be caused by the assays, but also by different extractant (ethanol and water). The different extractants and a higher ability to extract the antioxidant compounds, could cause the significant differences in the results.

There was a correlation between the total phenol content and the radical scavenging ability. The higher the content of the total phenols, the higher the antioxidant activity was found. The results obtained are in a good agreement with the literature data (6, 16, 23), where the authors determined positive correlations between the total phenolic content and DPPH radical scavenging ability for the grape skin, lemon balm and sage.

Typical phenolics that indicate antioxidant activity are known to be mainly phenolic acids and flavonoids (26). Our results confirm that the extract with the highest content of flavonoids (lemon balm, red grape) showed the highest antioxidant activity. This confirms the statement that flavonoids significantly affect the antioxidant activity (24).

The important role in protecting human health is to find effective natural antioxidants and replacement the widely used harmful synthetic antioxidants. The added amount of natural antioxidants could be as low as possible. Some antioxidants are able to potentiate the antioxidant activity of other ones and thereby they could be more effective in lower levels. The aim of our work was also to follow the synergic effect of red grape extract in combination with sage, agrimony and melissa extract, but none of them showed this effect. This combination of herbs in 0.2 % concentration was even less effective than the extracts alone.

CONCLUSION

This study demonstrated the high total phenols and flavonoids content and antioxidant activity of commercially prepared ethanolic extracts of; agrimony (*Agrimonia eupatoria* L.), lemon balm (*Melissa officinalis* L.) and red grape (*Vitis vinifera* L.) that can contribute to sustain antioxidant status and protect against free radical damage. It provides useful information for the production of safe food additives with important antioxidant properties. The synergic effect of combinations of extracts was not found.

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STUDIES ON CLINICAL ANATOMY OF THE MANDIBULAR AND MAXILLARY-FACIAL REGIONS OF THE BAKERWALI GOAT (*Capra hircus*)

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ABSTRACT

This study was conducted on the skulls of thirty eight apparently healthy adult Kagani goats without any skeletal disorders. Some parameters of the upper jaw and the mandibles of these animals were recorded. The distance from the facial tuberosity to the infraorbital canal was 2.36 cm and the distance from the infraorbital canal to the root of the alveolar tooth was recorded as 2.06 cm. The distance from the mandibular foramen to the base of the mandible and distance from the lateral alveolar root to the mental foramen were 3.35 cm and 1.94 cm, respectively. Similarly, the maximum height of the mandible was 3.07 cm and its total length was recorded as 15.60 cm. These data are discussed in regards to their clinical applications in various regional anesthetic procedures pertaining to the upper jaw and mandibular regions in the Kagani goat.

Key words: clinical anatomy; Kagani goat; mandible; maxillary-facial region

INTRODUCTION

The regional or topographic anatomy deals with the structure and relationship of all the organs present in particular parts or regions of the body. It constitutes a cooperative importance to its immediate application to clinical work and hence, regional anatomy is one of the foundations of clinical practice (1).The head consists of different vital structures such as the brain, tongue, nasal cavity, oral cavity, pharynx, horns and the skull. It also contains some foramina which are important from the clinical point of view, as these are the integral parts of the regional anesthetic procedures of the head (2). Lots of works have been done on the anatomy of the head of the domestic animals like the ox and horse (3), small ruminants (1) and pig and dog (4).

The kagani is a versatile breed of goat of the Jammu & Kashmir state of India, which is regarded for its tremendous contribution to the rural economy of the state by virtue of their meat and wool production. It is a strongly built animal, especially adapted for the hilly terrains of the state. Some work has been done on the anatomy of the bones of the skull of this animal (5), but no work has been conducted yet on the clinically important regional anatomy of the head.

This study is the first of its kind which aims to enlighten some of the clinically important anatomical parameters of the upper jaw and the mandible of the skull of the Bakerwali goat of the Jammu region of India.

MATERIALS AND METHODS

In this study, a total number of 38 skulls of Kagani goat were utilized. The heads of these animals were selected from an abattoir based on the apparent good health conditions and without any obvious skeletal deformities. After slaughter, the heads were severed at the atlanto-occipital joint and subsequently processed by the hot water maceration technique described by Tasbas and Tecirlioglu (6). At first, the heads were cleaned by enucleation the eyes and attaching skin and muscles. The heads were then put in polycaboxylate solution, anionic surfactant and small soap pieces and then heated over 80 °C for about 30 minutes. The skulls were boiled and subjected to running tap water and the remaining muscles were de-

tached with the help of knives. The skulls were put in detergent water for about 30 minutes and then again subjected to removal of the remaining muscles, ligaments etc. Subsequently, the skulls were kept in 1 % sodium hypochlorite solution for 24 hours and again the separation of residual muscles and ligaments were done. Then the skulls were left in the same solution for 48–72 hours and during this period, the solution was changed twice. Then the skulls were taken out of the solution and allowed to dry.

Measurements of the mandibular and maxillary-facial regions:

A. Distance between the facial tuberosity to the infraorbital canal. It was measured from the level of the most lateral bulging of the facial tuberosity to both extremities of the infraorbital canals.
B. Length of the mandible. It was estimated by measuring from the level of the cranial extremity of the alveolar root of the lower incisors to the level of the caudal border of the mandible.
C. Distance from the infraorbital canal to the root of alveolar tooth. The measurement was taken from the level of the ventral and dorsal ends of the infraorbital canal.
D. Distance from the lateral alveolar root to the mental foramen. It was estimated as the shortest distance from the mental foramen to the lateral extent of the alveolar root of the lower incisor.
E. Distance from the mental foramen to the caudal border of the mandible. It was taken from the level of the mental foramen to the extreme caudal border of the mandible.
F. Distance of the mandibular foramen to the base of the mandible. It was estimated as the vertical line from the ventral limit of the mandibular foramen to the base of the mandible.
G. Distance from the caudal border of the mandible to the middle of the vertical line produced by F as described above.
H. Height of the mandible from the condyloid fossa to the coronoid process.
I. Width/height of the mental foramen.
J. Distance from the condyloid fossa to the base of the mandible.
K. Maximum height of the mandible taken from the basal level of the mandible to the highest level of the coronoid process.

RESULTS

The line diagrams along with parameters undertaken for the skull and mandible are illustrated in Fig. 1, 2 and 3 and Table 1.

The distance between the most lateral bulging of the facial tuberosity to the infraorbital canal was 2.36 ± 0.03 cm. (Fig. 1). The distance from the infraorbital canal to the root of the alveolar tooth was recorded as 2.06 ± 0.03 cm in the skull of the Kagani goat. The facial crest is very prominent in Kagani goats and is located at the level of the dorsal margin in between the 4th and 5th upper cheek teeth. The infraorbital foramen was located on a depression at the level in between the 1st and 2nd cheek teeth. The Kagani goat has a mandibular length of 15.60 ± 1.89 cm (Fig. 3) with a height of 9.60 ± 1.23 cm. The distance from the lateral alveolar root to the mental foramen was recorded as 1.94 ± 0.03 cm and the distance from the mental foramen to the caudal border of the mandible was found to be 12.61 ± 0.07 cm.

In our study, the distance of the mandibular foramen to the base of the mandible was measured as 3.35 ± 0.07 cm and the distance from the caudal border of the mandible to the middle of the vertical line drawn downward from the middle of the mandibular foramen (Fig. 2) to base of the mandible was recorded as 1.44 ± 0.03 cm.

The maximum height of the mandible to the condyloid fossa, width or height of the mental foramen and distance from the condyloid fossa to the base of the mandible were recorded as 3.07 ± 0.03 cm, 1.0 ± 0.04 cm and 6.79 ± 0.03 cm, respectively.

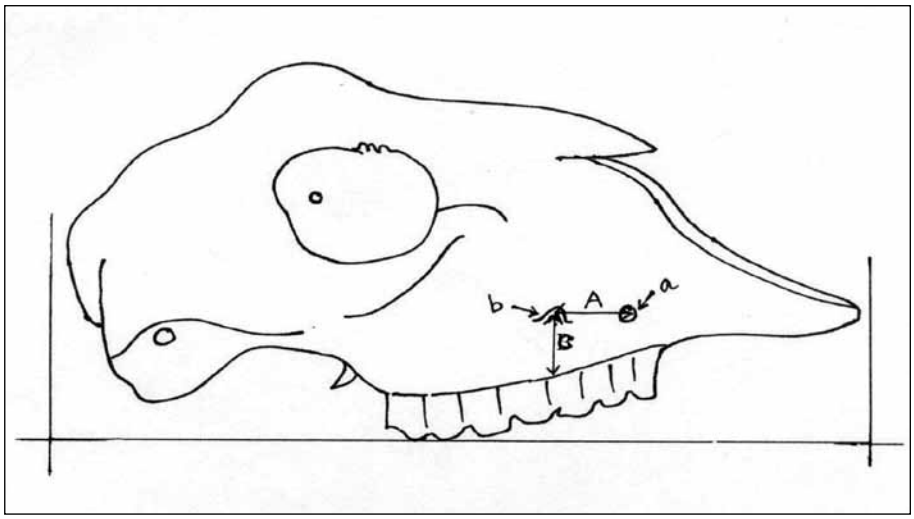

Fig. 1. Line diagram of the maxillary region of the skull of the Kagani goat showing:
A. Distance between the facial tuberosity to the infraorbital canal. B. Distance from the infraorbital canal to the root of alveolar tooth.
a – Infraorbital foramen; b – Facial tuberosity/crest.

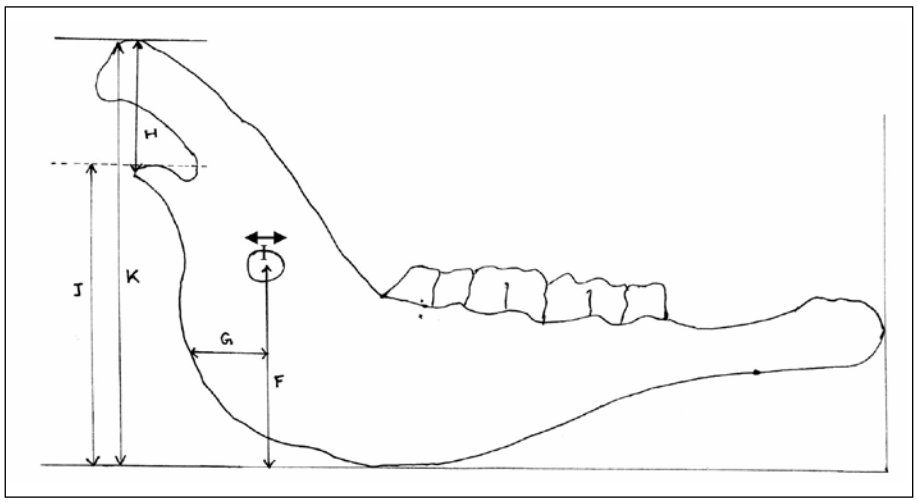

Fig. 2. Line diagram of the medial surface of the mandible of the Kagani Goat showing: F. Distance of the mandibular foramen to the base of the mandible. G. Distance from the caudal border of the mandible to the middle of the vertical line produced by F as described above. H. Height of the mandible to the condyloid fossa. I. Width/height of the mandibular foramen. J. Distance from the condyloid fossa to the base of the mandible. K. Maximum height of the mandible

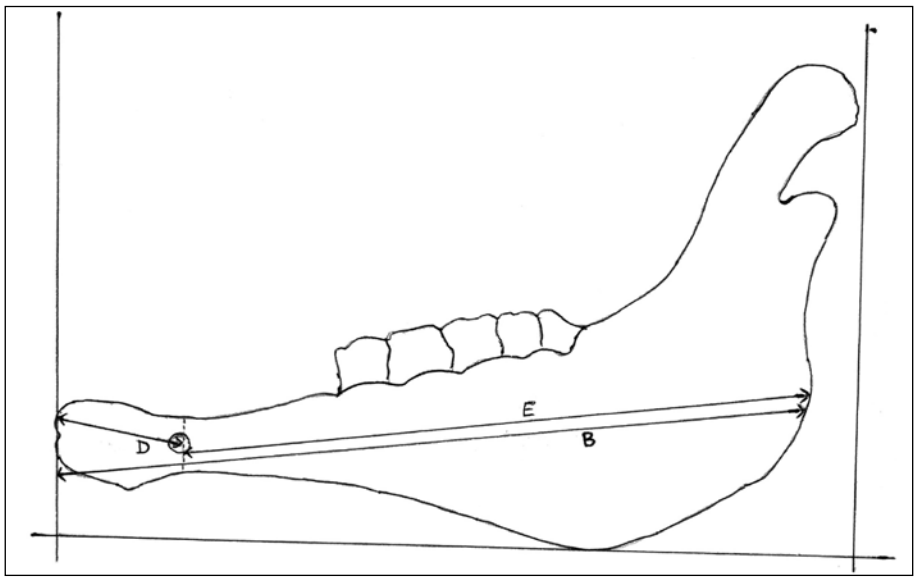

Fig. 3. Line diagram of the lateral surface of the mandible of the Kagani Goat showing: B. Length of the mandible. D. Distance from the lateral root to the mental foramen. E. Distance from the mental foramen to the caudal border of the mandible

DISCUSSION

The distances between the most lateral bulging of the facial tuberosity to the infraorbital canal was 2.36 ± 0.03 cm and the distance from the infraorbital canal to the root of the alveolar tooth was recorded as 2.06 ± 0.03. These data are of clinical importance for an infraorbital nerve block. The infraorbital nerve is the continuation of the maxillary division of the 5th cranial nerve and is entirely sensory, the desensitization of which leads to analgesia of the skin of the lip, nostril and the face on that side of the level of the foramen (7). The topographical location of the facial crest and its position in relation to the last few upper cheek teeth recorded in our study along with the observed distance between the facial tuberosity to the infraorbital foramen (2.36 cm) and distance from the infraorbital canal to the root of the alveolar tooth (2.06 cm) will help immensely to facilitate precise tracking of the nerve by tracing the facial tuberosity to perform regional anesthesia involving the infraorbital nerve in this Kagani breed of goat. The infraorbital canal in the horse has been recorded to be 4–5 cm along a line passing forward and downward from the anterior end of the facial crest (2)

Table 1. The measurements of the maxillary-facial region and the mandible of adult Bakerwali goat

Parameters*	Mean value (cm)	STD
A	2.36	0.03
B	15.60	1.89
C	2.06	0.26
D	1.94	0.03
E	12.61	0.07
F	3.35	0.07
G	1.44	0.03
H	3.07	0.03
I	1.00	0.04
J	6.79	0.03
K	9.60	1.23

* – As described in alphabetical order in Material and Methods

and in cattle, the infraorbital foramen is located 3 cm above the gum line of the first cheek tooth and slightly rostrad to it, as reported by Lahunta and Habel (8).

The various parameters pertaining to the mandible, viz. mandibular length, height, distance from the lateral alveolar root to mental foramen and distance from the mental foramen to the caudal border of the mandible recorded in our study will be useful to the clinicians for conduction of mental nerve blocks for the suturing of the lower lips and for operations on the lower incisors and first 1–2 premolars (7) and serve as tools for tracking the perfect location of the mental nerve in this species.

The mandibular nerve block is indicated for the demonstration of the mandibular nerve needed for clinical examination and surgical operations on the alveoli and teeth of the lower jaw in animals (8). In our study, the distance of the mandibular foramen to the base of the mandible was measured as 3.35 ± 0.07 cm and the distance from the caudal border of the mandible to the middle of the vertical line drawn downwards from the middle of the mandibular foramen to the base of the mandible was recorded as 1.44 ± 0.03 cm. The same data recorded in horses and dogs were 3.0 cm and 1.5–2 cm, respectively for the distance between the mandibular foramen and the base of the mandible (2). The data recorded in our study will facilitate the surgeons for locating the site for infiltration of anesthetic drugs for this particular regional anesthesia in Kagani goat. The maximum height of the mandible to the condyloid fossa, width or height of the mental foramen and distance from the condyloid fossa to the base of the mandible were recorded as 3.07 ± 0.03 cm, 1.0 ± 0.04 cm and 6.79 ± 0.03 cm, respectively. These findings will also serve as important guidelines for the clinicians practicing in this breed of goat for conducting different regional analgesic procedures involving the head.

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THE CORROSION TECHNIQUE IN STUDY OF ARTERIAL ARRANGEMENT OF THE CERVICAL SPINAL CORD IN RABBIT

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ABSTRACT

The aim of this study was to describe the arterial arrangement of the cervical spinal cord in rabbits using the corrosion cast technique, because it has been widely used to examine the pathophysiology of spinal cord injury. The study was carried out on 20 adult New Zealand white rabbits. We prepared corrosion casts of the arterial system of the cervical spinal cord. Spofacryl was used as the casting medium. The origin of the ventral spinal artery from the right vertebral artery was found in 40% of the cases. The origin from the left vertebral artery was found in 40% of the cases. In 20% of the cases the ventral spinal artery arose from the anastomosis of two ventral spinal arteries. The fusion of the bilateral vertebral arteries was found without fenestration, while some basilar arteries may demonstrate one longitudinal fenestration or two oval fenestrations. If the cervical spinal cord arterial arrangements in the different species of laboratory animals are not described in detail, it will be very difficult to determine the appropriate species for experiments in this field. The variations of the arterial arrangement may lead to biased or erroneous results in some studies.

Key words: basilar artery; corrosion cast; rabbit; spinal cord

INTRODUCTION

Injuries of the cervical spinal cord usually result in full or partial tetraplegia. However, depending on the specific location and severity of trauma, limited function may be retained; for example, loss of breathing, necessitating mechanical ventilators, phrenic nerve pacing, etc.

The rabbit has been widely used as a laboratory animal to test the effects of neuroprotective drugs and to examine the pathophysiology of spinal cord injuries (5). The arterial supply of the spinal cord in the cervical region has been described in only a few studies (3, 11). Research on the arrangement and variability of feeding arteries of the spinal cord in several species of laboratory animals (9, 12, 13, 14) and in man (1, 6) are more common.

The aim of this study was to contribute to the knowledge of the blood supply of the rabbit spinal cord, with particular focus on the cervical region, the arteries of which have an analogous arrangement to that of humans (11). The importance of the spinal cord blood supply in this region is in the origin of the arteries which run the entire length of the spinal cord, and arteries partially supplying the brain (16). We describe some variations in the organization of the main spinal cord arteries in the corresponding region.

MATERIALS AND METHODS

The study was carried out on 20 adult rabbits (age 140 days). We used New Zealand white rabbits (breed HY+) of both sexes (female n = 10; male n = 10) with an average weight 2.5 – 3 kg in an accredited experimental laboratory at the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15 – 20 °C, relative humid-

ity 45 %, 12 h light period), and fed with a granular feed mixture (O-10 NORM TYP). Drinking water was available for all animals *ad libitum*. The animals were euthanised by prolonged inhalation anaesthesia with ether. Immediately after euthanasia, the vascular network was perfused with a physiological solution. During manual injection through the ascending aorta, the right atrium of the heart was opened in order to lower pressure in the vessels to ensure a well perfused injection. Spofacryl (polymethylmethacrylate, SpofaDental, Czech Republic) in quantities of 35 ml was used as a casting medium. The maceration was carried out in 2–4 % KOH solution for a period of 5 days at 60–70 °C. This study was carried under the authority of decision No. 2647/07-221/5.

RESULTS

The cervical part has a more complicated blood supply than other segments of the spinal cord. The most cranial part of the cervical segment receives blood through small branches arising from the posterior inferior cerebellar artery, which is a branch of the vertebral artery. The vertebral artery enters the vertebral canal through the foramen vertebrale laterale of the atlas. On the caudal margin of the dorsal surface of the *pars basilaris ossis occipitalis*, it fuses together with the contralateral vertebral artery. This fusion forms the basilar artery. The fusion without fenestration was found in 50 % of the cases (Fig. 1), with one longitudinal fenestration in 30 % of the cases (Fig. 2), and with two oval fenestrations in 20 % of the cases (Fig. 3). The ventral spinal artery originated at the level of the formation of the basilar artery. We found the origin of the ventral spinal artery from the right vertebral artery in 40 % of the cases (Fig. 4), from the left vertebral artery in 40 % of the cases (Fig. 2) and from the anastomosis of two ventral spinal arteries, each coming from the medial flank of the vertebral artery on the same side in 20 % of the cases (Fig. 1). Using this technique it was not possible to describe the segmental arteries entering the ventral and dorsal spinal arteries.

Fig. 1. The origin of the ventral spinal artery from the anastomosis of two arteries, each coming from the medial flank of the vertebral artery on the same side. The formation of the basilar artery is without fenestration.
1 – basilar artery; 2 – left vertebral artery; 3 – right vertebral artery; 4 – ventral spinal artery. Dorsal view. Magn. × 8

DISCUSSION

Several reports describe the arterial arrangement of the cervical spinal cord as being similar in the rabbit and humans (11), but based on our study, we can conclude that there are different arterial arrangements compared with humans. We found the formation of the basilar artery without fenestration or with one or doubled fenestrations. The basilar artery in humans is formed by the fusion of bilateral vertebral arteries without fenestration (2). We found the origin of the ventral spinal artery (in humans the anterior spinal artery) from the right vertebral artery, from the left vertebral artery and from the anastomosis of two ventral spinal arteries, each coming from the medial flank of the vertebral artery on the same side. In humans, the anterior spinal artery is formed at the level of the foramen magnum only by the fusion of the anterior spinal branches of the vertebral arteries (10).

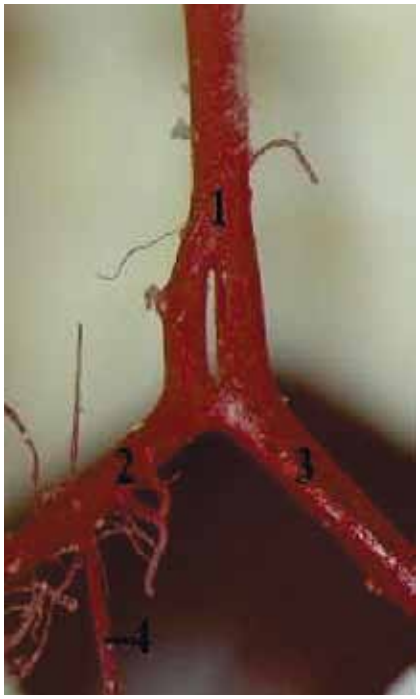

Fig. 2. The origin of the ventral spinal artery from the left vertebral artery. One longitudinal fenestration is visible on the basilar artery.
1 – basilar artery; 2 – left vertebral artery; 3 – right vertebral artery; 4 – ventral spinal artery. Dorsal view. Magn. × 12.5

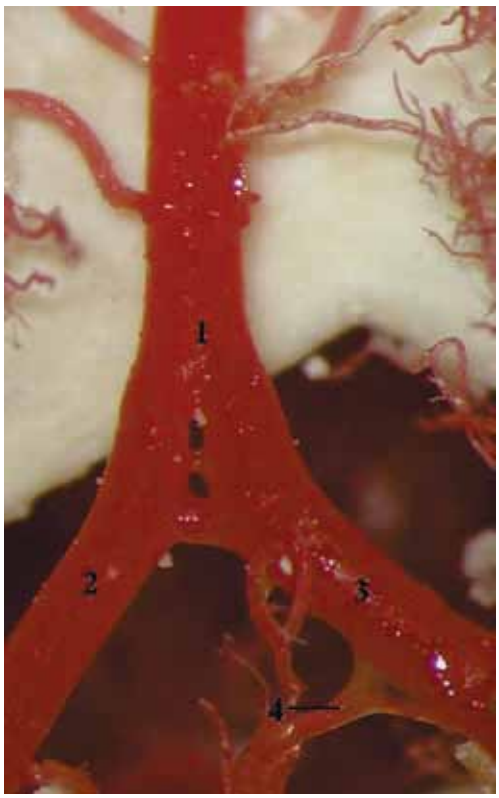

Fig. 3. The origin of the ventral spinal artery from the right vertebral artery. Two oval fenestrations are visible on the basilar artery.
1 – basilar artery; 2 –left vertebral artery; 3 – right vertebral artery; 4 – ventral spinal artery. Dorsal view. Magn. x×12.5

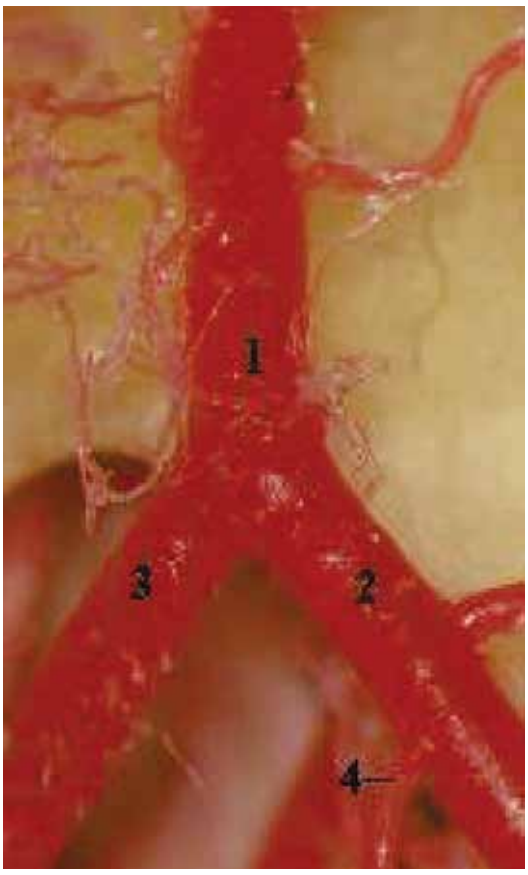

Fig. 4. The origin of the ventral spinal artery from the right vertebral artery.
1 – basilar artery; 2 – right vertebral artery; 3 – left vertebral artery; 4 – ventral spinal artery. Dorsal view. Magn. × 12.5

Chakravorty (3) studied the arterial blood supply of the spinal cord in the cervical region in monkeys, dogs, rabbits and rats. From the results obtained, it was not clear which variation was present in which species, and the origin of the ventral spinal artery was not recorded. In the majority of cases, the origin of the dorsal spinal arteries was from the posterior inferior cerebellar artery. In the remainder, it was found from the vertebral artery. We did not find the origin of the dorsal spinal arteries from the vertebral arteries in our specimens.

CONCLUSIONS

The study of the arterial patterns of the spinal cord based primarily on the use of an experimental animal might serve to elucidate the principles on which the blood vessels are distributed to the spinal cord; at the same time providing additional information concerning the manner of vascularization of the central nervous system in general (14, 15, 17).

Despite the presence of several variations in the formation of arteries, it seems that the rabbit is an appropriate animal for use in experiments on the cervical spinal cord.

The rabbit is often used as an experimental model for the study of spinal cord injury.

The cervical region is the experimental model for the study of several types of damage, e.g., spondylotic myelopathy (3, 4) and some others (7, 8).

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VOLTAMMETRIC DETERMINATION OF TRACE ELEMENTS IN INFUSIONS OF MEDICINAL PLANTS

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ABSTRACT

This work deals with the determination of biologically important trace elements, such as copper, zinc, cadmium and lead, in herbal infusions. Black elder flowers (*Sambuci flos*), birch leaves (*Betulae folium*), linden flowers (*Tiliae flos*), peppermint (*Menthae herba*) and sage (*Salviae herba*) were used as plant material for the infusion preparations. The method of differential pulse anodic stripping voltammetry was developed and applied for the determination of the above mentioned trace elements in digests of plant materials as well as in herbal infusions. The method of choice for digest preparation was the microwave assisted combustion in an oxygen atmosphere. Parameters of voltammetric measurements have been optimized. The concentrations of particular trace elements found in the digests; 36.6–226 μg.g^{-1} for zinc, 3.78–6.96 μg.g^{-1} for copper, 1.59–0.39 μg.g^{-1} for lead and 0.208–0.106 μg.g^{-1} for cadmium were compared with concentrations found in infusions: 32.7–1.86 μg.g^{-1} for zinc, 1.26–0.417 μg.g^{-1} for copper, 0.088–0.049 μg.g^{-1} for lead, and 0.076–0.021 μg.g^{-1} for cadmium. The ratios of the amounts for particular trace element transferred from plants into infusions ranged from 4% to 18%.

Key words: herbal infusion; microelements; stripping technique

INTRODUCTION

Electroanalytical methods are widely applied for determinations of metals in environmental and/or food analysis and in the analysis of water. The need for the determination of trace metals in human nutrition is important both from the point of their essentiality (copper, zinc) and toxicity (cadmium, lead). In the field of pharmacy, such methods are not used very often. Nevertheless, compared to optical methods, e.g. atomic absorption/emission spectrometry, their analytical properties are comparable in terms of the limits of detection, sensitivity, etc.

This work uses differential pulse anodic stripping voltammetry (DPASV) for the analysis of trace concentrations of copper, zinc, cadmium and lead in medicinal plants.

Infusions of widely recommended/used herbs were prepared under strictly defined conditions and the determinations of the above mentioned trace metals were done. The following herbs, often recommended in popular traditional medicine, have been tested. Flower of Black Elder (*Sambuci flos*), flower of Little-leaf Linden (*Tiliae flos*), leaf of White Birch (*Betulae folium*), haulm of Peppermint (*Menthae x piperitae herba*) and haulm of Common Sage (*Salviae officinalis herba*). According to traditional popular medicine, these herbs are able to palliate manifestations of common slight sicknesses.

The principles of DPASV can be found in Wang (23). The main applications of electroanalytical methods in pharmacy deal with the determination of the concentrations of drugs and their metabolites in body fluids (1, 2, 6–9, 11–13, 15, 22, 24, 26).

The objective of this study was to work up the electroanalytical method for the determination of concentrations of trace elements in digests and in infusions of often used herbs. The comparison of the total concentrations found after microwave supported combustion and concentrations found in infusions enabled calculation of a portion of a particular trace element under scope being transferred from the plant into the infusion.

MATERIALS AND METHODS

Herbs

For the determination of the individual mono-component samples; Black Elder (*Sambuci flos*) flowers, flowers of Little-leaf Linden (*Tiliae flos*), leaves of White Birch (*Betulae folium*), haulm of Peppermint (*Menthae x piperitae herba*) and haulm of Common Sage (*Salviae officinalis herba*) were used. The samples were purchased in the Léčivé rostliny (Medicinal Plants) shop in Brno-Královo Pole, Czech Republic.

Standards, solutions, buffers

Mixed standard solutions were prepared from stock standards of metals having the concentration of 1 000 $\mu g.g^{-1}$ (Analytika, Czech Republic). The concentration of particular metals in the working standards were 20 $\mu g.g^{-1}$ of Zn, 2 $\mu g.g^{-1}$ of Cu, 1 $\mu g.g^{-1}$ of Pb and 1 $\mu g.g^{-1}$ of Cd. Nitric acid, 2 $mol.l^{-1}$ (Suprapure, Merck, FRG) and water (GenPure, TKA GmbH, FRG) of high purity (specific conductivity < 1 $\mu S.m^{-1}$) were used for standards and sample preparations. Acetic acid (100 %) and sodium acetate (Suprapure, Merck, FRG) were used for the preparation of the acetate buffer. The pH range of the 1 $mol.l^{-1}$ acetate buffer was 5.5 – 5.7. Certified reference material BCR-62 Olive leaves (IRRM, Belgium) was used in order to check the accuracy of the determination after microwave supported combustion.

Instruments and glassware

The pH was measured by an Orion 4-Star Plus (Thermo-Scientific, USA) instrument. For the infusion preparations, the hot plate (Ika Labortechnik, FRG) was used. The filtrations were done using a medium sized filter paper and/or sterile gauze SteriluxES (10 x 10 cm, 8 layers, Hartmann, FRG). Voltammetric measurements were done using the AUTOLAB instrument (EcoChemie, The Netherlands) with the electrode system VA-Stand 663 (Metrohm, Switzerland). A 70 ml measuring cell was used and the electrode system consisted of the hanging mercury drop electrode (working electrode), the argentochloride electrode filled with 3 $mol.l^{-1}$ KCl (reference electrode) and the graphite rod (auxiliary electrode). All electrodes were produced by Metrohm (Switzerland). Medicinal oxygen (Linde Gas, Czech Republic) was used for combustion. In order to remove oxygen from the measured solutions in the polarographic cell, high purity argon 4.6 UN 1 006, GA260 was used (Linde Gas, Czech Republic). The evaluations of the measurements were done using the GPES software (EcoChemie, The Netherlands).

A microwave oven Multiwave 3 000 (A. PAAR, Austria) was used for the destruction of the sample matrix by combustion in an oxygen atmosphere to enable the determinations of the total analyte concentrations.

All glassware was cleaned prior to use. It was immersed in 10 % HNO_3 for at least 24 hours, then rinsed thoroughly several times with highly purified water and left to dry until used in a dust free environment.

SAMPLE PREPARATION

Infusions

Two g of herb samples were mixed with 8 ml of highly purified water in a grinding mortar and the mixture was left for 15 min with occasional grinding. The mixture was transferred into a 25 ml beaker, 10 ml of boiling water was added and the beaker content was heated on the hot plate for 5 min at 95 °C. Extraction continued for another 45 min by standing at room temperature. The extract was filtered into a 20 ml volumetric flask and finally water was added up to the mark. The prepared sample was immediately used for measurement and/or stored in a refrigerator at 4 °C for further use. The sample preparation procedure followed the requirements given in the literature (16, 21).

Microwave supported combustion

The total concentration of analytes was measured after microwave supported combustion of the samples in an oxygen atmosphere. A 0.2 – 0.3 g of a sample was placed in the thick walled combustion quartz cuvette using a special shaped holder. At the bottom of the combustion cuvette was 10 ml of 2 $mol.l^{-1}$ nitric acid. The cuvette was then closed and filled up with oxygen (1.8 MPa). The total combustion time was 23 minutes. The microwave power used for the combustion program ranged from 420 W to 1 400 W. After the combustion procedure was finished, the resulting solution was quantitatively transferred into a 25 ml volumetric flask, filled up to the mark with highly purified water, and refrigerated at 4 °C.

Voltammetric measurement procedure

Twenty ml of buffer was pipetted into the measuring cell. The standard addition technique was used for measurements and measurement evaluations. The whole measuring process consisted of the measurements of buffer, sample and two standard additions. As an analytical signal, maximum peak heights were evaluated at potentials corresponding to the particular metals. Measuring parameters are given in Table 1 and potentials of peak maxima for measured analytes are given in Table 2.

The statistical evaluation was done according to Eckschlager *et al.* (10). The data are expressed in term of 95 % confidence intervals.

Table 1. Measuring parameters used for DPASV determinations in acetate buffer

Buffer	1.0 $mol.l^{-1}$ acetate buffer
pH range	pH = 5.5 – 5.7
Accumulation potential	–1 400 mV
Accumulation time	600 s
Modulation interval	50 ms
Pulse interval	500 ms
Initial potential	–1 400 mV
Final potential	+200 mV
Step potential	5.1 mV
Modulation amplitude	25.05 mV

Table 2. Potentials of peak maxima in acetate buffer for DPASV determination of copper, zinc, cadmium and lead

Herb	Potential (mV)			
	Cu	Zn	Cd	Pb
Black Elder	−40 ± 5	−930 ± 3	−617 ± 3	−443 ± 5
Little-leaf Linden	−40 ± 0	−980 ± 3	−653 ± 3	−461 ± 3
White Birch	−66 ± 5	−949 ± 3	−602 ± 8	−461 ± 3
Peppermint	−45 ± 0	−975 ± 3	−627 ± 3	−463 ± 0
Common Sage	−38 ± 3	−949 ± 3	−587 ± 3	−455 ± 3

Table 4. Recovery studies in herbal infusions

Analyte	Recovery (%)			
	Cu	Zn	Cd	Pb
Black Elder	95 ± 6.9	93 ± 15.4	98 ± 10.0	102 ± 7.7
Little-leaf Linden	100 ± 21.4	103 ±11.1	86 ± 24.9	104 ± 13.8
White Birch	100 ± 15.9	94 ± 19.7	104 ± 15.2	99 ± 21.2
Peppermint	105 ± 22.1	104 ± 13.8	101 ± 6.0	110 ± 11.7
Common Sage	96 ± 15.2	96 ± 14.2	92 ± 11.2	108 ± 13.4

RESULTS

The limits of detection for copper, zinc, cadmium and lead were; 0.123 ng.g^{-1}, 0.516 ng.g^{-1}, 0.023 ng.g^{-1} and 0.027 ng.g^{-1} respectively, for the measurement procedure developed and used in this work.

The accuracy of the method was checked using the certified reference material: Olive Leaves (BCR-62) in the case of the microwave supported combustion. Table 3 gives the results for particular metals.

Table 3. Standard reference material BCR-62 results

Analyte	DPASV found value µg.g^{-1}	Certified value µg.g^{-1}
Zn	13.24 ± 3.30	16.0 ± 0.07
Cd	0.12 ± 0.05	0.10 ± 0.02
Pb	24.08 ± 1.70	25.0 ± 1.5

According to the results given in Table 3, there is not a systematic error in the procedure in use. In order to check the accuracy of the determinations of trace metals in herbal infusions, recovery studies have been done. The amount of added standards was approximately twice the concentration found for each particular analyte. The results can be seen in the Table 4.

It can be seen that, according to the recovery studies, there is not any statistically significant error in the determination of a particular trace element in the herbal infusions. Therefore, it can be concluded that the accuracy of both the microwave supported combustion and the herbal infusion measurements are satisfactory.

The precision of measurements calculated as a relative standard deviation varied in ranges from 10.0 % to 28.1 %. From the point of view of particular elements, no matter what sample preparation method was applied, the precision for copper was 14.3 %, for zinc was 13.5 %, for cadmium was 24.6 % and for lead was 20.1 %. On the other hand, the precisions from the point of view of different herbs are; 17.3 % for Black Elder, 18.9 % for Little-leaf Linden, 21.2 % for White Birch, 15.8 % for Peppermint and 17.9 % and for Common Sage, irrespective of the applied sample preparation method.

The values given in Table 5 and Table 6, are based on three replicated determinations for each herb. In Table 5 the results of DPASV measurements in herbs infusions can be seen.

Table 5. Concentrations of copper, zinc, cadmium and lead in infusions of different herbs (µg.g^{-1})

Analyte	Cu	Zn	Cd	Pb
Black Elder	0.4173 ± 0.030	4.20 ± 1.19	0.074 ± 0.441	0.079 ± 0.033
Little-leaf Linden	1.18 ± 0.59	3.08 ± 0.53	0.076 ± 0.052	0.049 ± 0.021
White Birch	0.609 ± 0.321	32.7 ± 9.24	0.039 ± 0.021	0.088 ± 0.053
Peppermint	1.26 ± 0.45	1.86 ± 0.36	0.022 ± 0.005	0.064 ± 0.041
Common Sage	0.611 ± 0.152	3.33 ± 0.44	0.021 ± 0.012	0.049 ± 0.024

The values can be compared with the total concentrations of the trace metals under investigation which were measured after microwave supported combustion of herb samples. The total concentrations are given in the Table 6.

Table 6. Total concentrations of copper, zinc, cadmium and lead in herb samples (μg.g^{-1})

Analyte	Cu	Zn	Cd	Pb
Black Elder	6.96 ± 0.79	49.4 ± 16.0	0.136 ± 0.073	0.82 ± 0.16
Little-leaf Linden	6.08 ± 1.60	60.5 ± 10.3	0.123 ± 0.05	0.95 ± 0.13
White Birch	3.78 ± 0.94	226 ± 77	0.208 ± 0.081	1.15 ± 0.21
Peppermint	6.94 ± 1.19	65.5 ± 9.9	0.123 ± 0.041	1.59 ± 0.36
Common Sage	5.34 ± 0.65	36.6 ± 4.8	0.106 ± 0.0032	0.39 ± 0.14

Table 7 shows the calculated ratios of the concentrations which were found in digests and infusions.

Table 7. Ratios of concentrations (digests/infusions)

Sample	Cu	Zn	Cd	Pb	Mean – Herb
Black Elder	16.69	11.76	1.84	10.3	**10.15**
Little-leaf Linden	5.15	19.64	1.62	19.39	**11.45**
White Birch	6.21	6.91	5.33	13.07	**7.88**
Peppermint	5.51	35.22	6.15	24.84	**17.93**
Common Sage	8.74	10.99	5.05	7.93	**8.18**
Mean – Element	**8.46**	**16.90**	**4.00**	**15.11**	

DISCUSSION

From the available literature, the following concentrations of the trace metals under investigation have been found: in infusions of flowers of Black Elder, 7.50 – 10.0 μg.g^{-1} of zinc (5); in infusions of White Birch leafs, 3.50 μg.g^{-1} (25) and 95.37 μg.g^{-1} of zinc (13), 0.75 μg.g^{-1} of copper and 0.041 μg.g^{-1} of lead (14); in infusions of flowers of Little-leaf Linden leaves, 3.45 – 9.90 μg.g^{-1} of zinc (4, 25), 3.85 μg.g^{-1} of copper (4) and, 0.046 – 0.125 μg.g^{-1} of lead (3); in infusion of the haulm of Peppermint, 0.50 – 8.75 μg.g^{-1} of zinc (3, 17, 18, 20, 25), 2.96 – 6.0 μg.g^{-1} of copper (17, 18, 20), 1.00 – 1.12 μg.g^{-1} of lead (20) and 0.008 – 0.038 μg.g^{-1} of cadmium (18, 20); in the haulm of Common Sage, 1.50 – 13.80 μg.g^{-1} of zinc (4, 25) and 2.70 μg.g^{-1} of copper (4).

In this work the highest concentrations of 226 μg.g^{-1} (in digests) and 32.7 μg.g^{-1} (in infusions) respectively, have been found for zinc in the White Birch sample. The lowest concentrations of 0.106 μg.g^{-1} (in digests) and 0.021 μg.g^{-1} (in infusions) have been found in the Common Sage sample. Generally, the concentrations of zinc were the highest in all herbs, whereas the lowest concentrations have been found for cadmium. As expected, concentrations in digested samples which represent the total concentration of a particular trace element were in all cases larger than the concentrations in herb infusions.

The ranges of total concentrations were 36.6 – 226 μg.g^{-1} for zinc, 3.78 – 6.96 μg.g^{-1} for copper, 1.59 – 0.39 μg.g^{-1} for lead, and 0.208 – 0.106 μg.g^{-1} for cadmium. The ranges for concentration which have been found in herb infusions are, 32.7 – 1.86 μg.g^{-1} for zinc, 1.26 – 0.417 μg.g^{-1} for copper, 0.088 – 0.049 μg.g^{-1} for lead, and 0.076 – 0.021 μg.g^{-1} for cadmium.

Based upon the data shown in the Table 7, it can be stated that cadmium is weakly bound in the organic matrix of herbs and therefore it can be easily transferred into infusions. The calculated ratios are relatively uniform with the average value of 4.00. On the other hand, zinc and lead are bound in herb matrices relatively strongly and therefore they cannot easily pass from herbs into the infusions. The calculated ratios are relatively uniform with the average values of 16.9 and 15.11 respectively. From the point of view of herbs under this study, the Black Elder can hold metals (except for cadmium) relatively strongly. The calculated ratio was 10.15; without the cadmium value, 13.0 even. On the other hand, a reduced ability to bind metals was found in White Birch (except for lead). The calculated ratio was 7.88, without the lead value, it was 6.15 even. It depends probably on the complexing agents present in a particular herb.

The dose of toxic trace elements is far below the 1% of the Allowed Daily Intake (19). Not any data was found in the available literature dealing with the portion of trace metals being transferred from plants into the infusions.

Generally, the amount of trace metals which can be taken in by the plant depends on many factors, e.g. composition and/or pH of soil, weather, watering and/or form in which the particular element is present in soil or water.

CONCLUSIONS

The highest amount of metals transferred from herb into the infusion was found for cadmium (34.2 %), followed by copper (14.2 %), zinc (8.0 %) and lead (7.8 %). The absolute amount of a metal being transferred from 1 g of the plant material is on average, 9.03 μg for zinc, 0.815 μg for copper, 6.6 ng for lead, and 4.6 ng for cadmium. These results could be further substanciated by analysis of more samples originating from different places. From the point of view of the usual daily amounts of herb infusions taken, they cannot present any danger in the case of toxic elements (Cd, Pb), as well as any important source for supplementation of essential trace elements (Cu, Zn).

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RESISTANCE TO ANTIBIOTICS OF COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM THE SOFT MEAT PRODUCTS

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ABSTRACT

The aim of this study was to identify and to assess the resistance of staphylococci isolated from soft meat products to eight antibiotics (penicillin, tetracycline, erythromycin, oxacillin, ampicillin, gentamicin, novobiocin and cefoxitin) using the agar dilution method. Isolates of staphylococci were tested for the production of plasma coagulase and deoxyribonuclease; none of these enzymes being confirmed in any of twenty isolates tested. The following species of staphylococci (*Staph.*) were identified with the help of MALDI BioTyper(TM) system; *Staph. vitulinus* (10), *Staph. equorum* (5), *Staph. cohnii* (2), *Staph. epidermidis* (1), *Staph. hominis* (1) and *Staph. warneri* (1). All isolates of *Staph. vitulinus* were resistant to oxacillin and novobiocin. The majority of them (80%) showed simultaneous intermediate susceptibility to cefoxitin. Isolates of both *Staph. equorum* and *Staph. cohnii* mainly showed multiresistance to four or five antibiotic; single isolate of *Staph. equorum* was polyresistant to seven out of eight of the antibiotics tested. Among isolates of *Staph. epidermidis* and *Staph. hominis* the resistance to erythromycin and oxacillin combined with intermediate sensitivity to cefoxitin were confirmed most frequently. Isolate of *Staph. warneri* was resistant to erythromycin and novobiocin.

Key words: agar dilution method; antibiotics; coagulase; meat product; resistance; staphylococci

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are ubiquitous commensals normally inhabiting animal and human skin and mucous membranes. However, many of them are also significant disease-causing organisms, responsible for infections in both hospital and community environments (12). Currently, increased antibiotic resistance in human bacterial pathogens is a major worldwide public health concern. Antimicrobial resistance (AMR) is the ability of microorganisms that cause disease to withstand attack by one or more antimicrobial medicines. It is a consequence of the use, particularly the misuse, of antimicrobial medicines and develops when a microorganism mutates or acquires a resistance gene (24). As reported, CoNS can serve as a reservoir for antimicrobial resistance genes and virulence factors to *Staphylococcus aureus* (2). Resistant bacteria can be spread from animal to animal, from animal to the environment and from animal to human by direct contact or *via* food (22). Moreover, enterotoxin-producing food-associated CoNS strains (including *Staph. equorum*) have also been described in the last decade (23).

Meat and meat products belong to the most important components in human nutrition. Coagulase-negative staphylococcal species are frequently isolated from different specimens in meat chain production, including the final meat products (17). Several studies reported the presence of *Staph. epidermidis*, *Staph. warneri*, S*taph. equorum* or S*taph. saprophyticus* in fermented sausages (4, 17). Thus, inspite of the heat processing, meat products can serve as a source of Gram-positive bacteria being resistant to one or more antibiotics.

Antimicrobial susceptibility testing can be performed by routine hospital, community, public health, food, and veterinary laboratories. The most common methods utilized are the disk diffusion susceptibility test method (also known as Kirby-Bauer) and minimal inhibitory concentration (MIC) determination (25). In this study, the occurrence, species identification and antimicrobial resistance to eight antibiotics of staphylococci isolated from the heat-pro-

cessed soft meat products were determined using the agar dilution method in order to highlight the possible risk for the consumer.

MATERIALS AND METHODS

Samples for microbiological investigation were taken aseptically from the heat-processed soft meat products distributed by the Billa chain of supermarkets in 2010. Sampling and isolation of staphylococci were performed in accordance with the requirements of valid food standards (19, 20, 21) using the Baird Parker agar medium (Hi-Media, India). The species of staphylococci were identified at the Institute of Animal Physiology of the Slovak Academy of Sciences in Košice with the help of MALDI BioTyper(TM) system (Bruker Daltonics, USA) based on protein "fingerprints" measured by MALDI-TOF mass spectrometry. Each isolate was further tested for the production of coagulase (Staphylo PK, Imuna Pharm, Slovakia). The production of deoxyribonuclease was determined after inoculation of the DNase agar (Oxoid, United Kingdom) according to the instructions of the producer.

The minimum inhibitory concentrations (MICs) of eight antibiotics (Sigma-Aldrich, USA) were determined by the agar dilution method on Mueller-Hinton agar according to CLSI document M7-A7 (5). The following concentrations of antibiotics were used: penicillin (Pen) 0.06; 0.125; 0.25; 0.5 $mg.l^{-1}$; tetracycline (Tet) 2.0; 4.0; 8.0; 16.0 $mg.l^{-1}$; erythromycin (Ery) 0.25; 0.5; 1.0; 2.0; 4.0; 8.0 $mg.l^{-1}$; oxacillin (Oxa) 0.125; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0; ampicillin (Amp) 0.25; 0.5; 1.0 $mg.l^{-1}$; gentamicin (Gen) 2.0; 4.0; 8.0; 16.0 $mg.l^{-1}$; novobiocin (Nov) 0.125; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0; 16.0 $mg.l^{-1}$ and cefoxitin (Cef) 2.0; 4.0; 8.0; 16.0; 32.0 $mg.l^{-1}$. Dilution series of individual antimicrobial agents were placed in the amount of 1 ml in Petri dishes and thoroughly mixed with 19 ml of melted Mueller-Hinton agar cooled to 55 °C added to each plate. The plates were further dried in an incubator at 37 °C for 20–30 minutes and inoculated as spots with a 1 µl of an overnight BHI broth culture of staphylococci previously adjusted to a density equivalent to a 0.5 McFarland standard. The results were read after a 24-hour incubation of inoculated plates at 37 °C. The lowest antibiotic concentration showing inhibition of visible bacterial growth was taken as the MIC for the specific strain. The susceptibility, intermediate susceptibility or resistance of individual staphylococcal isolates were determined according to the MIC breakpoints for *Staphylococcu*s spp. (Table 1) established by the CLSI document M100-S16 (6).

Table 1. Criteria for Agar Dilution Method testing of coagulase-negative staphylococci isolates (6)

Antibiotic	MIC ($mg.l^{-1}$)			Antibiotic	MIC ($mg.l^{-1}$)		
	S ≤	I =	R ≥		S ≤	I =	R ≥
Penicillin	0.12	–	0.25	**Erythromycin**	0.5	1–4	8
Ampicillin	0.25	–	0.5	**Gentamicin**	4	8	16
Oxacillin	0.25	–	0.5	**Novobiocin**	–	–	1.6
Tetracycline	4	8	16	**Cefoxitin**	8	16	32

S – susceptible; I – intermediately susceptible; R – resistant

RESULTS

Twenty isolates of staphylococci were obtained during microbiological examination of the heat-processed soft meat products, all of them being coagulase- and nuclease-negative. As shown in Table 2, six species of staphylococci

Table 2. Number of resistant (R), intermediately susceptible (I) and susceptible (S) species of staphylococci (*Staph.*) isolated from the soft meat product

Species	Number of isolates	Penicillin			Ampicillin			Oxacillin			Tetracycline			Erythromycin			Gentamicin			Novobiocin			Cefoxitin		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Staph. vitulinus	10	1	0	9	5	0	5	10	0	0	0	0	10	1	0	9	0	0	10	10	0	0	0	8	2
Staph. equorum	5	4	0	1	4	0	1	4	0	1	0	0	5	2	1	2	1	0	4	5	0	0	1	3	1
Staph. cohnii	2	2	0	0	2	0	0	2	0	0	0	0	2	0	0	2	0	0	2	2	0	0	2	0	0
Staph. epidermidis	1	0	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1	0
Staph. hominis	1	0	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1	0
Staph. warneri	1	0	0	1	0	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1
Total	20	7	0	13	11	0	9	18	0	2	0	0	20	6	1	13	1	0	19	18	0	2	3	13	4

Table 3. Numbers of resistant (R) and intermediately susceptible (I) isolates of staphylococci from the soft meat products to individual antibiotics tested

Antibiotics	Isolates of staphylococci		Minimum inhibitory concentrations (MICs)							
	n = 20		0.25 mg.l^{-1}	0.5 mg.l^{-1}	1.0 mg.l^{-1}	2.0 mg.l^{-1}	4.0 mg.l^{-1}	8.0 mg.l^{-1}	16.0 mg.l^{-1}	32.0 mg.l^{-1}
	R	I								
Penicillin	7	0	6	1						
Ampicillin	11	0		5	6					
Oxacillin	18	0		10	7			1		
Erythromycin	6	1				1			6	
Gentamicin	1	0								1
Novobiocin	18	0				1	8	3	6	
Cefoxitin	3	13							13	3

Table 4. Antibiotic profiles of mono-, multi- and polyresistance in staphylococcal isolates from the soft meat products

Antibiotics	Number of resistant isolates
Nov	1
Ery-Nov	1
Oxa-Nov	5
Ery-Oxa	2
Oxa-Amp-Nov	4
Pen-Oxa-Amp-Nov	2
Pen-Oxa-Amp-Nov-Cef	2
Pen-Ery-Oxa-Amp-Nov	2
Pen-Ery-Oxa-Amp-Gen-Nov-Cef	1
Total	20

were identified using the MALDI BioTyper(TM) system with the dominance of *Staph. vitulinus* (50 %) being followed with *Staph. equorum* ssp. *equorum* (25 %) and *Staph. cohnii* ssp cohnii (10 %). The resistance to oxacillin and novobiocin was observed in 18 out of 20 staphylococcal isolates (90 %), 11 isolates (55 %) showed resistance to ampicillin. On the other hand, all isolates were susceptible to tetracycline and the only isolate of *Staph. equorum* ssp. *equorum* (5 %) was resistant to gentamicin. Intermediate susceptibility to cefoxitin was determined in 65 % of the isolates. One strain of *Staph. equorum* ssp. *equorum* was inhibited by cefoxitin – the same isolate showed only intermediate susceptibility to erythromycin and monoresistance to novobiocin (Table 4) which made it the least resistant strain among all isolates of staphylococci tested in this study.

The most frequent minimum inhibitory concentrations for individual antibiotics were as follows: penicillin 0.25 mg.l^{-1}; ampicillin 1.0 mg.l^{-1}; oxacillin 0.5 mg.l^{-1} ; and novobiocin 4.0 mg.l^{-1} (Table 3). As seen in Table 4, nineteen out of twenty isolates showed simultaneous resistance to 2–7 antibiotics tested, the most frequent being multiresistance to two antibiotics (8 isolates). Each isolate of *Staph. vitulinus* was resistant to oxacillin and novobiocin, eight out of ten strains (80 %) also showed intermediate susceptibility to cefoxitin. Multiresistance to four antibiotics (Pen-Oxa-Amp-Nov) combined with intermediate susceptibility to cefoxitin was determined in two isolates of *Staph. equorum* ssp. equorum; simultaneous resistance to five antibiotics (Pen-Ery-Oxa-Amp-Nov) combined with intermediate susceptibility to cefoxitin occurred in two isolates (*Staph. equorum* ssp. *equorum* and *Staph. vitulinus*). Both *Staph. cohnii* ssp cohnii isolates showed multiresistance to five antibiotics (Pen-Oxa-Amp-Nov-Cef). Polyresistance to seven of out of eight antibiotics tested was found in one isolate of *Staph. equorum* ssp. *equorum*. Isolates of *Staph. hominis* and *Staph. epidermidis* showed resistance to erythromycin and oxacillin plus intermediate susceptibility to cefoxitin. The *Staph. warneri* isolate was resistant to erythromycin and novobiocin.

DISCUSSION

CoNS are considerably less virulent than *Staph. aureus* (13). Selected species of CoNS are considered technologically important in the manufacturing processes of various meat-

derived products, especially dry fermented sausages, where they are used as starters to ensure the quality and safety of the final product (3). On the other hand, approximately half of the identified CoNS species have been associated with human infections (14). Some of those species (*Staph. saprophyticus*, *Staph. epidermidis*, and *Staph. haemolyticus*) are opportunistic/emerging pathogens which can colonize animal and human tissues due to their ability to form protective biofilms and their ubiquitous occurrence in the environment (7).

Because of the increasing clinical significance of CoNS, accurate species identification is of great importance. As seen from the results of this study, six species of coagulase-negative staphylococci (*Staph. vitulinus*, *Staph. equorum* ssp. *equorum*, *Staph. cohnii* ssp *cohnii*, *Staph. hominis*, *Staph. epidermidis* and *Staph. warneri*) were identified in the sample of soft meat products using the MALDI BioTyper(TM) System. Currently, no data are available on species identification of CoNS food isolates with the help of this system. However, there is a generally accepted opinion that phenotypic methods used for species identification of CoNS isolates appear to be unreliable and unsatisfactory (10). Therefore, genotypic identification methods including species-specific PCRs (17), partial sequencing of the tuf gen (10) or the combination of real-time PCR and melt curve analysis (18), are reported as the best and reproducible methods for identification of CoNS species. However, the API Staph ID test is a reasonably reliable phenotypic alternative (10).

The prudent use of antimicrobial agents in food-producing animals is of great importance for the development of resistance in both emerging food borne pathogens and comensals (including coagulase-negative staphylococci) which can enter the food chain *via* raw material (9). Depending on their susceptibility to novobiocin, CoNS can be divided into two groups (novobiocin-resistant or novobiocin-susceptible). Our results show that only two out of twenty isolates obtained from heat-processed soft meat products (*Staph. epidermidis* and *Staph. hominis*) were novobiocin-susceptible. Nowadays, novobiocin susceptible CoNS, particularly *Staph. epidermidis*, are the major cause of nosocomial infections (11). Unlike coagulase-positive staphylococci isolated from the same healthy animal, the coagulase-negative strains are more frequently resistant to novobiocin (a specific feature of a number of the strains) and much less frequently resistant to benzylpenicillin and streptomycin (1) which facts correspond well with the results of this study (90 % of CoNS isolates showed resistance to novobiocin and only 35 % resistance to penicillin).

As shown in Table 3, 95 % of CoNS isolates from the heat-processed soft meat products were simultaneously resistant to 2–7 out of eight antibiotics tested. The occurrence of multi- or polyresistance among coagulase-negative staphylococci found in meat products was also confirmed by other authors. Mauriello *et al.* (15) isolated 42 strains of CoNS from Italian salami, these being specified as *Staph. xylosus*, *Staph. capitis*, *Staph. saprophyticus*, *Staph. hominis*, *Staph. simulans*, *Staph. cohnii* and as *Staphylococcus* spp. More than 64 % of isolates were resistant to lincomycin, penicillin G, amoxicillin, fusidic acid and novobiocin. All the strains were multiresistant and displayed at least three resistances. Resch *et al.* (16) studied the resistance of 330 CoNS associated with food or used in starter cultures against 21 antibiotics using the disk diffusion method. According to the results of this study, most strains of *Staph. equorum* (63 %), *Staph. succinus* (90 %) and *Staph. xylosus* (95 %) exhibited resistances against up to seven antibiotics. Resistances to lincomycin, penicillin, fusidic acid, oxacillin, ampicillin and tetracycline were most frequent. Moreover, resistances were often determined in strains of *Staph. equorum*, *Staph. succinus* and *Staph. xylosus* isolated from sausage (83 %). Even *et al.* (8) reported that safety hazards associated with CoNS were mostly limited to the presence of antibiotic resistance. Within four species of CoNS (*Staph. equorum*, *Staph. xylosus*, *Staph. epidermidis* and *Staph. saprophyticus*) which are frequently isolated from food or clinical environment, seventy-one percent possessed at least one gene encoding antibiotic resistance. Food safety hazards were more pronounced in *Staph. epidermidis* than in the three other species regardless of the food or clinical origin of the strains (69 % of strains carrying 5 or more antibiotic determinants belonged to *Staph. epidermidis* species).

Our findings show that despite heat processing, soft meat products can serve as a source of antibiotic-resistant staphylococci, suggesting the importance of resistance surveillance in the food production environment. Currently, permanent testing of the susceptibility of bacteria to antimicrobial drugs is very important in order to ensure food safety and to protect consumer´s health by the correct choice of antibiotics used in the therapy of infectious and food-borne diseases.

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EFFECTS OF CHEMICAL COMPOUNDS ON CELLS
(A Review)

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ABSTRACT

Chemical compounds can act on living cells at different levels. The three most significant and detailed levels are: cellular, subcellular and molecular. Examples of chemical compounds acting at targets in each of these levels are presented. An understanding of these levels of action may be very important for research on the chemicals mechanisms of action and the use of them as therapeutics in veterinary medicine. The knowledge about the sites of action is also crucial for toxicity studies and new drugs and antidote development.

Key words: cellular; level of action; molecular; subcellular

INTRODUCTION

Organisms are constantly subjected to the effects of different chemical substances. A great multiplicity of types of compounds is reflected in their diverse effects on cells. Among the numerous chemicals are, e.g. everyday substances (food ingredients, cosmetics, fuels, etc.), drugs, natural origin substances and compounds considered as pollutants. Understanding the mechanisms and levels of their action is very important for various areas of life and science.

We can distinguish a few levels of chemical actions, which coincide with different levels of biological organization. Thus, we can talk about action at the cellular, subcellular and molecular levels. Higher levels, such as tissue or organs, are not discussed this article. Between these scales of action there are interactions, due to their hierarchical structure. The most general and the most external is the cellular level and it is the first area of chemical action, after the exposure of the cell to the activity of the substance. The effects on the whole cell may be manifested as, e.g. apoptosis or necrosis induction, inhibition of proliferation, modulation of cell cycle and alteration or suppression of cell functions. The mechanisms of action are obviously situated inside the cell, but they have a molecular basis. The subcellular level of chemical actions concerns the interactions with the cellular organelles – most often the cellular membrane, nucleus, mitochondrion and cytoskeleton. The action on other organelles, such as endoplasmic reticulum, Golgi apparatus or lysosomes, has been less often reported. The third, elementary level is the molecular level. Molecular mechanisms of chemical compounds action cause observable effects first on the organelles, then the whole cells and finally tissues and the whole organism. Interactions at this degree may involve any target component of the cell, which can be receptors, macromolecules (enzymes, nucleic acids, signaling or transport molecules) or membrane lipids. The target molecules are usually considered in the case of drugs effects. The common drug targets are; enzymes, G protein-coupled receptors and other receptors and ion channels (84).

The identification of targets at all levels is very important for understanding the chemical's interactions with cells and have great implications in drug development processes or the search for toxin antidotes.

ACTION AT THE CELLULAR LEVEL

The cellular level is the very basic plane of action. In the case of a single-celled organism, the action may be essential for critical functions and its survival.

The most often observed response to various chemical compounds is the induction or modulation of programmed cell death. There are two types of cell death: apoptosis and necrosis. Apoptosis is an active, programmed physiological process, necessary in order to maintain tissue homeostasis and it is involved in the prevention of many pathological processes. Through apoptosis, the organism eliminates worn out or damaged cells, the presence of which may be associated with various disease states, including neurodegenerations, immunological problems and cancer (110). Apoptosis may be also induced by external factors of a chemical (therapeutic compounds, reactive oxygen species), physical (ionizing radiation, thermal shock) or biological (pathogens, signals from other cells) nature. Biochemical pathways leading to apoptosis are related to the most detailed and internal molecular levels of action. Necrosis is always induced by an external factor, especially infections, toxins and injuries of the cell. Cell death as a result of necrosis is premature and typically has negative effects in contrast to apoptosis, which is often beneficial for organisms. Necrotic cells are not removed from tissue by phagocytes and its components are released into the intracellular space. This results in an inflammatory response of tissue and results in necrosis.

A wide group of substances involved in apoptosis induction or modulation are metals, metalloids and their compounds. The apoptotic pathways induced by metallic compounds have been previously described by Pulido and Parrish (68) and Ranva (71). These reviews indicate, as a main apoptosis inductors, metals known to be carcinogenic: arsenic, antimony, beryllium, cadmium, chromium, cobalt, lead, mercury, nickel (68) and also copper and selenium, which are important trace elements (71). Furthermore, other metals or metalloids have been described as acting as apoptosis promoters. Organotellurium (IV) compounds can act on cancer cells through apoptosis induction, hence they have antitumor properties (1, 77). Sailer *et al.* (77) demonstrated, that a structural analogue of 2,2'-dimethoxydiphenyl ditelluride, not containing tellurium atom, does not have apoptotic effect on human leukemia (HL-60) cells. Also, gold(I) complexes, such as S-triethylphosphinegold (I)-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside and triethylphosphine gold(I) chloride can induce apoptosis in Jurkat cells (73). The exploration of metal-induced apoptosis mechanisms is very important for understanding carcinogenesis mechanisms. Arsenic compounds can also be potentially used in cancer therapy as a chemotherapeutics inducing agent for apoptosis in tumour cells (6).

The next group of compounds which may cause apoptosis are naturally occurring compounds, such as secondary plant metabolites and even vitamins. Among the very important plant metabolites are numerous groups of flavonoids. Flavonoids are known to be antitumor agents and their modes of action are different. They can, among other things, act as apoptosis modulators. It has been demonstrated that apoptosis may be induced by; genistein (67, 37, 93), daidzein (34, 37, 88), quercetin (23, 98), kaempferol (46, 53), luteolin (11, 43), and apigenin (82). Apoptosis can also be induced by flavonol glycosides, such as icariin (49) or diosmin (15). Also, other flavonoids, including some not yet discovered, may have apoptosis inducing activities. The mechanisms of their action are different and concern the molecular level. Besides the flavonoids, other groups of plant origin compounds have the proven action of apoptosis inductors. Among alkaloids, such activity was investigated by Rosenkranz and Wink (76). They have shown that several alkaloids of the isoquinolinone, quinolonone and indole type induce apoptosis in human leukemia cells (HL-60) (76). Moreover, noscapine, an alkaloid from the isoquinolinone group, derived from opium can induce apoptosis in colon cancer cells (104). A benzophenantridine alkaloid sanguinarine was examined as an apoptosis inductor in lung cancer cells (30). Apoptotic activity has been showed also for several polyphenolic compounds, for example curcumin (44) and resveratrol (present in grape skins) (105).

Among other chemical factors that may stimulate apoptosis can be found compounds used as drugs already. Apoptosis is one of the possible mechanisms for cancer therapy. Many chemotherapeutics are targeted by the induction of apoptosis in cancer cells and thus the inhibition of tumour growth. Among the well-known and used drugs involving that mechanism of action are: methotrexate, an antagonist of folic acid used in many cancer diseases (9); 5-azacytidine, a cytidine analogue (91); or paclitaxel (109). Similar activity can also be exhibited by different compounds, both organic and inorganic. Alcohols, especially ethanol, may induce apoptosis in various types of cells. Some effects of ethanol abuse may be related to this apoptotic activity (32, 107). It was also demonstrated that compounds like palmitate (86) or butyrate (108) act as apoptosis inductors in various cell types. Apoptosis are also involved in simple inorganic species like hydrogen peroxide (84, 54) or nitric oxide (48, 74, 96). Nitric oxide can also play an antiapoptotic function (48).

The second type of cell death caused by chemical factors is necrosis. The chemical compounds involved in necrosis induction generally can also act as apoptosis inducers. Usually observed is a concentration dependent response. Low concentrations of chemical factors usually lead to apoptosis induction, while at higher concentrations, necrosis is induced. Such dependence has been described for hydrogen peroxide (13), and metal ions (cobalt and nickel) (22).

Another mode of action at the cellular level is the effect on the cell cycle. The cell cycle is a sequence of consecutive events during the life of the cell. The cycle is controlled by a complex mechanism, which involves many enzymes. Interactions of chemical compounds with the enzymes can lead to cell cycle arrest or modulation. An example of a compound which can affect the cell cycle course is genistein, an isoflavone derived from soy. Genistein can disturb the cycle course and stop it at different points (67). Resveratrol, a phenolic compound present in grapes and wine and intensively studied because of its diverse biochemical and pharmacological properties can stop the cell cycle at the S phase checkpoint in HL-60 cells (70). A wide variety of natural occurring compounds have an effect on the cell cycle course. An alkaloid, lycorine, can arrest HL-60 cell cycle at the G2/M phase (51) just as with 5-methoxypsoralen, a furocoumarin isolated from natural oils (45). Many different natural compounds have the same action. The arrest of cell cycle at G2/M phase is induced also by metal compounds such as germanium oxide (10) or cadmium (102). Also, more organic compounds have cycle modulating activity, such as bisphenol A, which promotes cells to enter into the G2/M phase (100). The G2/M phase arrest is a crucial issue for cell proliferation. When the cell cannot enter the M phase it is not subjected to mitotic division and usually is directed on an apoptotic pathway. Thus, the various modes of action at the cellular level are interrelated.

ACTION AT THE SUBCELLULAR LEVEL

The subcellular level of chemicals action refers to their interactions with subcellular organelles. The organelles are specific structures in the cell's interior that have defined functions. Such specialized structures can be the targets for chemical agents corresponding to their specific action. The recognition of sites of action located in particular cellular compartments can lead to achievements in the field of drug design and their targeting to organelles which are involved in active mechanisms.

The first cellular component encountered by the chemical factor is a cellular membrane or in the case of plants, bacterial or fungi cells, a cell wall. It is the most external barrier by which the cell is contacting with the environment. The bacterial or fungal cell wall is a known target for antimicrobial and antifungal drugs. There are more chemical agents, including a wide group of antibiotics, which act as inhibitors of cell wall component biosynthesis. Inhibition of cell wall synthesis causes cell death by lysis. Similar mechanisms of action have numerous antifungal agents directed against fungal cell wall components like chitin (polyoxins and nikkomycins), glucan (papuklacandins and echinocandins) (21).

The cellular membranes consist of proteins and lipids and have specific properties among which the most important is selective permeability. This property allows for the control of substances moving into and out of the cell. Therefore, substances modulating the permeability of cell membranes can efficiently influence the various biochemical processes taking place in the cell interior. An example of impact on bilayer permeability is the action of DiMethylSulphOxide (DMSO). This agent decreases the thickness of the membrane and increases membrane core fluidity. At higher concentrations, DMSO acts as a transient pore inductor (25). It has also been shown that naturally occurring tannins can affect the membrane fluidity. Chinese hamster cells (CHO B14) treatment with tannins caused an increase of fluidity in the internal part of the membrane (40). The mechanism of action on cellular membrane may relate to the interactions with constituents of the membrane. Chemical agents can react with membrane lipids and proteins which are involved in numerous actions, such as, transmembrane transport. Various interactions with membrane constituents shows cis-platin, a widely used chemotherapeutic, acts on different subcellular structures. The consequence of these various actions is an increase of membrane fluidity and its destabilization (79).

The knowledge about modes of interactions of different chemicals with plasma membranes can help in the design of new strategies of drugs delivery to subcellular compartments because the step of transition through the membrane is an important factor in getting into the interior of the cell. The other cellular organelles are also membrane-enclosed and the membrane has a similar structure so the chemical compounds can affect it in a similar manner.

A very important cellular compartment is the cell nucleus. This organelle, occurring in eukaryotic cells, contains the genetic material of the cell and acts as a control centre for crucial cellular processes. The nucleus is a direct target for agents interacting with DNA. Direct interactions with the cell nucleus have been observed in case of methamphetamine which is distributed in the cytosol and also in the nucleus where it can interact with DNA-binding proteins (4). An alkaloid, sanguinarine, is also accumulated in the nucleus and it inhibits cell proliferation and induces apoptosis (81). The nucleus is certainly a place of action of many different agents acting at the cellular level as apoptosis promoters or cell cycle modulators. All of the effects related to DNA mutations, damage and generally genotoxic effects refer to the nucleus as the target of action. A metal-phenanthroline complex acting as apoptosis inductors and used as a fungistatic and fungicidal agents cause nuclear fragmentation as well as structural changes in other organelles, including mitochondria and the cytoplasm (12). An example of an interaction with plant cell nuclei, is the toxic effects of aluminium chloride, which induces various nuclear aberrations and disintegration of cell nuclei in the root tip cells of barley (*Hordeumvulgare*) (106).

Another essential organelle is the mitochondria occurring in most of the eukaryotic cells. The function of mitochondria is to generate energy (stored as ATP) for the whole cell. A characteristic feature of these organelles is the presence of genetic material inside. The mitochondria are targets for many drugs and other chemicals which act by disturbing the cell energetics processes. It has been also shown that mitochondria play a key role in the apoptosis course (94). The mitochondrial toxicants act mainly on the double membrane surrounding the organelle by changing its permeability. It leads to damaging the whole structure. The disruption of the mitochondria may cause complexing of metals with phenanthroline (12). The increase of the inner mitochondrial membrane permeability is a mode of action of salicylic and valproic acid (14). In turn, the increase of the outer membrane permeability was observed with cis-platin (79). Other molecular mechanisms involved in mitochondrial toxicity has been reviewed by Wallace and Starkov (94) and Czajk and Juszczyk (14). The possible mechanisms include mainly bioenergetics failures, mitochondrial DNA damage, and induction of oxidative stress (14, 94). A wide variety of action in mitochondria has been described following the clinical use of anthracyclines (36). It should be mentioned that mitochondria are the main subcellular target of the heavy metals. This has been shown, among other things, for cadmium (60) and lead (87) as well.

The endoplasmic reticulum (ER) is an organelle constructed of numerous channels and vesicles enclosed in membranes. Its functions include; the synthesis, modification and transport of cellular materials, especially proteins (rough endoplasmic reticulum), lipids and carbohydrates (smooth endoplasmic reticulum). The reticulum is an important target of chemical compounds used as chemotherapeutic drugs active by inducing ER stress. The ER stress activates unfolded protein response (UPR) which is manifested in accumulation of unfolded proteins in the ER. The primary aim of the UPR is to reestablish the normal action of the ER. If it is not achieved for a long time, the cell is directed to the apoptotic pathway (101). Thus, induction of ER stress by chemotherapeutics can be one of the possibilities of stimulating apoptosis in cancer cells. There are already known drugs acting as ER stress inductors resulting in apoptosis in melanoma cells – fenretinide and bortezomib (26). The ER is also a place of action for Eeyarestatin I, which blocks protein degradation associated with the ER. Its mode of action is similar to bortezomib and can synergize with it (95). Although in different ways, the ER stress can be induced by tunicamycin, thapsigargin and brefeldin A (7). The ER stress in yeast cells is induced by cadmium ions which also disturb calcium metabolism in the ER (19). Calcium ion concentration in the whole cell is the highest in the ER lumen and perturbations in its metabolism may have significant implications for ER functioning. An example of a plant-derived compound that can induce ER stress,

is the prenylated chalconxanthohumol (55). The ER stress may be also induced by ethanol and can be one of the mechanisms of the pathological responses in alcoholic diseases (33).

Another subcellular structure closely associated with the endoplasmic reticulum organelle is the Golgi apparatus. A Golgi complex has the form of a flattened cisternae set and is part of the cellular products modification and transport system. The action at the Golgi apparatus level can affect the secretory pathway of the cell compartments. There are several known natural compounds that affect the functioning of the Golgi complex. Monensin, a carboxylic ionophore can effect the pH in the Golgi complex lumen and disrupt protein transport processes (89). Norrisolide, a marine diterpene and its analogues can induce reversible fragmentation of Golgi apparatus (8, 24). Brefeldin A mentioned as an ER stress inductor, can inhibit protein secretion by interactions with the Golgi (7). Thus, the Golgi-ER network can become an important target for therapy of many diseases associated with secretory pathway disorders, including cancer therapy (99).

The lysosomes organelles are created from the Golgi apparatus. Lysosomes are the vesicles containing a set of digestive enzymes, capable of breaking down biomolecules. Their function in the cell is to degrade various molecules derived from both the outside and the inside of the cell. As shown, lysosomes are targets of the organochlorine pesticide pentachlorophenol with toxicity at the subcellular level. This compound causes lysosomal destabilization and degradation and as a result, there are adverse consequences at the cellular level, such as apoptosis or necrosis in mammalian Vero cells (16). The mycotoxin Enniatin B can act on cells through destabilization of the lysosomal system which, in combination with mitochondrial dysfunction, may lead to cell death (29). Similar effects has been found with yessotoxin, a phycotoxin derived from planktonic algae. It damages lysosomes which have been identified as the primary cellular component involved in apoptotic cell death (56). The lysosomal pathway of cell death can became a target for anticancer drugs. The release of lysosome content into the cytoplasm and then apoptosis induction may be a consequence of conventional anticancer drugs effect, such as etoposide, 5-fluorouracil or cis-platin (50, 79).

The interior of the cell is filled with cytoplasm. In this substance are suspended all the cellular organelles. The structure important for the cell division processes is the cytoskeleton. The cytoskeleton consists of microtubules, microfilaments and intermediate filaments. These protein structures help in cell shape support and movement of cell components during cell division. Effects on the cytoskeleton elements can lead to cell death or inhibition of mitotic cell division. A known neurotoxin and carcinogen, acrylamide, acts as intermediate filaments disruptor and thus an apoptosis promoter (3). Also, metals can interact with cytoskeletal proteins. Lead and mercury salts can affect the cytoskeletal motor proteins and cause genotoxic effects by a disturbance of chromosome segregation (90). Arsenic compounds by interacting with microtubules of the mitotic spindle, can induce a mitotic arrest of HeLa cells (27, 28). This makes the arsenites and arsenic trioxide potential anticancer agents. Similar activity has been known for the drug paclitaxel (35).

Other subcellular components are less frequently targets of chemical compounds action. However, there are known several examples of interaction with proteasome or peroxisomes. Proteasomes are organelles responsible for the degradation of undesirable or misfolded proteins inside the cell. One example of proteasome inhibition is the action of several pesticides: rotenone, ziram, diethyldithiocarbamate, endosulfan, benomyl and dieldrin. The proteasome inhibition may have a significant contribution in Parkinson disease pathogenesis (97). Otherwise, there are known and used drugs acting as proteasome inhibitors such as bortezomib, salinosporamide A and carfilzomib (65). These drugs can be used as anticancer agents.

Other organelles commonly occurring in cells are peroxisomes. These are single membrane enclosed structures containing numerous enzymes, such as catalase or peroxidases. The enzymes take part in cellular oxidative reactions. Peroxisomes rarely are places of the chemical compound's actions, but there are a few examples of their effects on peroxisomes. Antilipolytic drugs (3,5-dimethylpirazol and Acipimox) administrated to rats, caused changes in peroxisomal activities related to fatty acid oxidation. It was found that the changes may be the consequence of peroxisomes degradation by autophagy stimulated by antilipolytic drugs (52).

Summarizing, there are various types of subcellular actions and subcellular targets of chemical compounds. Almost all cellular organelles may be targeted structures for toxic or therapeutic activity. By the recognition of subcellular sites of action, there has already developed many strategies for drug distribution directly to its targets. This can be particularly seen in the case of anticancer drugs, acting as cell death promoters. The mechanisms of apoptosis involving organelles like mitochondria or lysosomes may eventually become effective therapeutic pathways.

ACTION AT MOLECULAR LEVEL

The molecular level means all detailed mechanisms ongoing inside the cell and the cellular compartments. It is the most basic and elementary level of action. Chemical molecules interact with a variety of molecular components of the cell. These interactions may be of a chemical or physical nature and may apply to any chemical component of the cell. The target molecules may be diverse: mainly receptors and other macromolecules. The vast majority of molecular targets are proteins. Typically, the first step of drug-target interaction is the binding. The molecular target usually has a specific site called the binding site. The active substance molecule can interact with this site through covalent binding or other non-covalent interactions, such as hydrogen bonds, electrostatic or ionic interactions, or van der Waals forces. Of great importance is the spatial structure of the active molecule and the electric field distribution on its surface. The steric effects may allow or hinder the binding with the active site.

The common molecular targets for drugs and other active substances are receptors. Receptors are molecules, usually proteins, which receives various chemical signals through binding a signal molecule, called a ligand. The interaction of a ligand with its corresponding receptor, initiates the cellular response. There are many classes of receptors among which the most important are; G-protein coupled receptors, receptors linked to ion channels or enzymes, and nuclear receptors modulating gene expression (41). The chemical compounds can interact with receptors as agonists or as antagonists. The agonists have a high affinity for the receptor and causes a specific response. In contrast, antagonists do not induce

a biological response and block access for agonists. In addition to ligands naturally acting as agonists or antagonists, such activity may include other compounds, such as drugs, as well as pollutants.

Another example is resveratrol, which can act as an oestrogen receptor agonist. It can bind with the receptors and induce responses similar to the normal estradiol ligand. The action of resveratrol in phytoestrogen may be linked to its activity as a cancer chemopreventive (20, 47). Resveratrol can also bind to Ah (aryl hydrocarbon) receptors (63). The ability to bind to oestrogen receptors has been shown also by certain flavonoids, e.g. genistein. Genistein can act through oestrogen receptor mediated pathways by regulating some gene expression (67). Xenoestrogens can act also as different chemical compounds of several groups. It has been shown that many chemical pollutants with different structures have significant affinity to the oestrogen receptors and may be involved in various oestrogen receptors mediated processes. The xenoestrogens can interfere with normal endocrine functions and induce significant effects in cells (5). Other compounds can modulate the affinity of natural ligands to its receptors. In glioma cells, the presence of lead acetate can decrease the affinity of glucocorticoids to glucocorticoid receptors. It may lead to blocking of signal transduction processes which is one of the effects of lead toxicity on the nervous system (92).

Enzymes are a secondary significant target molecule for chemicals actions. In each cell there are a huge varieties of enzymes controlling virtually all cellular processes. Chemical compounds can act first of all as enzyme inhibitors but they can also modify the enzymes structure. Well known inhibitors of acetylcholinesterase (AChE) are organophosphates, including pesticides such as diazinon, cypermethrin (17), malathion (38), and other compounds e.g. sarin and soman. AChE inhibitors like tacrine, donepezil, galantamine or metrifonate can be used in Alzheimer's disease therapy (57, 58). Acetylcholinesterase inhibitor activity can also cause toxic effects of indium nitrate on aquatic organisms (111). Different enzymes may be inhibited by metallic compounds. It has been shown that lithium and nickel can act as modulators of different enzymes activity in mouse neuroblastoma cells. Lithium can stimulate lactate dehydrogenase, succinate dehydrogenase and acetylcholinesterase, and nickel can inhibit lactate dehydrogenase (72). A significant decrease of cytochrome oxidase and succinate dehydrogenase activity was observed in rat mitochondria exposed to lead acetate (87). Lead acetate can also inhibit the activity of protein kinase C of C6 glioma cells (92).The frequently mentioned resveratrol can inhibit hydroperoxidase and cyclooxygenase (31).

Enzymes are often drugs targets in the therapy of diseases associated with enzyme activity deficiency or excess. Potential inhibitors can be used for reducing the activity of malfunctioning enzymes. Similar way of action can also be seen with antibacterial and antiviral agents, inhibiting the enzymes responsible for bacterial cell wall synthesis or viral genetic material synthesis and replication.

Action at the molecular level may also affect nucleic acids molecules. Xenobiotics influence on DNA or RNA molecules may involve physicochemical interactions or covalent chemical bounding. The common mechanism is intercalation – the fitting of intercalator molecule between base pairs in the nucleic acid helix. The intercalators may be very often aromatic, heteroaromatic and polyaromatic compounds such as anthracyclines (61), carbazole, acridine and anthracene derivatives (78), porphyrines (66), naphtalimides (103), ethidium (59). The intercalatating agents are used as antitumor compounds due to its topoisomerase inhibitory activity (103). For this reason, novel DNA intercalators are still being developed as potential chemotherapeutics (62). Other compounds may bind to DNA molecules through covalent bonds and in this way act as DNA cross-linking agents. An example of a DNA-binding drug, is cis-platin, which causes replication and cell division processes disruptions. This action finally leads to cell death (18). Action at the nucleic acids level includes also mutagenic activity. The mechanisms of mutagenicity are diverse but one of the most common is DNA alkylating. The alkylating agents can also form crosslinks and form monoadducts with alkyl groups. Examples of these agents are busulphan, cyclophosphamide, chlorambucil, dacarbazine, mechlorethamine, melphalan, mitomycin, nitrosoureas and thiotepa (80). The consequences of the action at the DNA level can also be manifested as chromosome aberrations. Such activity has been seen with metallic compounds as well. Mercury compounds exhibit genotoxicity related to DNA damage and structural chromosome aberrations (42, 83). Similar changes were induced in mammalian lymphocytes by vanadium (IV) compounds (75). Numerical and structural chromosome aberrations can be affected also by arsenium compounds – diphenylarsenic acid and dimethylarsenic acid (64). Gene mutations, chromosomal aberrations and other genetic disorders can be induced by useful drugs as has been shown for cyclophosphamide (29).

Other molecular targets of chemical's action can be transport proteins or structural proteins. Carrier proteins play a significant role in the life of a cell transferring various molecules into and out of cells. An important class of carriers are ATP ion pumps, which provide appropriate value for the membrane potential. These ion pumps may be inhibited by diverse drugs used in the therapy of diseases related to gastric disorders. An example is omeprazole which inhibits H^+K^+ ATPase in gastric parietal cells. Its activity is also related to the inhibition of the gastric mucosa carbonic anhydrase, isoform IV (69). Also, other types of carrier proteins can be affected, for example, intestinal glucose transporter may be inhibited by flavonoids, such as quercetin. This gives the possibility of the use of flavonoids in obesity treatment (39). Action on structural proteins is closely connected to the subcellular level of action. Chemical compounds affecting particular organelles, usually act on creating proteins.

Action at the molecular level can also include various biochemical mechanisms, related to the overlap of chemical reactions and physicochemical interactions.

CONCLUSIONS

The collected examples of chemical compounds action on cells, demonstrate a clear division of the action into three main levels. Starting from the lowest, most detailed molecular level by subcellular level related to action on cellular organelles up to the most general cellular level of action, the three levels are interrelated and every action at the molecular level is located in a particular subcellular compartment and induces specific cellular responses. The recognition of the accurate level and site of action may be helpful for knowledge about various diseases mechanisms and assist in the choice of appropriate drugs or to design novel drugs with definite targets.

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THORACIC LAMINECTOMY TECHNIQUE IN MINIPIGS

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ABSTRACT

This study deals with the surgical modified and standardized approach to the caudal part of the thoracic vertebral canal in minipigs, developed as a prerequisite for experiments, designed to study the different aspects of spinal cord injuries. The experimental operations in 16 female Göttingen-Minnesota-Liběchov pigs weighing 25–35 kg were performed between the 1st of January 2011 and the 30th of June 2012. The standard technique of laminectomy, adapted according to the anatomical characteristics of these animals, was used. Three potential surgical problems appeared: the procedure required special neurosurgical skills; a consistent determination of the correct place of intervention using external landmarks was not possible; the scar formation between the spinal cord and epidural tissues following decompressive durotomy prevented the development of a cerebrospinal fluid fistulae (this potentially could result in secondary damage of neural structures). In order to solve these problems, it was necessary to: acquire the help of a fully qualified neurosurgeons; use plain x-rays in lateral projections in order to locate the correct vertebrae for laminectomy; and recognize the need of a more widely opened spinal dural sac to prevent the fixation of the spinal medulla to the epidural and paraspinal structures.

Key words: dural sac reconstruction; laminectomy; minipigs; plain x-rays

INTRODUCTION

Spinal cord injuries (SCI) in humans are some of the most debilitating pathological conditions affecting not only the individual patient, but they also drastically reduce the quality of life of the entire family (3, 8, 11). The study of the complex actions evoked by and accompanying SCI, necessitates the utilization of animal experimentation. The most frequently used animals in the basic research to study this condition have been mice, rats, and rabbits (12, 18). Recently, preclinical and clinical data have demonstrated that the therapeutic measures which have produced positive results in rodent spinal cord traumas, have not been effective in humans (1, 13). This fact has shifted the interest of neuroscientists studying SCI in animal models, to seek out more appropriate animals models which more closely mimics the situation in humans (1, 9, 14–17, 20).

In our experiments with spinal cord ischemia we found dogs very suitable for this purpose (15, 16). However, due to emotional reasons we abandoned the model and tried to find a different experimental subject. Several reasons led us to the decision to carry out the SCI experiments on minipigs. An initial prerequisite for the study was to creation an adequate surgical approach to a caudal part of the thoracic spinal cord, i. e. thoracic laminectomy. The procedure is not common in these animals and requires considerable surgical skill.

MATERIALS 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 Administration in Bratislava (Decision No. 1749/10-221), as well as by the Ethical Commission of the Institute of Neurobiology, Slovak Academy of Sciences in Košice.

Sixteen female minipigs, originating from Göttingen-Minnesota (G-M) and Liběchov farm (Czech Republic) crossbreeding strains

weighing 25–35 kg, were used at the comencement of this study. Four of them were assigned for the purpose of practicing different technical details, necessary for the accomplishment of the whole complex experimental procedures. Twelve were assigned to the actual study group. Three days before the planned experiments, all animals were pre-treated by a combination of penicillin and streptomycin (*procaini benzylpenicillinum monohydricum* 100 mg + *dihydrostreptomycini sulphas* 125 mg in 1 ml of solution – "Norostrep", Norbrook Laboratories, Northern Ireland) administered intramuscularly in a dose of 0.5 ml × 30 kg^{-1} once a day. Premedication – 2 $mg.kg^{-1}$ of azaperonum (*azaperonum tartaricum* – "Stresnil", Janssen Pharmaceutica, Belgium) and 0.5 $mg.kg^{-1}$ of atropin (*atropinum sulphuricum* – "Atropin", Biotika, Slovakia) were administered intramuscularly 30–40 min before anesthesia, which was induced by the intravenous administration of 10 $mg.kg^{-1}$ of thiopental in 5 % solution (*thiopentalum natricum* – "Thiopental Valeant, Czech Pharma, Czech Republic). Following the induction of general anesthesia, the animals were intubated by endotracheal cannulae with a diameter 5.0–6.0 mm (according to a size of their laryngeal orifices) and placed on a volume-cycled ventilator. The anesthesia was further maintained by inhalation of 1.5 % sevofluran (fluoromethyl-ethyl-ether – "Sevoflurane", Baxter, Czech Republic) with oxygen. Analgesia was supported by the administration of 0.4 $mg.kg^{-1}$ butorfanol i. v. (*butorphanolum tartaricum* – "Butomidor", Richter Pharma, Austria). Catheters for the administration of infusions and medicaments were inserted bilaterally into the cephalic and auricular veins.

After stabilization of the normal cardiovascular and respiration parameters, the location of a skin incision and surgical approach to the vertebral canal, was originally determined by palpation of the last caudal ribs (in 4 practice and 9 experimental animals); later on, the location was determined with the help of plain x-rays of a lateral projection (Fig. 1). The animals were then fixed in an immobilization apparatus consisting of a basal oval steel platform (50 × 90 cm) with four vertical bars (3 × 9 cm, 23 cm long) supplemented by four horizontal bars (2 cm in diameter), adjustable by screws. The apparatus was placed on a standard operating table for larger animals. The minipigs were fixed in a prone position by horizontal bars slid bilaterally against the lateral portion of their paravertebral muscles in the lumbar region. The thoracic and pelvic extremities of the experimental animals were supported by surgical sheets or textile bands (Fig. 2).

Following shaving and disinfection of the operating field, the midline skin and subcutaneous fat incision extending one level cranially and one level caudally from a planned exposure of spinal cord was performed. The subcutaneous fat was released from the fascia and tips of the spinous processes. The paraspinal and intrinsic dorsal muscles were separated bilaterally from the posterior vertebral bony structures (*processus spinosi*, and *arcus vertebrae*). Bleeding was stopped by bipolar electrocoagulation. Then, self-retaining retractors were inserted to provide in depth visibility of the operating field. The interspinous ligaments (*ligamenta interspinalia*) were cut and the spinous processes (*processus spinosi*) resected by Liston and Luer (Lekssel) instruments ("Aesculap", Tübingen, Germany). The yellow ligaments (*ligamenta flava seu interarcualia*) were incised, and then together with the vertebral arches (*arcus vertebrae*) removed by Kerrison rongeurs, taking care to avoid injury to the dura mater (*saccus durae matris spinalis*) and/or neural structures (*medulla spinalis*). In this manner, a suitable and sufficient dorsal approach to the spinal vertebral canal was achieved. Following the removal of the epidural fat by muslin swabs and/or surgical spoons, the dural sac containing the spinal medulla, surrounded by cerebrospinal fluid (CSF), was exposed (Fig. 3).

The SCI was performed by a computer operated device with a metallic rod indirectly (through the intact spinal dural sac) compressing neural structures with a prearranged force and velocity. After the spinal cord compressive injury, hemostasis was ensured in the epidural space, paraspinal muscles were brought together by several stitches, and the skin incision was closed by temporary sutures (Fig. 4). Six hours later, still under continuous general anesthesia, the surgical wounds were re-opened by removal of the temporary stitches, and the spinal medulla was decompressed by longitudinal durotomy. The opened dura mater was fixed to the paraspinal muscles. The exposure of the spinal cord was performed with the intention of appling different medicaments (or stem cells) directly to the damaged part, by use of a special chamber with an external catheter. After finishing the intraspinal part of the intervention, the surgical wound was resutured in the same manner as described previously.

Aseptic conditions were maintained during all of the surgical procedures. During the postoperative period, the animals were housed in single pens under controlled conditions and their neurological status was monitored. They were administered penicillin with streptomycin (*procaini benzylpenicillinum monohydricum et dihydrostreptomycini sulphas* – "Penstrepten", Biotika, Slovakia) daily for another 10 days. After recovery from anesthesia, the animals were offered drinking water *ad libitum* and a full diet. Twelve animals representing the study group were euthanasied in a deep thiopental anesthesia by transcardial perfusion with 5 000 ml of heparinized saline and fixed by the same volume of 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Their vertebral columns together with neural structures were dissected, the operation sites examined, and then the spinal cords were removed and examined for histological changes.

RESULTS

All 16 minipigs survived laminectomies, spinal cord traumas, long-term general anesthesias, decompressive durotomies and reconstruction of the surgical wounds. Four test animals perished after completing the entire experimental procedure (two within 12 hours, one the next day, and the last one on the 3rd day), but their deaths (established by pathologist at post-mortem dissections) had extrasurgical causes. None of the minipigs from the study group showed any complication related to the laminectomies and durotomies performed 6 hours later. No cerebrospinal fluid (CSF) fistula developed in any of 12 experimental animals. At the end of the survival period their surgical wounds were healed per *primam intentionem*.

Following the dissections of the vertebral columns and the preparation of the spinal cords for thorough macroscopical examination and histological processing, a firm densely adhering scar was found on the damaged medulla. The scar which developed completely overlapped the decompressive durotomies, fixed the neural structures to the paraspinal muscles and

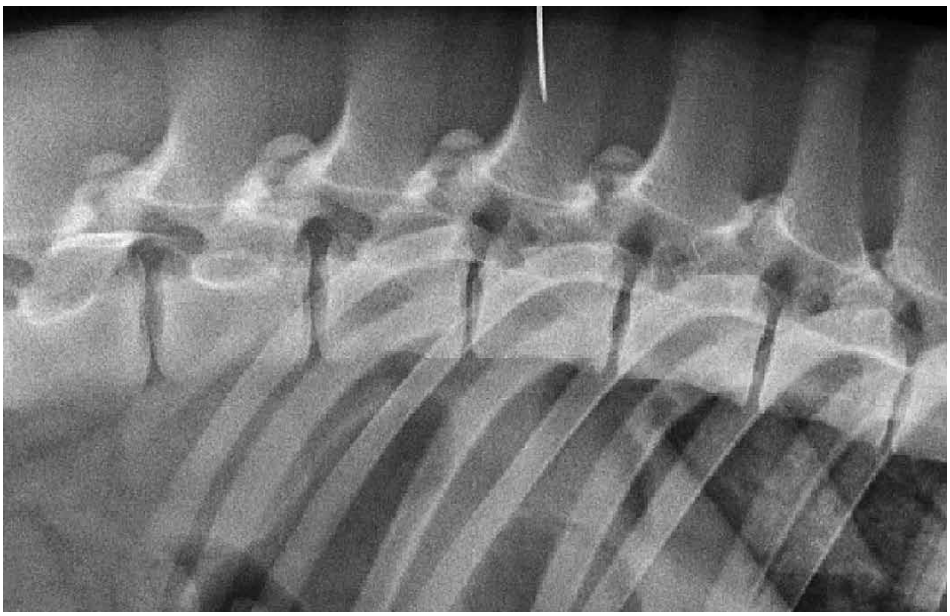

Fig. 1. Plain x-ray of thoraco-lumbar part of vertebral column with a needle used as a mark improving identification of the correct vertebra for the laminectomy

Fig. 2. Fixation of a minipig in a spinal immobilization apparatus before the surgical phase of the experimental procedure

impeded the free movement of the spinal medulla in the dural sac and vertebral canal (a secondary tethered spinal cord syndrome, a very harmful condition for the spinal cord).

DISCUSSION

Despite the fact that small animal models are very useful in biological research, they may not accurately emulate processes characteristic for the diseases in humans (7, 18, 19). That is why new experimental models in larger animals have been proposed in the past several years (3, 5, 9, 13, 15, 17, 20). One of the more important technical details to solve is the surgical approach to the spinal cord. On the 29th of December, 1882, the outstanding Czech surgeon, Karel Maydl, performed the first successful intraspinal operation, consisting of removal of spinous processes and vertebral arches (parts of vertebral canal addressed as *laminae* in med-

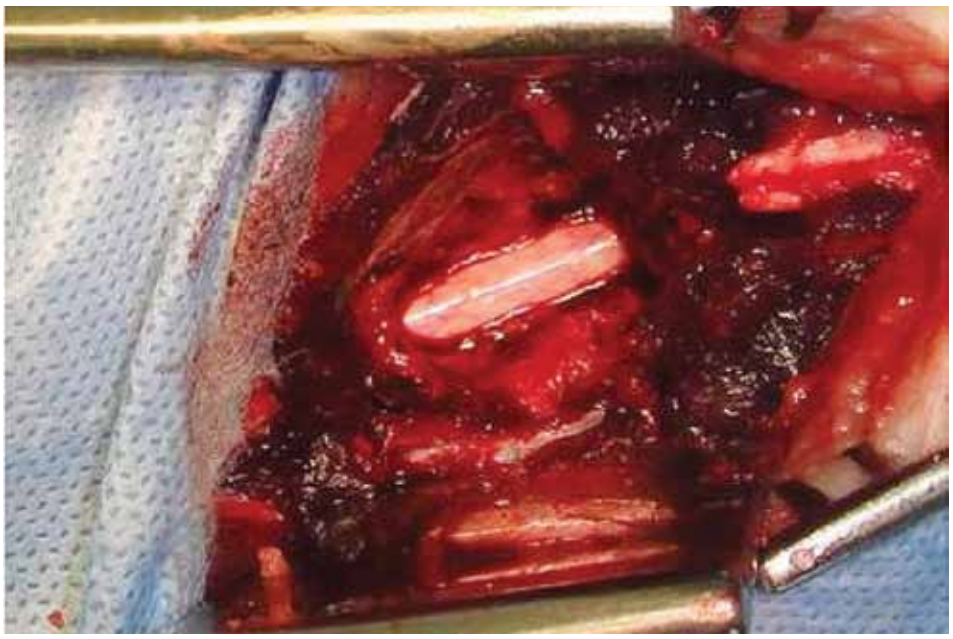

Fig. 3. State after laminectomy of Th15. The skin and paraspinal muscles are held by self-retaining retractors. The spinal cord with its blood vessels is visible through an intact dura mater

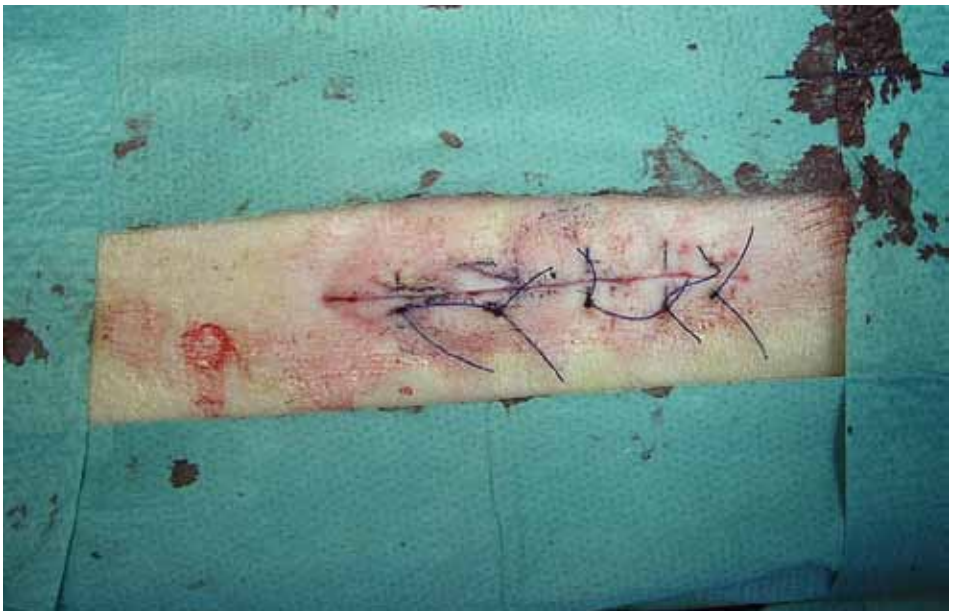

Fig. 4. The surgical procedure completed by a suture of the skin incision

ical literature) in a young carpenter suffering from complete paraplegia due to a compressive fracture of the vertebral column. Since then, the laminectomy has become the standard approach to different lesions located in the spinal canal (6). The method was first introduced to human neurosurgery and subsequently to veterinary surgery. We have had very positive experiences using this method in dogs (15). However, there are only a few reports in the literature reporting upon its utilization in pigs (9, 17).

It is well known that pigs share many anatomical and physiological characteristics with humans (2, 5, 9). They have a relatively large central nervous system, which makes them suitable for studies: of neurological diseases; brain and spinal cord imaging; assessment of new pharmaceuticals; for surgical purposes; and for disease models (2, 4, 10, 14). Their potential for modeling human brain and spinal cord disorders has been widely recognized (5, 9, 17). In view of these features, the minipig crossbreeding strains produced by the

Göttingen-Minnesota and Liběchov farm appeared to be the most suitable experimental animals to study processes developing in the spinal cord following compression injury and to establish the efficacy of different therapeutic measures (17). Moreover, the progeny of cross-breeding G-M and domestic pigs (Liběchov, Czech Republic) are characterized by a dense whole body hair growth. This makes paraplegic minipigs more resistant to the development of decubitus ulcerations, which is critical for long-term survival of experimental animals subjected to SCI.

On the other hand, the porcine model does have some limitations. One is the lack of fine neuroanatomical as well as the functional organization of the motor system, which is characteristic for primates and humans (9). The other, is the non-consistent number of thoracic vertebrae in pigs, fluctuating from 13 to 17 (10, 14). This could prove to be a real problem when the reproducibility of experimental procedures is considered. We were able to identify the correct vertebrae only by the use of plain x-rays in a lateral projection and recommend the utilization of this method whenever suitable x-ray equipment is available.

For placement of laminectomies in pigs, it is more suitable to use the lumbar or caudal thoracic part of the vertebral column. This is because of the much longer spinal processes and the thick paravertebral muscles in the mid- and upper-thoracic areas; plus the cervical region could constitute an obstacle for the surgical approach to the spinal medulla.

The epidural scar formation following the laminectomies and the fixation of the bare spinal cord in the durotomy by tough connective tissue, imply that only watertight dural closure could prevent the excessive scar formation and its potentially harmful influence on the soft neural structures. After decompressive durotomy (which is effective only when the incision in the spinal dural sac is long and wide), it is possible to close the dura mater only by help of fascial patches.

CONCLUSIONS

The experimental operations performed in 16 Göttingen-Minnesota-Liběchov minipigs revealed that the caudal thoracic laminectomy can be a standard procedure in the hands of trained neurosurgeons. The correct skeletotopic location is possible only by the use of a portable x-ray apparatus. The development of a secondary tethered spinal cord syndrome and damage of soft neural structures by excessive epidural scarring is preventable by the meticulous reconstruction of spinal dural sac – if necessary with the help of a fascial patch.

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THE ROLE OF ENDOTHELIAL AND KUPFFER CELLS IN THE LIVER (A Review)

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ABSTRACT

We review the morphological characteristics and physiological functions of the hepatic sinusoidal endothelial cells and Kupffer cells. We discuss the major implications of these cells in the pathophysiology of the liver. The dysfunction of the hepatic sinusoids leads to parenchymal impairments due to insufficient supplies of oxygen and the lack of several various substrates. Recent studies have demonstrated that each of the sinusoidal cell types has the capacity to become "activated" and release pro-inflammatory and cytotoxic mediators that may contribute to hepatotoxicity.

Key words: endothelial cells; hepatotoxicity; Kupffer cells; liver

BACKGROUND

The structural characteristics of the liver are mainly determined by its circulatory system. Approximately 30 % of the entire blood volume passes through the liver per minute (20). The liver is supplied by blood from the *arteria hepatica* and the *vena portae*. Approximately 70 % of the blood entering the liver is venous blood from the *vena portae*. After entering the liver, the vessels gradually branch and enter the connective tissues between the individual lobuli. The luminal diameters of the branches from the *vena portae* are much larger than those of the branches of the *arteria hepatica*. The veins in the portobiliary space are considered distribution veins. Their walls consist of endothelium and a thin layer of smooth muscle cells. These veins give off additional branches, the so-called supply venules (*venulae afferentes*) located on the surface of the hepatic lobule, and open directly into the liver sinusoids. Their terminal parts consist exclusively of endothelial cells capable of contracting and thus regulate the volume of blood flowing into the sinusoids.

The interlobulary artery in the portobiliary space contains, besides endothelial cells, also a reduced muscle layer. In the portobiliary space, the interlobular artery gives off abundant branches, some of them supplying directly to the interlobular vein (*periportal plexus*) and others providing blood and nourishment to all of the interlobular hepatic ducts (*peribiliar plexus*). Only terminal branches open into the liver sinusoids. Before their entry into the sinusoids, the wall consists only of endothelial cells.

The blood sinusoids form richly branched slit-like spaces between the hepatic trabeculae. Their lumen is 9–12 μm wide and their wall consists of a single-layer of epithelium formed by the endothelial cells. Between the endothelial cell and hepatocyte there is an irregular perisinusoidal space named the space of Disse. In this space, there are bundles of collagen and reticular fibres that strengthen the wall of the blood sinusoids. Sporadically, Ito cells, the so-called lipocytes, are found in the space of Disse (13, 19).

On the basis of studies of the rat liver, Wisse published in 1970 the first detailed description of the liver ultrastructure (25), focusing on the endothelial cells (EC). In their walls he observed open pores or fenestrae, with approximately 0.1 μm space. They occurred in clusters (each containing 10 to 50 fenestrae) and occupied 6–8 % of the endothelial surface. They were not "passive sieves", but constituted a highly dynamic structure, varying in size and density depending upon their location in the hepatic lobule. This heterogeneity was observed in the direction from the *v. centralis* towards the portobiliary space. The pores could contract or dilate under the influence of various substances (8), such as; alcohol, serotonin or endotoxins (22). In this process, the cytoskeleton of the cell played an important role. Experiments demonstrated that the EC cytoplasm

contained actin and myosin filaments that can form a calmodulin-actin complex which participates in the regulation of the size of these pores (15).

There are phenotypic differences between individual EC depending upon their location in the liver. The branches between the *v. porte* and terminal venules are lined with spindle-shaped, non-fenestrated EC. Short microvilli cover the apical portion of the cytoplasmic membrane. Endothelial cells in the transient zone between the terminal venules and hepatic sinusoids are bigger, smoother and contain many actin fibres. These EC function as an entry "sphincter", most likely regulating and controlling the blood flow into the hepatic sinusoids.

Terminal branches of the *a. hepatica* also open into the sinusoids. It has been assessed that the arterial pressure is 20 to 40-fold greater than the blood pressure in the hepatic sinusoids. The decrease in pressure between the arterioles and sinusoids is caused by the so-called pre-capillary sphincter which consists of tall EC and smooth muscle cells. Presumably, the fenestrae in the EC also contribute to the decrease in the blood pressure, because their diameter in the periportal space is the biggest which enables the rapid passage of blood plasma into the space of Disse (1, 14, 24). This is supported also by the absence of the *lamina basalis* in the blood sinusoids (25), which facilitates both the infiltration of blood plasma into the space of Disse and the passage of macromolecules, such as; lipoproteins, albumins and fibrinogen, released from the hepatocytes (25). However, a basal membrane can gradually develop, which is due to chronic damage to the liver. This phenomenon is called capillarization of the sinusoids and is associated with a reduction of the pores in the EC (2).

In addition to the previous described functions, the EC are capable of removing from the blood, some soluble macromolecules, individual components of tissues and waste substances. These unique properties of EC are indicated by the presence of vesicles and lysosomes in their cytoplasm (23). The EC eliminate particularly; hyaluronic, chondroitin sulphate, collagen, denaturated albumin, ovoalbumin and other modified or denatured macromolecules. This process involves receptor-mediated endocytosis and many of the receptors have already been identified (4, 6, 21).

The EC also produce mediators that affect other cells in the blood sinusoids and the hepatocytes themselves; this involves namely, IL-1, IL-6, interferon, lysosome enzymes, nitrogen oxides and reactive oxygen species (ROS), for example, superoxide anions (7, 9, 11, 16). The exposure of the liver to the influence of hepatotoxin induces the production and release of IL-1 and IL-6. In this manner, the cells become involved in inflammatory and immune responses. The mediators and ROS released from the endothelial cells can directly damage the vessels and the surrounding liver parenchyma (10).

The hepatic sinusoids comprise another very important cell, the so-called Kupffer cell (KC). They are located in the sinusoidal lumen and sometimes also in the space of Disse. They have an irregular shape with numerous cytoplasmic projections. The cytoplasm of the Kupffer cells contains a great number of lysosomes and phagosomes. They have, a well-developed endoplasmic reticulum, the Golgi system, as well as secretory vesicles. Their distribution in the hepatic lobuli varies. The KC are found particularly in the periportal and central parts of the lobuli. Their localization in the periportal space is advantageous in view of the effective elimination of harmful substances from the blood (18). KC migrate slowly along the hepatic sinusoids, frequently causing temporary disturbances of the blood flow or sometimes even its cessation of flow. This facilitates closer contacts between macrophages and lymphocytes. In addition, Kupffer cells pass into the space of Disse and are involved in the phagocytosis of apoptotic hepatocytes (18). They are the first cells coming into contact with xenobiotics and harmful substances originating from the digestion system (3).

Xenobiotics that enter an organism may induce direct or indirect liver damage. Xenobiotics or their metabolites do not have to cause direct damage to hepatocytes, They can act on endothelial cells, Kupffer cells and leukocytes. Their activation results in the release of inflammatory mediators, ROS, nitrogen radicals, proteases as well as metabolites of lipids. These bioactive molecules can act directly on hepatocytes and cause the death of cells or indirectly, by the activation of other cells (5, 11, 12). The released mediators induce proliferation of endothelial cells and thus strengthen the toxic effect of xenobiotics (10). Long-term effects of xenobiotics may cause changes resulting in liver fibrosis. Sinusoidal capillarization in liver fibrosis is associated with a marked decrease in the number of fenestrae and the development of a continuous basal membrane (17). These changes result in the decreased diffusion of oxygen and the transport of substances through the sinusoids which may have negative effects on the hepatic functions.

CONCLUSIONS

The blood flowing through the sinusoids comes into contact with Kupffer and endothelial cells. The blood flow in the liver is slow which allows both types of cells to eliminate various macromolecules and xenobiotics. While the EC remove soluble substances through receptor-mediated endocytosis, the Kupffer cells phagocytise particles. Both EC and Kupffer cells are crucial for immunological and inflammatory processes that take place in the liver. While the non-parenchymal hepatic cells form one integrated unit, they are absolutely necessary for the maintenance of the normal functions of this organ.

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