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FOLIA VETERINARIA, 58, 4, 2014

CONTENTS

FOLIA VETERINARIA, 58, 4: 203—205, 2014

STUDY OF ENDOGENOUS FLUORESCENCE OF VIPER VENOM

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ABSTRACT

This study characterizes the endogenous fluorescence of venom from *Vipera berus*. Simple comparison of synchronous fluorescence fingerprints of the analysed Slovak and Polish male and female viper venom samples revealed differences in venom composition and showed a higher fluorescence of venom from Polish vipers in comparison with the venom of Slovak vipers. Moreover, the synchronous fluorescence analysis displayed a higher endogenous fluorescence of the female viper venom in comparison with the venom of male vipers. Our results confirmed a different molecular structure between the male and female venom of *Vipera berus berus*.

Key words: autofluorescence; synchronous fluorescence fingerprint; venom; viper

INTRODUCTION

The Northern viper *Vipera berus berus* is a wild venomous snake living in Central Europe. Vipers with longer fangs occur at altitudes about 300—680 metres above sea level [2], but vipers with shorter fangs can be found also at altitudes about 100 metres above sea level in the Polish lowlands [6].

Viper venom is a multicomponent mixture which contains more than 100 different components [9]. More than 90 % of the dry weight of the viper venom are proteins, comprising a variety of enzymes, nonenzymatic polypeptide toxins, and nontoxic proteins. However, the venom does not contain paralytic postsynaptic neurotoxins, cardiotoxins, myotoxins or necrotizing agents. The highly neurotoxic substance PLA_2 (phospholipase A_2), occurs in the venom of some subspecies of vipers. PLA_2 acts presynaptically and may affect innervation of facial muscles. Haemocoagulation and cytotoxic agents may be present in the viper venom in negligible quantities. Venoms of several poisonous snakes contain clinically dangerous haemoragins and circulating toxins which, if injected into the bloodstream, increase capillary permeability and cause haemorrhages. The constituent proteins of snake venom can affect the breakdown of tissue proteins and peptides. A lethal dose of viper venom for a human body is 15—20 mg, which is similar to the lethal dose of venom from the cobra *Naja naja* [9]. The nonprotein ingredients include; carbohydrates and metals (often part of glycoprotein metalloprotein enzymes), lipids, free amino acids, nucleosides and biogenic amines, such as serotonin and acetylcholine, polysaccharides, low molecular weight substances and ions [5], [10]. The fluorescence properties of the particular venom components are the consequence of their molecular structure.

These investigations are a continuation of the study of the structure of various venoms of all kinds of African mamba of the genus *Dendroaspis* [1] and two species of the African cobra *Naja ashei* and *Naja nigricollis* [6]. The aim of this study was to characterize and compare the endogenous fluorescence of the venom of *Vipera berus* males and females living in Poland and Slovakia.

MATERIALS AND METHODS

The samples of venom from the Northern viper (*Vipera berus berus*) female and male (one sample from each gender) were collected in the Slovak Republic and Poland. The vipers were captured at altitudes about 650—750 metres above sea level in the Slovak Republic and at altitudes about 115—130 metres above sea level in Poland. The snakes were induced to bite through a thin latex membrane covering the measuring vial, and pressure was applied to the venom glands. The venom was collected in the vial, stored in transport cryogenic microtubes under liquid nitrogen temperature (–196 °C) and finally kept in a freezer (–70 °C).

Viper venom samples were analysed by synchronous fluorescent fingerprints (SFF) and a simple synchronous excitation fluorescence spectra using a spectrophotometer Perkin-Elmer LS 55 (Waltham, Massachusetts, USA). Each synchronous excitation spectrum was measured from $\lambda = 200$ until $\lambda = 600$ nm at room temperature. The resulting SFF was created from 20 scans of simple synchronous excitation spectrum (number of scans at different excitation wavelengths) with constant 20 nm in distance increment among all viper venom with increment 20.

The data were processed by software WinLab (PerkinElmer, Waltham, Massachusetts).

RESULTS AND DISCUSSION

Fluorescence spectra of male viper venom (Fig. 1b, 1d, 2b, 2d) show different maximum intensity of fluorescence in comparison with the spectra of female viper venom (Fig. 1a, 1c, 2a, 2c).

The comparison of SFF (Fig. 1b and 1d) revealed a similar structure and fluorescence centres in viper male venom from Slovakia (50/294 nm, F = 540) and Poland (50/294 nm, F = 573), but detailed examination of the fluorescent centres showed some differences. Fluorescence of venom from the viper female (Fig. 1a and 1c) from Slovakia (50/310 nm, F = 1000) and Poland (50/300 nm, F = 1000) had different SFF structure, but similar localization of centres with maximum fluorescence (Fig. 2a and 2c).

The mixture of organic molecules present in natural snake venom forms a multi-fluorescent viscous liquid and can be studied by fluorescence spectroscopy [6], [8]. The main organic fluorophores responsible for the final fluorescence of venom include; enzymes, amino acids and proteins. Obviously, serotonin, nucleosides and biogenic amines can influence the venom fluorescence intensity as well. Most venom contains fluorescent l-amino acid oxidase with prosthetic cofactor FMN or FAD [7], which is responsible for the yellow colour of many types of venom. From the literature it is known that riboflavin (vitamin B_2) and cofactors (FMN and FAD) are active fluorophores [3], [4].

Vertical cuts of SFF (Fig. 1) represent fluorescence emission spectra of the viper venom. These spectra revealed differences in the structure of individual viper venom (Fig. 2).

Vertical cuts of male Slovak and Poland venom SFF at $\lambda_{ex} = 294$ nm (Fig. 1b,d) showed a maximum of the fluorescence intensity at $\Delta\lambda = 50$ and 70 nm (Fig. 2b,d) in comparison with female Slovak and Poland venom which exhibited the maximum of fluorescence intensity at $\Delta\lambda = 30$ and 50 nm (Fig. 2a, c). The vertical cut of Slovak female venom SFF at $\lambda_{ex} = 309$ and Poland female venom SFF (Fig. 1a, c) at $\lambda_{ex} = 300$ exhibited F = 1000 (Fig. 2a, c).

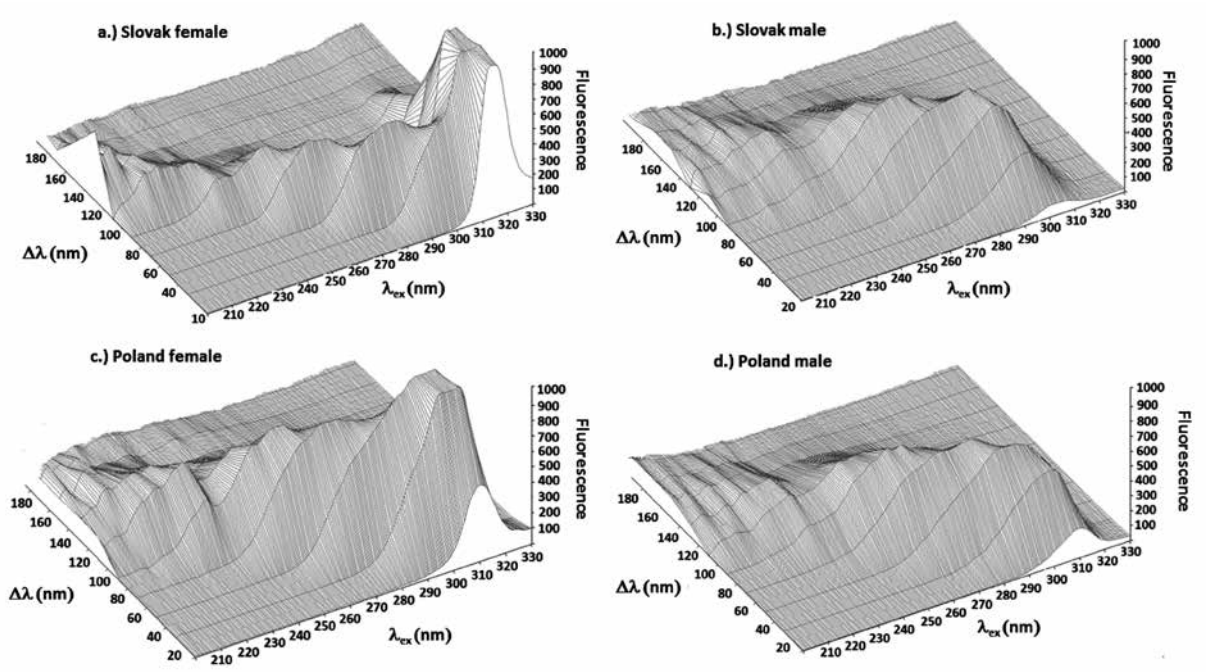

Fig 1. Synchronous fluorescence fingerprints of viper venom (*Vipera berus berus*) of a) Slovak female; b) Slovak male; c) Poland female; d) Poland male. The graphs show dependence of Δ (nm) (difference between emission and excitation wavelengths) on excitation wavelength λ_{ex} (nm); z axis represents intensity of fluorescence

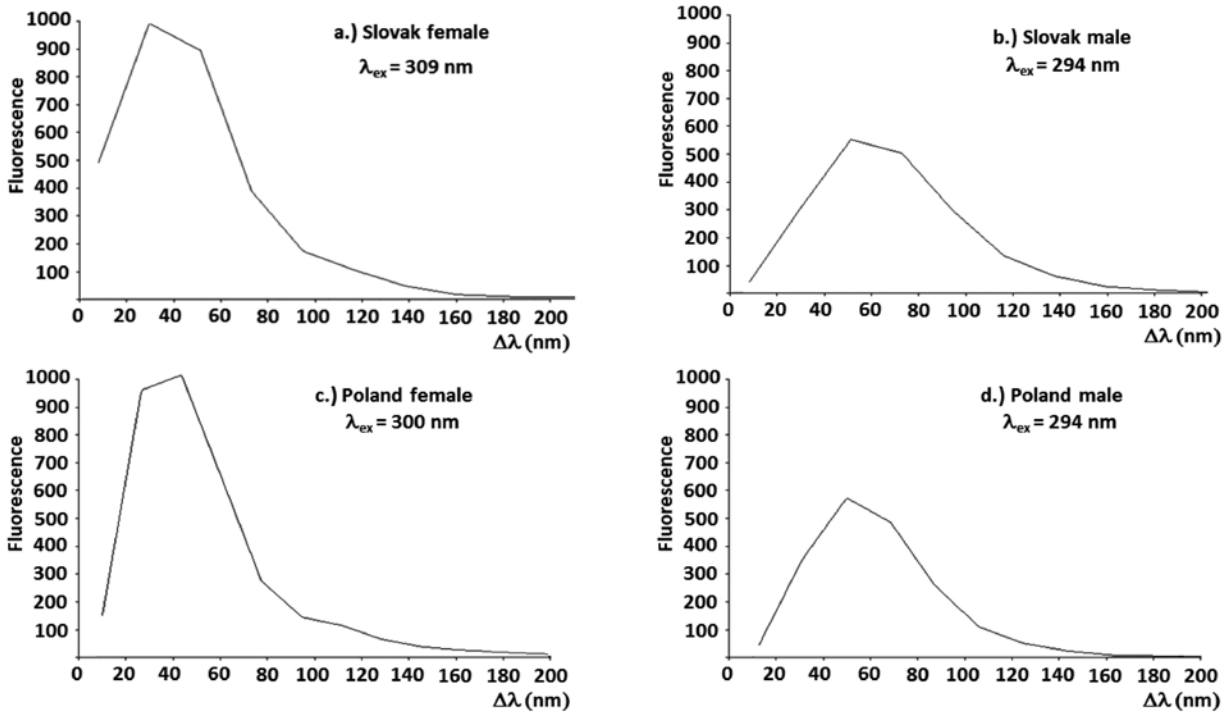

Fig. 2. The emission fluorescence spectra (comparison of fluorescence maxima in the excitation synchronous spectrum) of *Vipera berus berus* venom from: a) Slovak female; b) Slovak male; c) Poland female; d) Poland male. Δλ = difference between emission and excitation wavelength; λ_{ex} = excitation wavelength

CONCLUSIONS

This experimental study examined (and compared) the differences between viper venoms from males and females of *Vipera berus berus* living in Poland and Slovakia by means of fluorescence spectroscopy. The different amounts of individual fluorophores in viper venoms were revealed together with different locations and fluorescence intensities in the SFF which manifested unique structures of the individual venoms. The different composition of male and female venoms of *Vipera berus* living in Poland and Slovakia was probably influenced by the different biotopes.

The method used in this study might become an important tool for the analytical identification of novel and unknown venoms taken from different species of snakes.

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THE SUPERFICIAL VENOUS DRAINAGE OF THE RAT HEAD

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ABSTRACT

Considering the high variability in the incidence of vein anastomosis and due to alternative venous drainage, the aim of this study was to present the details in the venous vasculature of the rat head, and the existence of new and variable venous drainage pathways of the circulatory system presented with an emphasis on the anatomical variations. We used the corrosion cast method and radiography for the visualization of the venous system in 32 Wistar rats. Our results demonstrated the variability in the superficial head veins as manifested by differences in the size, mode of opening and formation of the common root. In 77 % of the cases, the *v. dorsalis nasi* formed an anastomosis with the *v. labialis superior*, and as observed in 26 % of the cases, the *vv. laterales nasi* formed mutual connections. The *v. transversa faciei* emptied into the *v. temporalis superficialis* in 81 % of the cases, but in 19 % of the cases, the *v. transversa faciei* directly entered into the *v. maxillaris*. The *v. occipitalis* entered to the point of the confluence of the *v. linguofacialis* with the *v. maxillaris* (61 %). In 31 % of the cases, the *v. occipitalis* emptied more caudally into the *v. jugularis externa*, and in 8 % of the cases, the *v. occipitalis* entered into the *v. maxillaris*. All of the information concerning the drainage pathways of the veins are important in reconstructive surgery to provide the proper controls and the precautions necessary for clinical practitioners who are required to perform procedures such as; cannulation, intravenous infusion, central venous pressure monitoring, or intravenous therapies in order to avoid the destruction of the venous system.

Key words: anastomosis; corrosion casting; head; rat; variability; venous system

INTRODUCTION

Extensive head deformities, including bone and soft tissue defects, are always challenging for reconstructive surgeons. The application of the face/scalp transplantation in rats´ model requires information about the vascularization when using this model to test new reconstructive options for head and neck deformities [14].

The rat has been used as a model for techniques that are crucial for microvascular en block tissue transfer and replantation. Preparation of the rat for the replantation requires familiarity with the vascular anatomy and the careful tissue handling with atraumatic dissection of the arterial and venous vessels [5].

Although the general distribution of the head venous vasculature in humans and animals has been reported [20], [21], it is surprising that the venous system in small laboratory animals such as mice, rats or guinea pigs have been only insufficiently described. The emphasis has been placed on the blood supply of the rat; mandible, molar teeth and their supporting structures [11]. Some articles pay attention to the cerebral venous drainage, intracranial connections between cerebral veins and dural venous sinuses, and the vascular structures that constitute the main parts of the selective brain cooling [4]. The mechanism of the selective brain cooling is mobilized when the organism is overheated; blood is transported

by the superficial nasal veins (*v. dorsalis nasi*), *v. angularis oculi* and by the ocular veins for this process to occur. Morphological examinations conducted on rabbits, pigs, and sheep, show that the *v. angularis oculi* and the *v. facialis* cools blood from the nasal area that may be directed either to the *sinus cavernosus* or through the *v. facialis* and the *v. maxillaris* veins to the jugular vein [23].

The only variations in the origin and termination of the *v. jugularis externa* and the *v. facialis* have been well documented in humans.

The purpose of this paper was to describe the details of the superficial venous drainage of the rat head. In this regard, radiographic and corrosion cast methods were used to reveal the anatomical variants of these veins

MATERIALS AND METHODS

We studied the anatomy of the venous system in 32 healthy adult Wistar rats of both sexes (20 females, 12 males), at 6 month of age with an average weight of 320 g. Animals were obtained from an accredited Laboratory of Research Biomodels, University of P. J. Safarik in Kosice. Immediately after transport, the animals were sacrificed with an intraperitoneal injection of sodium pentobarbital (50 mg.kg^{-1}, Thiopental Valeant, Valeant Czech Pharma, Czech Republic). We cannulated the *v. jugularis externa dextra* et *sinistra* and after heparin administration (50 000 IU.kg^{-1}, Heparin Leciva, Zentiva, Czech Republic), we perfused the venous system of the head with a 0.9 % saline solution [16]. We prepared the corrosion casts with Spofacryl® resin (SpofaDental Ltd, Jicin, Czech Republic). The casting medium in an amount of 15 ml was injected through the

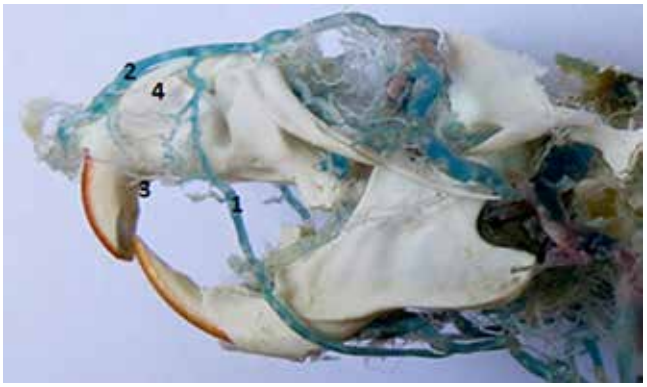

Fig. 1. Anastomoses among the *v. dorsalis nasi*, *vv. laterales nasi* and *vv. labiales superiores*, corrosion cast, lateral view. 1) *v. facialis;* 2) *v. dorsalis nasi;* 3) *vv. labiales superiores;* 4) *vv. laterales nasi*

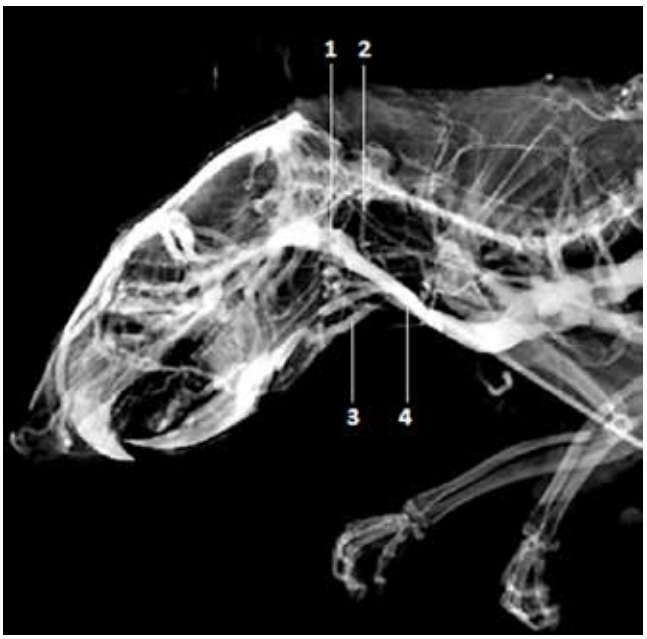

Fig. 2. *V. occipitalis* emptied into the *v. maxillaris*, lateral view, X-ray image. 1) *v. maxillaris;* 2) *v. occipitalis;* 3) *v. linguofacialis;* 4) *v. jugularis externa*

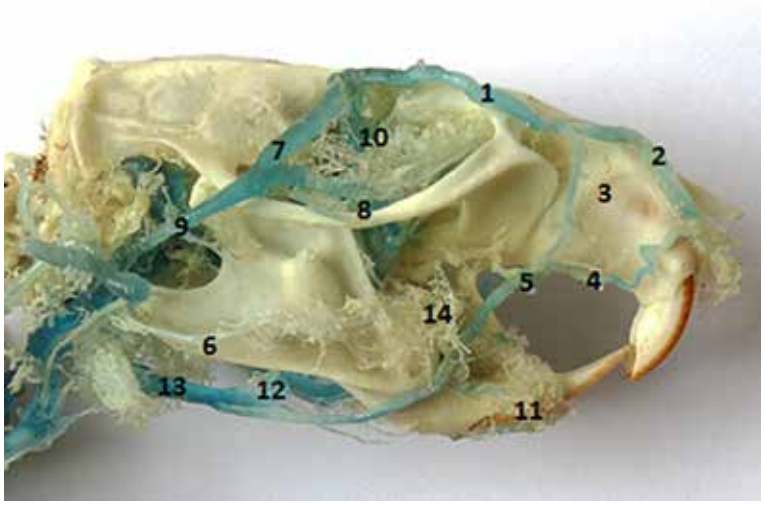

Fig. 3. The entire superficial venous drainage of the rat head. *V. transversa faciei* joined with *v. temporalis superficialis*. lateral view, corrosion cast. (1) *v. frontalis;* (2) *v. dorsalis nasi;* (3) *vv. laterales nasi;* (4) *vv. labiales superiores;* (5) *v. facialis;* (6) *v. masseterica caudalis;* (7) *v. temporalis superficialis;* (8) *v. transversa faciei;* (9) *v. maxillaris;* (10) *vv. ophthalmicae;* (11) *plexus mentalis;* (12) *v. lingualis;* (13) *v. linguofacialis;* (14) *v. buccalis*

plastic cannulae inserted and fixed in the bilateral v. jugularis externa. The soft tissue removal by maceration was done in potassium hydroxide solution (2—4 % KOH), from 3 to 4 days, at a temperature from 60 to 70 °C [17]. For radiography, we used a contrast agent of Urografin 76 % sol. inj. (Bayer Pharma AG, Germany) at a dose of 10 ml per rat. After intravenous application of the contrast agent into the bilateral *v. jugularis externa*, we used X-ray DRTECH FDxD 810 (DRTECH Corp., Korea) for radiography. The anatomical nomenclature of the veins were identified in accordance with the Nomina Anatomica Veterinaria [6].

RESULTS

The dominant venous drainage of the neck and the head was provided by the *v. jugularis externa*. In the rat, it was formed by the fusion of the *v. maxillaris* and *v. linguofacialis* which originated by the fusion of two large veins, i. e., *v. lingualis* and *v. facialis*.

The v. facialis commences at the side of the nasal root and runs downward to the *incisura vasorum facialium*. It was created rostral to the medial canthus under the union of the *v. frontalis, the v. angularis oculi medialis*, and the *v. dosalis nasi*. The *v. dorsalis nasi* served as the venous drainage from the dorsal and lateral region of the nose leading up to the nostrils. In 77 % of the cases, the anastomosis was established rostroventral with the *v. labialis superior* that drained the blood from the upper lip (Fig. 1). We noted two veins, the *vv. labiales superiores* in 56 % of the cases and observed the *vv. laterales nasi* (in number 2—4) between the *v. dorsalis nasi* and the *v. labialis superior.* In 26 % of the cases, the *vv. laterales nasi* formed mutual connections. The v. *labialis inferior* arose from the venous tangle plexus mentalis, that was present beside the bed of the lower incisors entering into the *v. facialis*. The *v. submentalis* drained towards the intermandibular area and entered in the *v. facialis* in 81 % of the cases, while the remaining 19 % merged with the *v. lingualis*. Other branches of the *v. facialis* were very thin veins; particularly, the *v. bucalis* branching from the *regio bucalis*, and *ramus massetericus* that drained the ventral part of the *m. masseter* without existing variations.

The *v. frontalis* began on the forehead which ran superficially and passed rostral to the medial angle of the orbit to join the facial vein, and caudally it communicated with the *v. temporalis superficialis*. At this point, it received the *v. angularis oculi lateralis*. The *v. frontalis* and the *v. temporalis superficialis* communicated with the ophthalmic veins. The ophthalmic veins occurred in numbers 3—4 and were conducted towards the medial wall of the orbit entering the *sinus periopticus* that had a direct connection with *sinus cavernosus* and thus with other intracranial dural venous sinuses.

The v. palpebralis inferior ran along the ventral side of the orbit circumference up to the *margo infraorbitalis* and entered into the *v. temporalis superficialis* in 72 % of the cases. In 28 % of the cases, it was observed as a tributary flow of the *v. transversa faciei*. The v. *palpebralis superior* entered the branch of the *v. supraorbitalis* in 53 % of the cases and in 47 % of the cases it was a tributary which flowed into the *v. temporalis superficialis*.

The v. transversa faciei ran from the caudal parts of the upper lip along the *facies lateralis ossis zygomatici*. It drained the blood from the *angulus oris*, the dorsal parts of the *m. masseter*, and emptied into the *v. temporalis superficialis* (Fig. 3). In 19 % of the cases, the *v. transversa faciei* directly entered into the *v. maxillaris*.

The *v. maxillaris* was the caudoventral continuation of *v. temporalis superficialis* consisting of the main maxillary blood vessel and its extensions. The *v. maxillaris* received blood from the thin veins of the *plexus pterygoideus*, palate and the pharynx, as well as from the auricles, and the *v. auricularis rostralis* et *caudalis.* The v. *masseterica caudalis* collected blood from the middle third of the mandible and emptied into the *v. maxillaris* in all studied cases.

The *v. occipitalis* began as a plexus at the caudal aspect of the skull from the *squama occipitalis*. Within the *v. occipitalis* we noted three types of venous drainage patterns. It frequently entered to the point of the confluence of the *v. linguofacialis* with the *v. maxillaris* in 61 % of the cases. The v. *occipitalis* terminated more caudally into the *v. jugularis externa* in 31 % of the cases, or it drained into the *v. maxillaris* in 8 % of the cases (Fig. 2).

DISCUSSION

It should be emphasized that veins are not only passive canals responsible for blood outflow from the tissues, but also they constitute a very specific multifunctional system. Depending on their location, veins perform different functions [18]. Within the head they participate in the regulation of the intracranial pressure. The superficial *v. facialis* take part in the process of selective brain cooling in humans and other animals [23].

Most of the superficial veins are used for various diagnostic and therapeutic procedures. Deviation from the normal pattern in the vascular system is a common feature, and it is more common in the veins than in the arteries [10]. Angiographic and anatomical studies in humans show a wide anatomical variability and varying degrees of jugular and non-jugular venous drainages. The variations in the origin and termination of the *v. jugularis externa* and the *v. facialis* in humans are well documented. Variations have been observed in the pattern of the termination of the facial vein into the external jugular vein. Thereafter, the unusual drainage of the common facial veins have been reported when blood drained into the subclavian vein, with the absence of external jugular veins bilaterally.

Another interesting variation was that the *v. facialis* opening into the *v. jugularis interna*, or the facial veins have been found to open into the *v. jugularis externa* at varying distances from the base of the mandible [3]. Thus, also in humans, an unusual course of the facial vein in combination with an anomalous termination of the vein was found. After almost a horizontal course, the *v. facialis* ended in the *v. temporalis superficialis* and cranial to the retromandibular

vein formed by the confluence of the maxillary with superficial temporal veins [22]. However, there is another variation: the persistence of the anastomotic channel and also failure of the retromandibular vein to drain into the linguofacial trunk [15]. According to Enjolras et al. [7], the variations in findings are much more frequent in patients with head and neck venous malformations than in the general population. But in the present study, we observed a high degree of variations even across the healthy rats.

In our study, the major vein providing the main venous drainage from the head was the *v. jugularis externa* as it is in other animal species [2], [20], [21], whereas in humans, the dominate vein represents the *v. jugularis interna* [2]. The *v. jugularis externa* in the rat originated by the confluence of the *v. linguofacialis* with the *v. maxillaris*, just as it has been described by Popesko et al. [21] and Hebel and Stromberg [9].

Within the superficial head veins we observed a high variability, where the *v. dorsalis nasi* in 77 % of the cases anastomosed with the *v. labialis superior*, and where in 56 % of the cases, 2 veins formed. We observed the *vv. laterales nasi* in varying numbers. They were presented as 2—4 veins that in 26 % of the cases created mutual anastomoses. The communication of the veins in the facial area of the brain, together with anastomosis between these veins, may be important in venous outflow during the brain cooling. We think that the existence of other drainage pathways could help blood flow and could lead to earlier cooling of the brain. There were two mains venous pathways, leading from thermolytic surfaces of the head to the intracranial cooling system, consisting of dural sinuses via the dorsal nasal and frontal veins and through the diploic network of the nasal bones [4].

According to Krzymovski et al. [13], the specific structure and function of the nasal and facial veins were connected, not only with the process of the selective brain cooling, but may also have served to receive pheromonal chemical signals, which were then subsequently sent to the *sinus cavernosus*, hypophysis and finally to the brain. Following this hypothesis, the structural adjustment and functional reactions of the nasal and facial veins can result in the activity of pheromones, or maybe also steroid hormones [13].

In the rat, we observed different points of entry by the *v. transversa faciei* that entered into the *v. maxillaris* in 19 % of the cases, while in 81 % of the cases it entered into the *v. temporalis superficialis*. Similarly, the *v. submentalis* discharged into the *v. facialis* in 81 % of the cases and in 19 % of the cases it merged with the *v. lingualis*.

Especially interesting was the different venous drainage from the upper and lower eyelids. Whereas the *v. palpebralis inferior* joined with the *v. transversa faciei* in 28 % of the cases, in 72 % of the cases it connected with the *v. temporalis superficialis*. The *v. palpebralis superior* fused with the *v. supraorbitalis* in 53 % of the cases, but in 47 % of the cases it emptied into the *v. temporalis superficialis*. According to Nejedlý [19], the *v. palpebralis inferior* entered into the *v. transversa faciei*, and as described by Popesko et al. [21], the *v. palpebralis inferior* was a tributary of the *v. temporalis superficialis*. Whereas, the communication among the *v. temporalis superficialis* and other veins may be essential, variations in their tributary flow played an important role, because this vein was often used in reconstruction surgery. Use of this vein for scalp and face reconstruction is reliable and safe. The superficial temporal vein should be considered as the primary recipient vessel in microsurgical reconstruction of the upper two-thirds of the face and/or scalp [8]. In humans, the *v. temporalis superficialis* may be divided into one, two or three major branches [12].

A high variability in the mode of opening demonstrated that the *v. occipitalis* entered into the *v. jugularis externa* in 31 % of the cases, whilst, the *v. occipitalis* entered into the point of confluence of the *v. linguofacialis* with the *v. maxillaris* in 61 % of the cases, and occasionally it entered in the *v. maxillaris* in 8 % of the cases. The v. *occipitalis* vein was connected to the *sinus occipitalis* and internal vertebral venous plexus. However, if it was blocked, it could cause problems with the lungs, heart or brain.

CONCLUSIONS

The results demonstrated the presence of unusual connections between superficial veins of the head, and revealed various drainage alternatives within these veins. Knowledge of the varying venous pattern in the head is important for clinical practitioners who perform procedures like cannulations, intravenous infusions, central venous pressure monitoring, or those who provide intravenous therapies. It is important for surgeons during neck and head surgery, as well as, for radiologists during catheterization and for internists in general [1].

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BRANCHES OF SUBCLAVIAN ARTERY IN THE GUINEA PIG

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ABSTRACT

The aim of this study was to describe the possible anatomical arrangements in branches arising from the *arteria subclavia dextra* and the *arteria subclavia sinistra*. The study was carried out on twenty-seven English self guinea pigs. The arterial system of the entire body was injected by Batson's corrosion casting kit No. 17. The branches of the *arteria subclavia dextra* showed atypical arrangements as follows: *arteria intercostalis suprema* in 13 animals, *arteria cervicalis superficialis* in 2 animals, *arteria thoracica interna* in 1 animal, *arteria vertebralis* in 9 animals, *arteria scapularis dorsalis* in 26 animals and *arteria cervicalis profunda* in all animals. The arrangement of the branches of the *arteria subclavia sinistra* were found as follows: *arteria intercostalis suprema* without atypical arrangement, *arteria cervicalis superficialis* atypical in 10 animals, *arteria thoracica interna* in 9 animals, *arteria cervicalis profunda* in 26 animals, *arteria scapularis dorsalis* and *arteria vertebralis* in all animals. Based on the results we can conclude that there is rather a high variability of the branches of both the subclavian arteries in guinea pigs.

Key words: *arteria subclavia*; corrosion cast; guinea pig; origin

INTRODUCTION

Guinea pigs have a special position in research. Together with rats, they are the most commonly used laboratory animals. Their small size, cleanliness, docility and relatively easy maintenance are the characteristic benefits that account for their usage as experimental animals [8].

Although there is frequent use of guinea pigs in biomedical research, there is still a significant gap in the scientific literature concerning the gross morphology of the guinea pig arterial system. Some published works deal with the arterial system of these animals in general [6], or more specifically [1], [2]. Only a few authors have concentrated on the investigation of their arterial system in detail [5], [7].

The aim of this study was to describe all possible variations in the arrangement of the branches arising from the *arteria subclavia dextra* and the *arteria subclavia sinistra*. They give off important branches participating in the blood supplying the head, neck, thorax and thoracic limbs.

MATERIALS AND METHODS

This study was carried out on 27 adult (age 220 days) English self guinea pigs of both sexes (female n = 14; male n = 13) weighing 0.8—1 kg in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Košice. The animals were kept in cages under standard conditions (temperature 15—20 °C, relative humidity 45 %, 12-hour light period), and provided with a granular feed mixture (FANTASIA, Tatrapet, Liptovský Mikuláš, Slovak Republic). Drinking water was available to all animals *ad libitum*. The animals were injected intravenously

with heparin (50 000 IU.kg^{-1}) 30 min before they were sacrificed by the intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}; Eurochem, Prague, Czech Republic).

Immediately after euthanasia, the vascular network was perfused with physiological saline solution. During the manual injection through the ascending aorta, the right atrium of the heart was opened in order to lower the pressure in the vessels in order to ensure a well perfused injection. Batson's corrosion casting kit No. 17 using a volume of 50 ml (Dione, České Budějovice, Czech Republic) was used as the casting medium. After polymerization, the maceration was carried out in 2–4 % KOH solution for a period of 2 days at 60—70 °C [3], [4]. The study was carried out under the authority decision No. 2647/07-221/5.

RESULTS

The *arteria subclavia dextra* arose from the *truncus brachiocephalicus* (Fig. 1) and the *arteria subclavia sinistra* was an independent branch coming from the *arcus aortae* (Fig. 4, 5).

Arteria subclavia dextra

The *arteria intercostalis suprema* originated as an independent branch in 14 animals (Fig. 1). It gave off 5 *arteriae intercostales dorsales* in 10 animals and 6 in 4 animals. The a*rteria cervicalis superficialis* was found as an independent branch in 25 animals (Fig. 1, 2, 3) and the *arteria thoracica interna* in 26 animals (Fig. 1, 2, 3). The a*rteria vertebralis* as a single artery was present in 17 animals (Fig. 1, 2), with independent origin in 8 animals. In 10 animals, it was formed by fusion of the *arteria vertebralis* I and the *arteria vertebralis* II (Fig. 3). The a*rteria vertebralis* II was found as an independent branch in 4 animals. In all cases, the fusion of the *arteria vertebralis* I and the *arteria vertebralis* II was located in the space between the sixth and seventh cervical vertebra (Fig. 3). The a*rteria scapularis dorsalis,* with independent origin, was present in 1 animal. In all cases, the *arteria scapularis dorsalis* and the *arteria cervicalis profunda* arose as a common trunk with a different place of origin.

The following trunks originated from the *arteria subclavia dextra*: the *arteria cervicalis profunda* and the *arteria intercostalis suprema*, which gave 5 *arteriae intercostales dorsales* in 1 animal; the *arteria cervicalis profunda*, *arteria scapularis dorsalis* and *arteria intercostalis suprema*, gave off 4 *arteriae intercostales dorsales* in 1 animal and 5 in 2 animals; *arteria cervicalis profunda*, *arteria scapularis dorsalis* and *arteria vertebralis* in 5 animals (Fig. 1); *arteria cervicalis profunda* and *arteria scapularis* dorsalis in 5 animals; *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* and *arteria intercostalis suprema* (Fig. 2), which gave off 3 *arteriae intercostales dorsales* in 1 animal and 5 in 2 animals; *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis*, *arteria cervicalis superficialis* and *arteria*

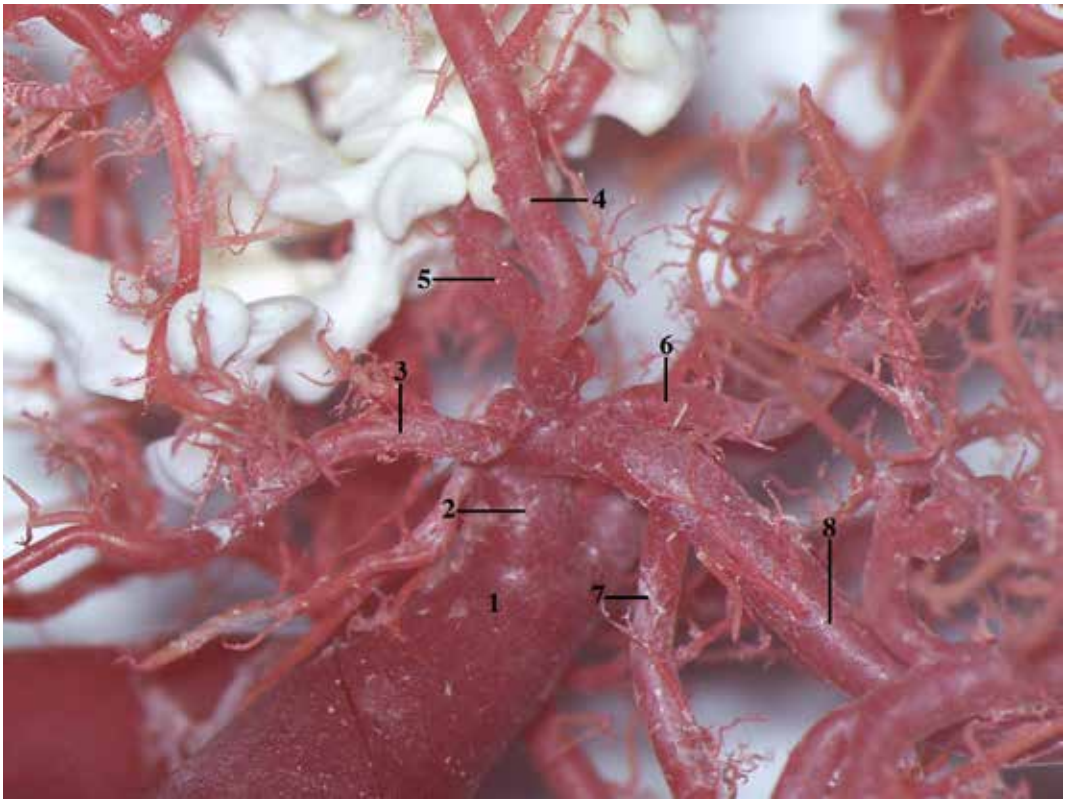

Fig. 1. Arrangement of branches of the *arteria subclaviadextra* with the presence of a common trunk formed by *the arteria cervicalis profunda*, the *arteria scapularis dorsalis* and the *arteria vertebralis*. (1) *truncus brachiocephalicus;* (2) *arteria subclavia dextra;* (3) *arteria intercostalis suprema;* (4) common trunk divided into the *arteria cervicalis profunda* and the *arteria scapularis dorsalis;* (5) *arteria vertebralis;* (6) *arteria cervicalis superficialis;* (7) *arteria thoracica interna;* (8) *arteria axillaris.* Caudoventral view. Magn. ×8

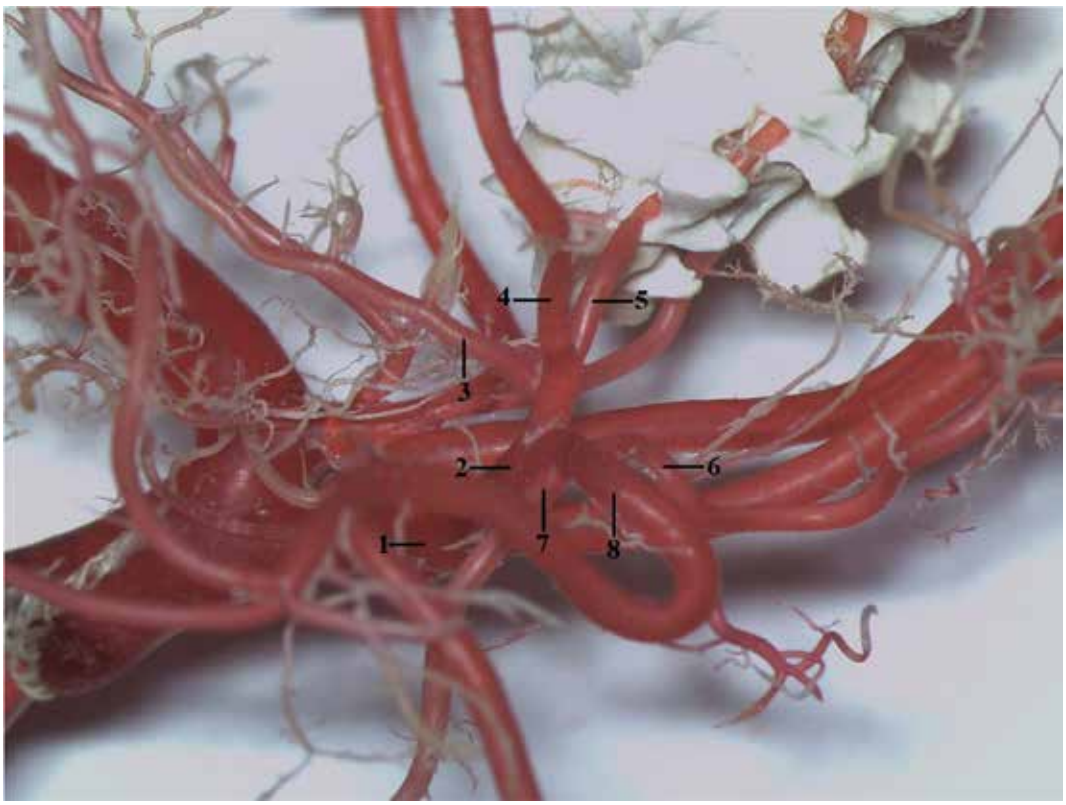

Fig. 2. Arrangement of the branches of the *arteria subclavia dextra* with the presence of a common trunk formed by the *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* and *arteria intercostalis suprema*. (1) *truncus brachiocephalicus;* (2) *arteria subclavia dextra;* (3) *arteria intercostalis suprema;* (4) common trunk divided into *arteria cervicalis profunda* and *arteria scapularis dorsalis;* (5) *arteria vertebra lis;* (6) *arteria cervicalis superficialis;* (7) *arteria thoracica interna;* (8) *arteria axillaris*. Lateral view. Magn. ×5

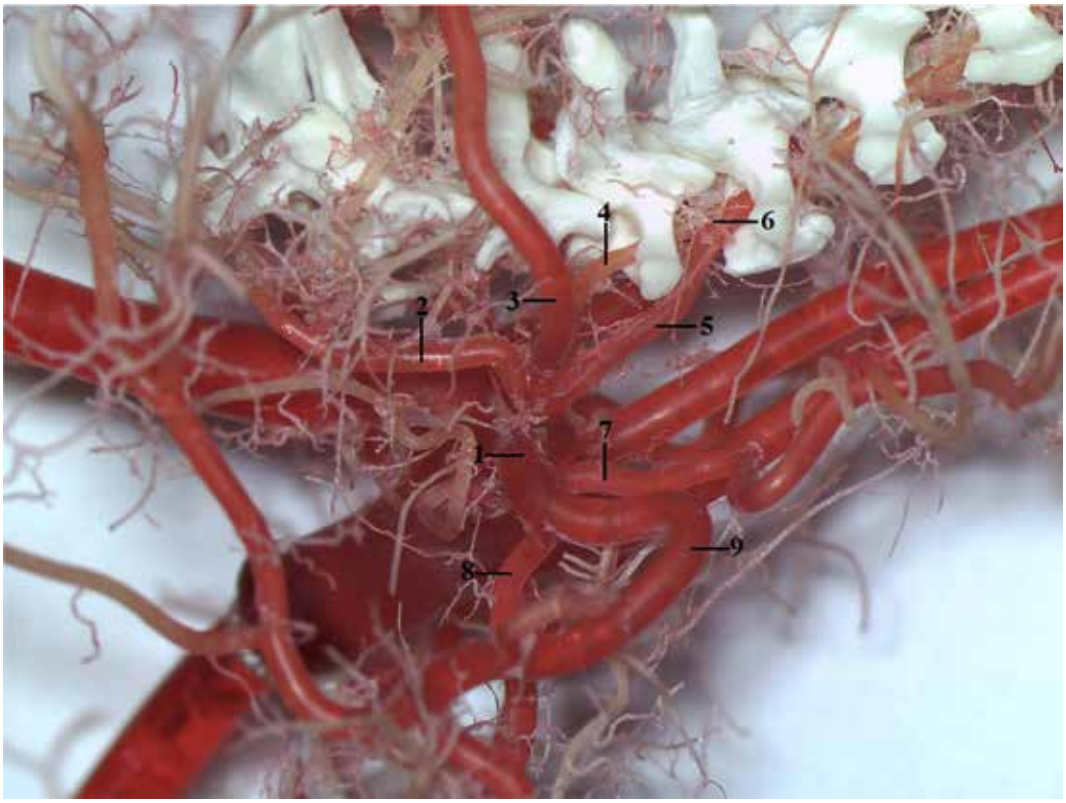

Fig. 3. Arrangement of the branches of the *arteria subclavia dextra* with the presence of a common trunk formed by the *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I, *arteria vertebralis* II and *arteria intercostalis suprema*. (1) *arteria subclavia dextra;* (2) *arteria intercostalis suprema;* (3) common trunk divided into the *arteria cervicalis profunda* and the *arteria scapularis dorsalis;* (4) *arteria vertebralis* I; (5) *arteria vertebralis* II; (6) fusion of the *arteriae vertebrales;* (7) *arteria cervicalis superficialis;* (8) *arteria thoracica interna;* (9) *arteria axillaris*. Lateral view. Magn. ×5

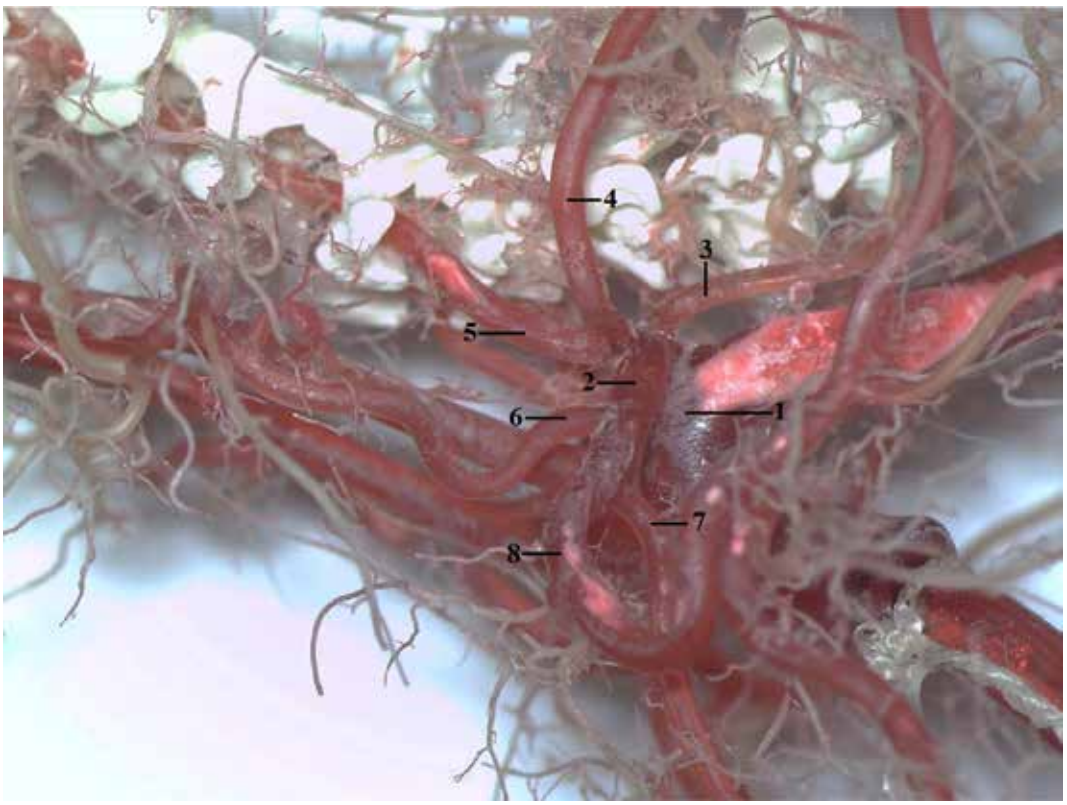

Fig. 4. Arrangement of the branches of the *arteria subclavia sinistra* with the presence of a common trunk formed by the *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I and *arteria vertebralis* II. (1) *arcus aortae;* (2) *arteria subclavia sinistra;* (3) *arteria intercostalis suprema;* (4) common trunk divided into the *arteria cervicalis profunda* and the *arteria scapularis dorsalis;* (5) *arteria vertebralis* I; (6) *arteria vertebralis* II; (7) fusion of the *arteriae vertebrales;* (8) *arteria cervicalis superficialis;* (9) *arteria thoracica interna;* (10) *arteria axillaris*. Lateral view. Magn. ×5

Fig. 5. Arrangement of the branches of the *arteria subclavia sinistra* with the presence of a common trunk formed by the *arteria cervicalis profunda* and *the arteria scapularis dorsalis*. (1) *arcus aortae;* (2) *arteria subclavia sinistra;* (3) *arteria intercostalis suprema;* (4) common trunk divided into the *arteria cervicalis profunda* and the *arteria scapularis dorsalis;* (5) *arteria vertebralis;* (6) *arteria cervicalis superficialis;* (7) *arteria thoracica interna;* (8) *arteria axillaris*. Lateral view. Magn. ×5

thoracica interna in 1 animal; *arteria vertebralis* I and *arteria vertebralis* II in 1 animal; *arteria cervicalis profunda*, *arteria scapularis dorsalis* and *arteria vertebralis* I in 2 animals; *arteria cervicalisprofunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I and *arteria vertebralis* II in 1 animal; *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I, *arteria vertebralis* II and *arteria intercostalis suprema* (Fig. 3), which gave off 8 *arteriae intercostales dorsales* in 1 animal, 6 in one animal and 5 in 1 animal; *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I and *arteria intercostalis suprema*, which gave off 6 *arteriae intercostales dorsales* in 2 animals, and *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria cervicalis superficialis*, *arteria vertebralis* I, *arteria vertebralis* II and *arteria intercostalis suprema*, which gave off 5 *arteriae intercostales dorsales* in 1 animal.

Both the *arteriae subclaviae* continue laterally around the first rib as the *arteriae axillares*. They proceeded ventrally to supply the thoracic limbs.

Arteria subclavia sinistra

The a*rteria intercostalis suprema* was present as an independent branch in all animals (Fig. 4, 5). Six *arteriae intercostales dorsales* were present in 8 animals, 5 in 16 animals and 4 in 3 animals. The a*rteria cervicalis superficialis* was found as an independent branch in 17 animals (Fig. 4, 5) and the *arteria thoracica interna* in 18 animals (Fig. 4, 5). The a*rteria vertebralis,* as a single artery, was found in 10 animals (Fig. 5) and as an artery formed by the fusion of the *arteria vertebralis* I and the *arteria vertebralis* II in 17 animals (Fig. 4). The a*rteria vertebralis* I, as an independent artery, was discovered in 1 animal and the *arteria vertebralis* II in 6 animals. In all specimens, the fusion of the *arteria vertebralis* I and the *arteria vertebralis* II was located in the space between the sixth and seventh cervical vertebra (Fig. 4). The *arteria cervicalis profunda* was present as an independent artery in 1 animal. In all cases, the *arteria scapularis dorsalis* and the *arteria cervicalis profunda* arose as a common trunk which had a different place of origin. There were formed the following trunks arising from the *arteria subclavia sinistra*: *arteria cervicalis profunda* and *arteria scapularis dorsalis* in 3 animals (Fig. 5); *arteria cervicalis profunda*, *arteria vertebralis* and *arteria cervicalis superficialis* in 1 animal; *arteria cervicalis profunda* and *arteria vertebralis* in 3 animals; *arteria cervicalis superficialis* and *arteria thoracica interna* in 1 animal; *arteria cervicalis profunda*, *arteria vertebralis*, *arteria scapularis dorsalis*, *arteria cervicalis superficialis* and *arteria thoracica interna* in 6 animals; *arteria scapularis dorsalis* and *arteria vertebralis* I in 5 animals; *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria vertebralis* I and *arteria vertebralis* II in 11 animals (Fig. 4), and *arteria cervicalis profunda*, *arteria scapularis dorsalis*, *arteria cervicalis superficialis* and *arteria thoracica interna* in 2 animals.

DISCUSSION

Arteria subclavia dextra

The origin, course and direction of the *arteria subclavia dextra* in our specimens were similar to the observations of Popesko et al. [5] and Shively and Stump [7].

The *arteria intercostalis suprema* and the *arteria cervicalis profunda* were described as branches arising from the *truncus costocervicalis*, which was a branch of the *arteria subclavia dextra* [7]. Popesko et al. [5] described it as an independent branch. We found the *arteria intercostalis suprema* as an independent branch of the *arteria subclavia dextra* in 14 animals. In the rest of the cases, it originated as a common trunk with other branches of the *arteria subclavia dextra*. It gave off 5 the *arteriae intercostales dorsales* in 17 animals, 6 in 7 animals, 3 in 1 animal, 4 in 1 animal and 8 in 1 animal. Popesko et al. [5] found 5 *arteriae intercostales dorsales.* Shively and Stump [7] described 4 to 7 *arteriae intercostales dorsales*. They found the first and occasionally the second *arteria intercostalis dorsalis* as arteries originating directly from the *truncus costocervicalis*. Only in 1 animal, we found the same *truncus costocervicalis,* similar to Shively and Stump [7].

In all cases, the *arteria cervicalis profunda* originated by means of a common trunk with the other branches of the *arteria subclavia dextra*. Popesko et al. [5] described it as a branch originating together with the *arteria vertebralis* I and Shively and Stump as a branch of the *truncus costocervicalis* [7].

The *arteria cervicalis superficialis* originated directly from the *arteria subclavia dextra* in 25 of our animals and in the rest, two as a common trunk with the other branches of the *arteria subclavia dextra*. Popesko et al. [5] and Shively and Stump [7] found it only as an independent branch in all of their cases.

Only in 1 animal, did the *arteria thoracica interna* originate as a common trunk with the other branches of the *arteria subclavia dextra*. In the remaining cases, it was an independent branch. Popesko et al. [50] and Shively and Stump [7] found it only as an independent branch in all of the cases.

The *arteria scapularis dorsalis* arose as an independent branch in 1 animal. In 26 cases it arose as a common trunk with other branches of the *arteria subclavia dextra*. Shively and Stump [7] described a common trunk formed by the *arteria scapularis the dorsalis*, *arteria vertebralis* I and the *arteria vertebralis* II. Popesko et al. [5] did not describe the *arteria scapularis dorsalis.*

In 8 animals, the *arteria vertebralis* was present as a single independent artery arising from the *arteria subclavia dextra* and in 9 animals, it arose as a common trunk with the other branches of the *arteria subclavia dextra.* In 10 animals, the *arteria vertebralis* originated in form of two branches. In

10 animals, the *arteria vertebralis* I arose as a common trunk with the other branches of the *arteria subclavia dextra. The arteria vertebralis* II originated as an independent branch in 4 animals and in 23 cases it formed a common trunk with other branches of the *arteria subclavia dextra.* Popesko et al. [5] described the *arteria vertebralis* as a fusion of the *arteria vertebralis* I and the *arteria vertebralis* II. The a*rteria vertebralis* I arose as a common trunk with the *arteria cervicalis profunda* and the *arteria vertebralis* II originating from the *arteria subclavia dextra*. Their fusion was described between the sixth and seventh cervical vertebra, also. Shively and Stump [7] found a common trunk formed by the *arteria vertebralis* I, the *arteria vertebralis* II and the *arteria scapularis dorsalis* with the same place of fusion.

Arteria subclavia sinistra

The origin, course and direction of the *arteria subclavia sinistra* in our specimens have been covered previously by the descriptions of Popesko et al. [5] and Shively and Stump [7].

We found *the arteria intercostalis suprema* as an independent branch in all animals. The same findings were presented by Popesko et al. [5]. Shively and Stump [7], who described it as a branch of the *truncus costocervicalis* and the *arteria cervicalis profunda*. It gave off 6 *arteriae intercostales dorsales* in 8 animals, 5 in 16 animals, and 4 in 3 animals. Popesko et al. [5] described 5 *arteriae intercostales dorsales*. Shively and Stump [7] found 4 to 7 *arteriae intercostales dorsales*, but the first and occasionally the second *arteria intercostalis dorsalis* were branches of the *truncus costocervicalis*.

The *arteria cervicalis profunda* was present only in 1 animal as an independent branch and in the rest of the cases it formed a common trunk with other branches of the *arteria subclavia sinistra*. Shively and Stump [7] described it as a branch of the *truncus costocervicalis* and Popesko et al. [5] found a common origin with the *arteria vertebralis* I.

The arteria cervicalis superficialis was present as an independent branch in 17 animals and in 10 it formed a common trunk with other branches of the *arteria subclavia sinistra*. As an independent branch, it was described by Popesko et al. [5] and Shively and Stump [7].

In 18 animals, the *arteria thoracica interna* was present as an independent branch. In 9 animals, it formed a common trunk with other branches of *arteria subclavia sinistra*. Its independent origin was found by Popesko et al. [5] and Shively and Stump [7] in all of their specimens.

In all of our cases, the *arteria scapularis dorsalis* formed a common trunk with other branches of the *arteria subclavia sinistra*. Popesko et al. [5] did not mention the *arteria scapularis dorsalis*. Shively and Stump [7] described a common trunk which divided into the *arteria scapularis dorsalis*, *arteria vertebralis* I and *arteria vertebralis* II.

The *arteria vertebralis,* as a single branch, was present in 10 our animals and originated forming a common trunk with another branches of the *arteria subclavia sinistra*. In 17 animals, it was formed by the fusion of the *arteria vertebralis* I and the *arteria vertebralis* II. The arteria vertebralis I was present as an independent branch in 1 animal and the *arteria vertebralis* II in 6 animals. Popesko et al. [5] described the *arteria vertebralis* as a common trunk after fusion of the *arteria vertebralis* I with the *arteria vertebralis* II. The a*rteria vertebralis* I originated together with *arteria cervicalis profunda,* forming a common trunk and the *arteria vertebralis* II was an independent branch of the *arteria subclavia dextra*. Their fusion was located between the sixth and seventh cervical vertebra also. Shively and Stump [7] found a common trunk formed by the *arteria vertebralis* I, the *arteria vertebralis* II and the *arteria scapularis dorsalis* with the same place of fusion.

CONCLUSIONS

The anatomical details found by our investigation strongly suggests that the corrosion cast technique is still an important part of teaching anatomy and provides an excellent opportunity for the integration of anatomy, clinical medicine and experimental medicine.

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FORMATION OF VERTEBROBASILAR SYSTEM IN THE GUINEA PIG

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ABSTRACT

The aim of this study was to describe the arterial arrangement of the cervical spinal cord in guinea pigs using the corrosion cast technique. The study was carried out on 20 adult English self guinea pigs. We prepared corrosion casts of the arterial system of the cervical spinal cord. Spofacryl was used as a casting medium. The ventral spinal artery branched from the right vertebral artery in 40 % of the cases, from the left vertebral artery in 35 % of the cases and from the anastomosis of two medial branches of the vertebral artery on the corresponding side in 25 % of the cases. The fusion of the bilateral vertebral arteries was found without a triangular gap, with one longitudinal triangular gap and with the presence of a communicating branch between the bilateral vertebral arteries in the place of their fusion. The implications of our results will help in planning and executing experimental procedures in this species, and interpreting the data obtained, especially when the aim is to extrapolate the information to humans.

Key words: basilar artery; corrosion technique; guinea pig; spinal cord

INTRODUCTION

It is well known that anatomical differences exist in species in many organ systems and the cardiovascular systems [2], [7]. Guinea-pigs are frequently used in many research areas including; heart failure, abnormalities of the circulatory system, blood supply to organ systems and ischemia. Sufficient anatomical knowledge of the vascular distribution to organ systems may help to analyze the experimental findings from a practical viewpoint.

The literature regarding the distribution of arteries supplying the spinal cord of the guinea-pig is limited [3]. Therefore, the aim of this study is to investigate the blood supply of the guinea pig spinal cord with a focus on the cervical region.

MATERIALS AND METHODS

The study was carried out on 20 adult guinea pigs (age 220 days). We used English self guinea pigs of both sexes (female n = 10; male n = 10) with an average body weight of 0.8—1 kg in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15—20 °C, relative humidity 45 %, 12-hour light period), and fed with a granular feed mixture (FANTASIA). Drinking water was available to all animals *ad libitum*. The animals were injected intravenously with heparin (50 000 IU.kg^{-1}) 30 min before they were sacrificed by intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}).

Immediately after killing, 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 the pressure in the vessels to ensure an optimal injection

distribution. Spofacryl, in a volume of 50 ml (polymethylmethacrylate, SpofaDental, Czech Republic), was used as the casting medium. After polymerization, the maceration was carried out in a 2—4% KOH solution for a period of 2 days at 60—70 °C [4], [5]. This study was carried out under the authority decision of No. 2647/07-221/5.

RESULTS

The blood supply of the cervical spinal cord was more complicated than in the other spinal regions. The posterior inferior cerebellar artery arose bilaterally from the corresponding vertebral artery and gave off numerous small branches supplying the most cranial part of the first cervical segment.

The vertebral artery emerged from the thoracic cavity through the thoracic inlet. It then passed through the transverse opening of the sixth cervical vertebra and continued cranially through the transverse canal of the cervical vertebrae. Its spinal branches supplied the cervical part of the spinal cord. Bilateral vertebral arteries entered the vertebral canal through the *foramen vertebrale laterale* of the first cervical vertebra. The bilateral vertebral arteries were fused together on the caudal margin of the dorsal surface of the *pars basilaris ossis occipitalis* forming the basilar artery. The basilar artery participated in the formation of the cerebral arterial circle.

The fusion of the bilateral vertebral arteries was found in 60% of the cases without a triangular gap (Fig. 1) and in 30% of the cases with one longitudinal triangular gap. In 10% of the cases, was found a communicating branch between the bilateral vertebral arteries (Fig. 2). The rostral origin of the ventral spinal artery was located at the level of formation of the basilar artery. The ventral spinal artery branched from the right vertebral artery in 40% of the cases (Fig. 2), from the left vertebral artery in 40% of cases, and from the anastomosis of two medial branches of the vertebral artery on the corresponding 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.

DISCUSSION

The arterial supply to the spinal cord of the guinea pig is basically similar to that in the human [3]. However, based on our study, we can conclude that the guinea pig has a somewhat different arterial arrangement compared that found in the human. We found the formation of the basilar artery without a triangular gap, with one longitudinal triangular gap or with a communicating branch between the bilateral vertebral arteries in the place of their fusion. In humans, the basilar artery is formed by the fusion of bilateral vertebral arteries without a triangular gap [1].

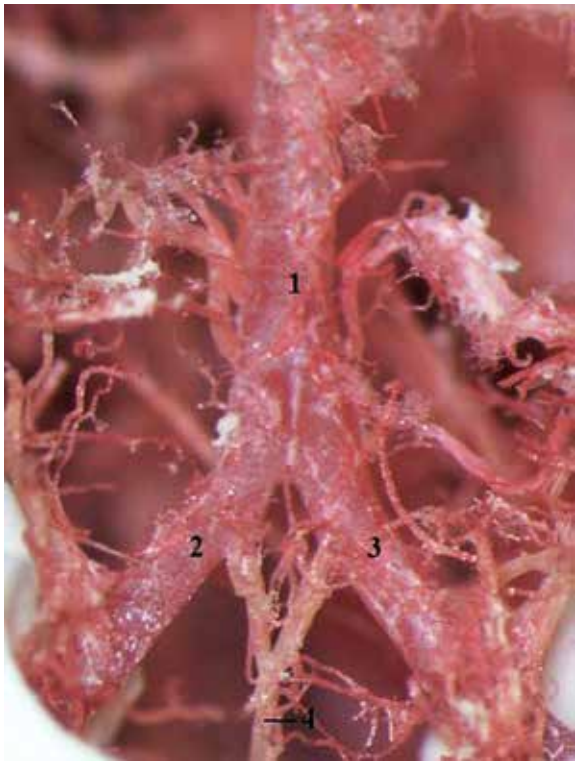

Fig. 1. Formation of the ventral spinal arteries from the anastomosis of two arteries, each coming from the medial border of the corresponding vertebral artery. (1) basilar artery; (2) left vertebral artery; (3) right vertebral artery; (4) ventral spinal artery. Dorsal view. Magn. × 12.5

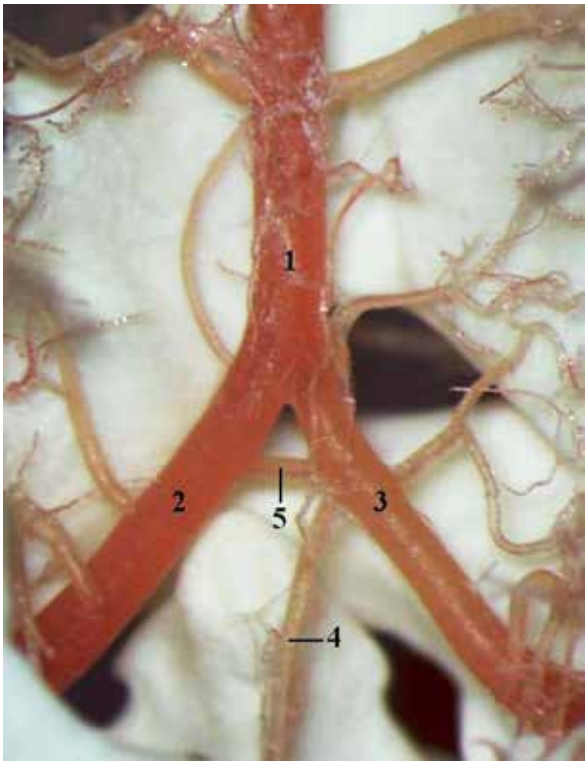

Fig. 2. Origin of the ventral spinal artery from the right vertebral artery, the presence of the communicating branch between the bilateral vertebral arteries. (1) basilar artery; (2) left vertebral artery; (3) right vertebral artery; (4) ventral spinal artery; (5) communicating branch. Dorsal view. Magn. × 12.5

The ventral spinal artery (in humans, the anterior spinal artery) rostrally originated from the right vertebral artery, from the left vertebral artery and from the anastomosis of two medial branches of the vertebral artery on the corresponding sides. The anterior spinal artery in the human is formed only by the fusion of the anterior spinal branches of the vertebral arteries at the level of the foramen magnum [6].

In the majority of the cases, the rostral origin of the dorsal spinal arteries was from the posterior inferior cerebellar artery. In the remainder, it was formed from the vertebral artery. We did not find the connection of the dorsal spinal arteries to the vertebral or cerebellar arteries in our specimens. The most cranial part of the first cervical segment was supplied by small branches arising from the posterior inferior cerebellar artery.

The study of the arterial patterns of the spinal cord are based primarily on the use of an experimental animal which may serve to elucidate the principles upon 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 [8].

CONCLUSIONS

In the guinea pig, the frequency of individual radicular branches reaching the cervical spinal cord is greater than is the case in the human. Thus, arteries supplying the cervical spinal cord have a more segmented pattern in guinea pigs than in humans. According to our results, it can be concluded that the higher resistance to ischemic damage by the interruption of ventral and spinal arteries, is because of the presence of radicular arteries in almost every segment.

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ARTERIES OF THE CERVICAL SPINAL CORD IN THE GUINEA PIG

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ABSTRACT

The aim of this study was to describe the arrangement of arteries supplying the cervical spinal cord in guinea pigs. The study was carried out on 20 adult English self guinea pigs. The arteries of the cervical spinal cord were injected by using Batson's corrosion casting kit no. 17. The presence of the ventral radicular branches of the *rami spinales* entering the ventral spinal artery in the cervical region was observed in 42 % of the cases on the right side and in 58 % of the cases on the left side. There were two dorsal spinal arteries located on the dorsal surface of the cervical spinal cord in 60 % of the cases, three in 30 % of the cases and there were highly irregular vascular patterns in 10 % of the cases. The presence of the dorsal radicular branches of the *rami spinales* that reached the spinal cord, was observed in 63 % of the cases on the left side and in 37 % of the cases on the right side. Based on our results, we can conclude that there is a high variability of the blood supply to the cervical spinal cord in the guinea pig. The number of radicular branches supplying the spinal cord is greater in guinea pigs than in humans.

Key words: guinea pig; dorsal spinal artery; segmental artery; ventral spinal artery

INTRODUCTION

The guinea pig has been used as an experimental model in the study of spinal cord injuries and the effects of various neuroprotective drugs on the damaged spinal cord [3], [7], [12]. The arterial blood supply of the cervical spinal cord in guinea pigs has been described in only a few studies [4], [9]. Research on the arterial arrangement and the variability of arteries feeding the spinal cord has been reported in several species of laboratory animals [8], [10], [11], [13].

The aim of this study was to contribute to the anatomical knowledge of the blood supply of the guinea pig spinal cord with a focus on the cervical region. We describe some variations in the arrangement of the segmental spinal arteries in the cervical spinal cord of the guinea pig.

MATERIALS AND METHODS

The study was carried out on 20 adult guinea pigs (age 220 days). We used English self guinea pigs of both sexes (female n = 10; male n = 10) with an average weight of 0.8—1 kg in an accredited experimental laboratory of the University of Veterinary Medicine and Pharmacy in Kosice. The animals were kept in cages under standard conditions (temperature 15—20 °C, relative humidity 45 %, 12-hour light period), and fed with a granular feed mixture (FANTASIA). Drinking water was available to all animals *ad libitum*. The animals were injected intravenously with heparin (50 000 IU.kg^{-1}) 30 min before they were sacrificed by intravenous injection of embutramide (T-61, 0.3 ml.kg^{-1}).

Immediately after death, 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 the pressure in the vessels to ensure an optimal injection distribution. Batson's corrosion casting kit No. 17 using a volume of 50 ml (Dione, Česke Budějovice, Czech Republic) was used as the casting medium. After polymerisation of the medium, 4 % formaldehyde was injected into the vertebral canal between the occipital bone and the first cervical vertebra, and between the sixth and seventh cervical vertebra to fix the spinal cord. After 1-week fixation, the vertebral canal was opened by removing the vertebral arches in the cervical spinal region. Also, the occipital bone was partly removed. The prepared spinal cord was fixed in 4 % formaldehyde. This study was carried under authority decision No. 2647/07-221/5.

RESULTS

The ventral spinal artery was present as a single trunk located on the ventral surface of the spinal cord. Two or three dorsal spinal arteries were present on the dorsal surface. The ventral and dorsal spinal arteries had rostral terminations at the vertebral arteries. They received along their course several dorsal and ventral radicular branches entering the intervertebral foramina. Some radicular branches significantly contributed to the spinal cord blood supply.

The ventral spinal artery was located along the ventral median fissure of the spinal cord. The *rami spinales* arose from the bilateral vertebral arteries and entered the vertebral canal through the intervertebral foramen. After entering the vertebral canal, they sent ventral and dorsal radicular branches to the spinal cord. Some ventral radicular branches entered the ventral spinal artery. The frequency of occurrence of individual ventral radicular branches reaching the spinal cord is shown in Table 1. The left-sided ventral radicular branches entering the ventral spinal artery were present in 58 % of the cases, the right sided ventral radicular branches were present in 42 % of the cases.

Two dorsal spinal arteries were located on the dorsal surface of the cervical spinal cord in 60 % of the cases (Fig. 1); there were three in 30 % of the cases (Fig. 2), and they formed highly irregular vascular pattern in 10 % of the cases. Two longitudinal dorsal spinal arteries were formed by the fusion of the small cranial and caudal branches arising from the dorsal radicular branches of spinal arteries. In the case of the highly irregular vascular pattern of the dorsal spinal arteries, the cervical part of spinal cord received the blood by means of dorsal radicular branches of spinal arteries with a very irregular arrangement (Fig. 3). We found no rostral origin of dorsal spinal arteries in the area of the formation of the basilar artery. The frequency of the occurrence of the individual dorsal radicular branches reaching the spinal cord is shown in Table 2. The left-sided dorsal radicular branches were present in 63 % of the cases, the right-sided ventral radicular branches were present in 37 % of the cases.

DISCUSSION

Based on our results, we can conclude that there is a high variability of the blood supply pattern in the cervical part of the spinal cord in guinea pigs. Others have described only a uniform rostral origin of the ventral spinal artery [4], [9]. Dogs, rats, pigs and guinea pigs are often used in the study of cervical spinal cord injury. In the dog, the blood supply of the cervical spinal cord has been studied in detail, including variations in the rostral origin of the ventral spinal artery and the frequency of occurrence of spinal arteries [6]. The blood supply of the rat spinal cord is probably the most profusely documented, but the results were often variable [1], [9], [13], [14]. In the pig, only variations of the spinal cord blood supply and the presence of extrasegmental arteries have been described [10], [11]. The frequency of the occurrence of the segmental spinal arteries was higher on the left than on the right side, i. e., opposite the case in dogs [6].

Table 1. Frequency of occurrence of ventral radicular arteries of the cervical spinal cord that contributed significantly to the spinal cord blood supply

	Right	Left
C1	0	0
C2	30	30
C3	0	0
C4	30	60
C5	50	50
C6	90	30
C7	0	100
C8	30	50
	42 %	**58 %**

C — Cervical segment of the spinal cord

Table 2. Frequency of occurrence of dorsal radicular arteries of the cervical spinal cord that contributed significantly to the spinal cord blood supply

	Right	Left
C1	50	50
C2	0	50
C3	50	60
C4	30	90
C5	50	50
C6	60	100
C7	0	50
C8	50	50
	37 %	**63 %**

C — Cervical segment of the spinal cord

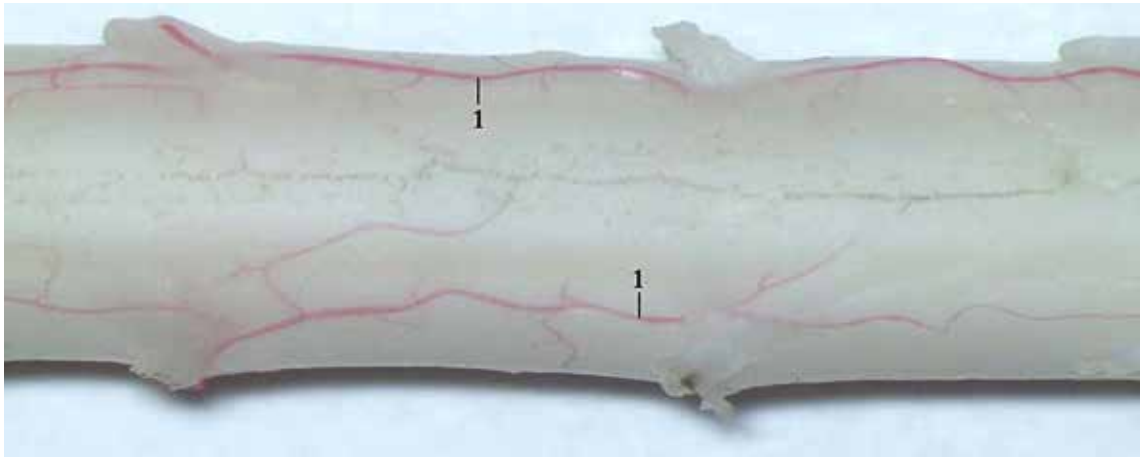

Fig. 1. Presence of two dorsal spinal arteries. (1) Dorsal spinal artery. Dorsal view. Magn. × 12.5

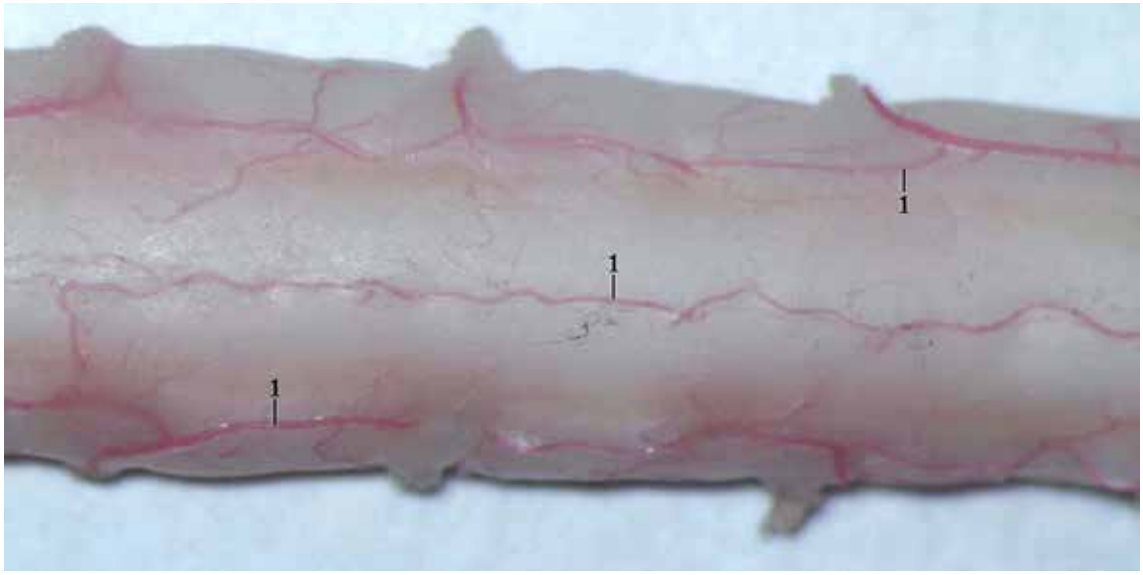

Fig. 2. Presence of three dorsal spinal arteries. (1) Dorsal spinal artery. Dorsal view. Magn. × 12.5

Fig. 3. Dorsal spinal arteries forming highly irregular vascular pattern. Dorsal radicular branches of spinal arteries with irregular arrangement. Dorsolateral view. Magn. × 12.5

Knox-Macaulay et al. [4] studied the arterial blood supply to the spinal cord of the guinea pig. From the results obtained, the frequency of the occurrence of the segmental arteries in the cervical region is not clear. They found 2 to 5 ventral radicular branches entering the ventral spinal artery. The ventral spinal artery was described as sometimes interrupted in its course and at the cranial end connected to the vertebral arteries. We found the ventral spinal artery to be an interrupted single trunk with its rostral origin at the level of the junction of bilateral vertebral arteries, but with different arrangements.

We found right-sided radicular ventral branches entering the ventral spinal artery in 42 % and the left-sided in 58 % of cases. Melissano et al. [5] described in humans 2 or 3 ventral radicular branches entering the anterior spinal artery.

On the dorsal surface we found two, three, or a highly irregular vascular pattern of dorsal spinal arteries (in human the posterior spinal arteries). In humans, the posterior spinal arteries are normally rostral and caudal continuing trunks which supply the posterior third of the spinal cord [2].

CONCLUSIONS

The guinea pig is often used as an experimental model for the study of spinal cord injury. The cervical region in laboratory animals is the experimental model for the study of several types of damage [3], [7], [12]. The more detailed description of the arterial arrangement of the cervical spinal cord contributes toward a better understanding of the pathophysiology in several experimental studies and by this way, it may serve to eliminate biased or erroneous results in such studies.

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DOSE- AND TIME-DEPENDENT *IN VITRO* EFFECTS OF QUERCETIN ON BOVINE SPERMATOZOA ACTIVITY AND SUPEROXIDE PRODUCTION

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ABSTRACT

This study was designed to investigate the dose- and time dependent *in vitro* effects of quercetin (QUE), a natural flavonoid with an array of biological properties on bovine spermatozoa during selected short-term (0 h, 2 h, 6 h) or long-term (12 h, 24 h) *in vitro* culture periods. Semen samples were collected from 20 adult breeding bulls and diluted in physiological saline solution containing 200, 100, 50, 10, 5 and 1 μmol.l^{-1} QUE. The spermatozoa motion parameters were assessed using the SpermVision™ and theCASA (Computer Assisted Semen Analyzer) system. The cell viability was examined using the metabolic activity MTT assay and the nitroblue-tetrazolium (NBT) test was applied to quantify the intracellular superoxide formation. The CASA analysis revealed that QUE supplementation was able to prevent a rapid decline of spermatozoa motion parameters, especially in the case of concentrations ranging between 10 and 100 μmol.l^{-1} ($P < 0.001$ with respect to Times 12 h and 24 h). At the same time, supplementation of 50 and 100 μmol.l^{-1} of QUE led to a significant preservation of the cell viability throughout the short term ($P < 0.05$ in the case of Time 2 h), as well as long-time periods of the experiment ($P < 0.01$ with respect to Time 12 h, and $P < 0.001$ in the case of Time 24 h). Fifty and 100 μmol.l^{-1} of QUE exhibited antioxidant characteristics which translated into a significant reduction of the intracellular superoxide production, particularly notable at Times 12 h ($P < 0.01$ in the case of 100 μmol.l^{-1} QUE; $P < 0.05$ with respect to 50 μmol.l^{-1} QUE) and 24 h ($P < 0.001$). The results indicate that QUE supplementation to bovine spermatozoa, particularly applying concentrations ranging between 10 and 100 μmol.l^{-1}, could be beneficial for a complex enhancement of the spermatozoa activity and the protection against the deterioration resulting from an exposure to an *in vitro* environment.

Key words: bulls; motility; oxidative stress; quercetin; spermatozoa; viability

INTRODUCTION

Contemporary evidence substantiates that oxidative stress (OS) plays an important role in male reproductive dysgenesis. The increased production of abnormal spermatozoa generating free radicals (FRs) and a compromised antioxidant activity of semen, frequently reported in animals and humans, are two major factors underlying seminal oxidative stress [3], [31]. Meanwhile, a characteristic cellular structure leaves a spermatozoon to be particularly vulnerable to oxidative damage. Typically, spermatozoal membranes contain large quantities of polyunsaturated fatty acids, while their cytoplasms lack considerable amounts of FR scavengers [9], leading to increased oxidative insults, and consequently, a decrease of motility and viability [12]. Additionally, OS may often result in increased morphological defects, with alterations to the sperm capacitation and acrosomal reaction, all of which are related to a compromised reproductive performance [2].

Studying the benefits of antioxidant supplementation on spermatozoa physiology and male fertility has become the spotlight of

diverse scientific reports [1], [13]. Nonetheless, currently available data illustrating the *in vitro* effects of antioxidants on the sperm cell behaviour have been shown to be contradictory. Examinations of the *in vitro* antioxidant potential of different compounds has repeatedly demonstrated that antioxidants may offer protection to the male germ cell against oxidative injury and subsequent structural or functional alterations. Additional *in vitro* protection of spermatozoa, provides an extra advantage during procedures designed for long-term spermatozoa preservation (cryoconservation) or protocols related to assistance of reproductive techniques. The *in vitro* environment represents an additional hazard to the sperm survival, as it may allow optimal conditions for FR overproduction and a subsequent damages to the spermatozoa [27].

This study provides more feedback on the activity of quercetin (3,30,40,5,7-pentahydroxylflavone; QUE), a flavonol-type flavonoid, which is ubiquitous in plant-derived foods and drugs. Quercetin has been reported to exhibit anti-inflammatory [16], anti-aggregatory [26] and vasodilating [15] effects. At the same time, quercetin has been shown to be an excellent *in vitro* antioxidant. It is believed to be a potent scavenger of both, reactive oxygen, as well as nitrogen species [15]. Moreover, the compound is suggested to substantially empower the endogenous antioxidant shield, due to its contribution to the total antioxidant capacity [11]. The potential of QUE as a cryoprotective agent has been proposed by S i l v a et al. [28], based on the observation that QUE administration was able to preserve the spermatozoa survival, DNA integrity and post-thaw functionality.

In order to define an optimal concentration of QUE for future experiments, this study was designed to assess the efficacy of different QUE concentrations on bovine spermatozoa motility, viability and superoxide radical formation during a 24 hour *in vitro* culture.

MATERIAL AND METHODS

Bovine semen samples were collected from 20 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed**,** and were obtained on a regular collection schedule using an artificial vagina. After collection, the samples were stored in the laboratory at room temperature (22—25 °C).

Each sample was diluted in a physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italy) with various concentrations of QUE (Sigma-Aldrich, St. Louis, USA; A — 200; B — 100; C — 50; D — 10; E — 5; F — 1 $\mu mol.l^{-1}$) and solubilized in 0**.**5 % dimethyl sulfoxide (DMSO; Sigma, St. Luis, MO, USA), using a dilution ratio of 1 : 40. The samples were cultured at room temperature (22—25 °C). We compared the control (Ctrl) group (medium without QUE supplementation) with the experimental groups at specific cultivation times of 0 h, 2 h and 6 h (models suitable for a short-term *in vitro* culture), as well as 12 h and 24 h (models suitable for a long-term *in vitro* culture).

The motility and progressive motility analysis was carried out using the CASA (Computer Assisted Semen Analyzer) system equipped with the SpermVision™ program (MiniTub, Tiefenbach, Germany) and the Olympus BX 51 microscope (Olympus, Japan). Each sample was placed into the Makler Counting Chamber (depth 10 µm, Sefi-Medical Instruments, Israel) and the percentage of motile (motility > 5 $\mu m.s^{-1}$; MOT) and progressively motile spermatozoa (motility > 20 $\mu m.s^{-1}$; PROG) was evaluated. A range of 1000—1500 cells were assessed in each analysis [22].

The viability of the cells exposed to QUE *in vitro* was evaluated by the metabolic activity (MTT) assay [20], [25]. This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can then be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to QUE). The results from the analysis were collected during five repeated experiments at each concentration.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of the superoxide radical [14]. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by the reduction of the membrane permeable, water-soluble, yellow-colored, nitrobluetetrazolium chloride (2,2´-bis(4-Nitrophenyl)-5,5´-diphenyl-3,3´-(3,3´-dimethoxy-4,4´-diphenylene) ditetrazolium chloride; Sigma-Aldrich, St. Louis, USA) and superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to QUE). The results from the analysis were collected during five repeated experiments at each concentration [32].

The statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). The descriptive statistical characteristics (mean, standard error) were evaluated at first. As we focused to evaluate the impact of different QUE concentrations on the spermatozoa activity (experimental groups) in comparison to the Control, rather than changes taking place over the course of the *in vitro* culture, thus taking one factor only into consideration, one-way ANOVA was used for the specific statistical evaluations. The Dunnet test was used as a follow-up test to the ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$).

RESULTS

The CASA assessment of the motion parameters showed a gradual decrease of spermatozoa motility and progressive motility in all groups over the course of a 24 h *in vitro* culture (Table 1, Table 2). The MOT and PROG examinations revealed a motion-promoting tendency of QUE, specifically in all experimental groups at Time 0h, followed by experimental groups B (100 $\mu mol.l^{-1}$ QUE), C (50 $\mu mol.l^{-1}$ QUE), D (10 $\mu mol.l^{-1}$ QUE) and E (5 $\mu mol.l^{-1}$ QUE) at Time 2 h. Furthermore, the administration of 200 $\mu mol.l^{-1}$ QUE (group A) revealed an inhibiting trend towards the spermatozoa motil-

ity parameters. After 6h, spermatozoa motion characteristics were significantly higher in the group B ($P < 0.01$ in case of MOT and $P < 0.05$ in terms of PROG) in comparison with the Control. The examination at 12 h of the *in vitro* culture, showed that the spermatozoa motility and progressive motility were significantly higher in groups B, C and D ($P < 0.001$ for MOT and $P < 0.05$ in case of PROG, respectively), when compared to the Control. At the end of the experiments (24 h), a significantly higher spermatozoa motility was observed in all experimental groups supplemented with a range of 1—100 μmol.l^{-1} QUE ($P < 0.001$ in case of the experimental groups B, C and D, and $P < 0.05$ with respect to groups E and F). Similarly, QUE concentrations ranging from 10 to 100 μmol.l^{-1} led to significantly improved values for spermatozoa progressive motility ($P < 0.001$ in the group B, and $P < 0.05$ in case of groups C and D). Meanwhile, both MOT as well as PROG revealed a decreased bias in the group A, supplemented with the highest concentration of QUE, after a comparison with the Ctrl group (Table 1, Table 2).

According to the MTT assay, the immediate QUE supplementation had no significant effects on the sperm cell viability in any of the experimental groups (Figure 1). At 2 h it was revealed that 100 μmol.l^{-1} QUE (group B), 50 μmol.l^{-1} QUE (group C) as well as 10 μmol.l^{-1} QUE (group D) had a stimulating and vitality-promoting effects on the bovine spermatozoa, alongside with statistically significant results ($P < 0.05$) when compared to the Control group (0 μmol.l^{-1} QUE; Figure 1). The significance of the favourable effects of QUE on the mitochondrial activity were further extended at 6 h ($P < 0.01$ in the case of group B; $P < 0.05$ in terms of groups C and D). After 12 h of the *in vitro* experiments, the cell viability remained significantly improved in the experimental groups B—D ($P < 0.01$ in the case of groups B and C; $P < 0.05$ in group D), and this advantageous effect remained notable and statistically relevant towards the end of the spermatozoa culture (Time 24 h; $P < 0.001$ in the case of experimental groups B and C; $P < 0.05$ in the group D; Figure 1). Similar to the CASA analysis, the MTT test revealed an inhibitory tendency in the cell viability caused by the administration of 200 μmol.l^{-1} QUE (group A; Figur 1).

Although QUE had no effects on the oxidative balance within the *in vitro* spermatozoa culture at Times 0 h or 2 h (Figure 2), examinations following a 6 h cultivation revealed that the administration of 100 and 50 μmol.l^{-1} of QUE led to a significant decline of the superoxide formation in comparison to the Control (0 μmol.l^{-1} QUE; $P < 0.05$). Starting with Time 12 h and following the rest of the *in vitro* incubations, it was shown that QUE concentrations ranging from 10 to

Table 1. Spermatozoa motility (%) in the absence (Ctrl) or presence (A—F) of quercetin during different time periods (Mean ± SEM; n = 20)

Groups	Ctrl	A	B	C	D	E	F
Time 0h	87.25 ± 1.12	91.31 ± 1.30	91.79 ± 1.27	88.80 ± 0.82	91.04 ± 1.25	87.90 ± 0.96	87.74 ± 1.03
Time 2h	84.09 ± 1.77	81.71 ± 2.27	89.47 ± 1.66	87.40 ± 1.68	85.77 ± 1.38	85.06 ± 1.08	83.40 ± 1.24
Time 6h	71.06 ± 3.99	69.76 ± 2.00	85.04 ± 1.67**	79.13 ± 1.73	77.71 ± 2.05	75.11 ± 1.59	72.49 ± 2.09
Time 12h	62.51 ± 1.83	61.43 ± 1.35	80.19 ± 1.10***	79.61 ± 1.90***	76.01 ± 1.89***	67.34 ± 2.80	66.77 ± 2.78
Time 24h	42.56 ± 2.88	36.99 ± 1.67	57.95 ± 3.00***	52.52 ± 3.14***	53.58 ± 3.38***	51.77 ± 2.91*	51.04 ± 2.94*

*** — ($P < 0.001$); ** — ($P < 0.01$); * — ($P < 0.05$)
The comparison was carried out between the Control and Experimental groups within a specific timeframe

Table 2. Spermatozoa progressive motility (%) in the absence (Ctrl) or presence (A—F) of quercetin during different time periods (Mean ± SEM; n = 20)

Groups	Ctrl	A	B	C	D	E	F
Time 0h	79.46 ± 1.37	83.96 ± 1.42	82.26 ± 1.22	79.04 ± 1.44	79.13 ± 1.46	79.93 ± 1.58	79.38 ± 1.66
Time 2h	76.59 ± 1.89	74.78 ± 3.48	78.99 ± 1.63	78.93 ± 1.43	77.85 ± 1.39	77.91 ± 1.21	77.70 ± 2.30
Time 6h	67.31 ± 2.36	65.67 ± 1.86	75.91 ± 1.88*	74.57 ± 1.71	73.84 ± 1.93	69.39 ± 1.59	69.17 ± 1.54
Time 12h	60.77 ± 1.72	58.73 ± 1.58	70.72 ± 1.71*	68.97 ± 2.10*	68.70 ± 2.10*	64.40 ± 2.13	62.40 ± 2.23
Time 24h	36.15 ± 2.16	34.31 ± 2.60	51.18 ± 2.06***	49.51 ± 2.14*	47.84 ± 2.03*	46.08 ± 2.02	43.65 ± 2.22

*** — ($P < 0.001$); ** — ($P < 0.01$); * — ($P < 0.05$)
The comparison was carried out between the Control and Experimental groups within a specific timeframe

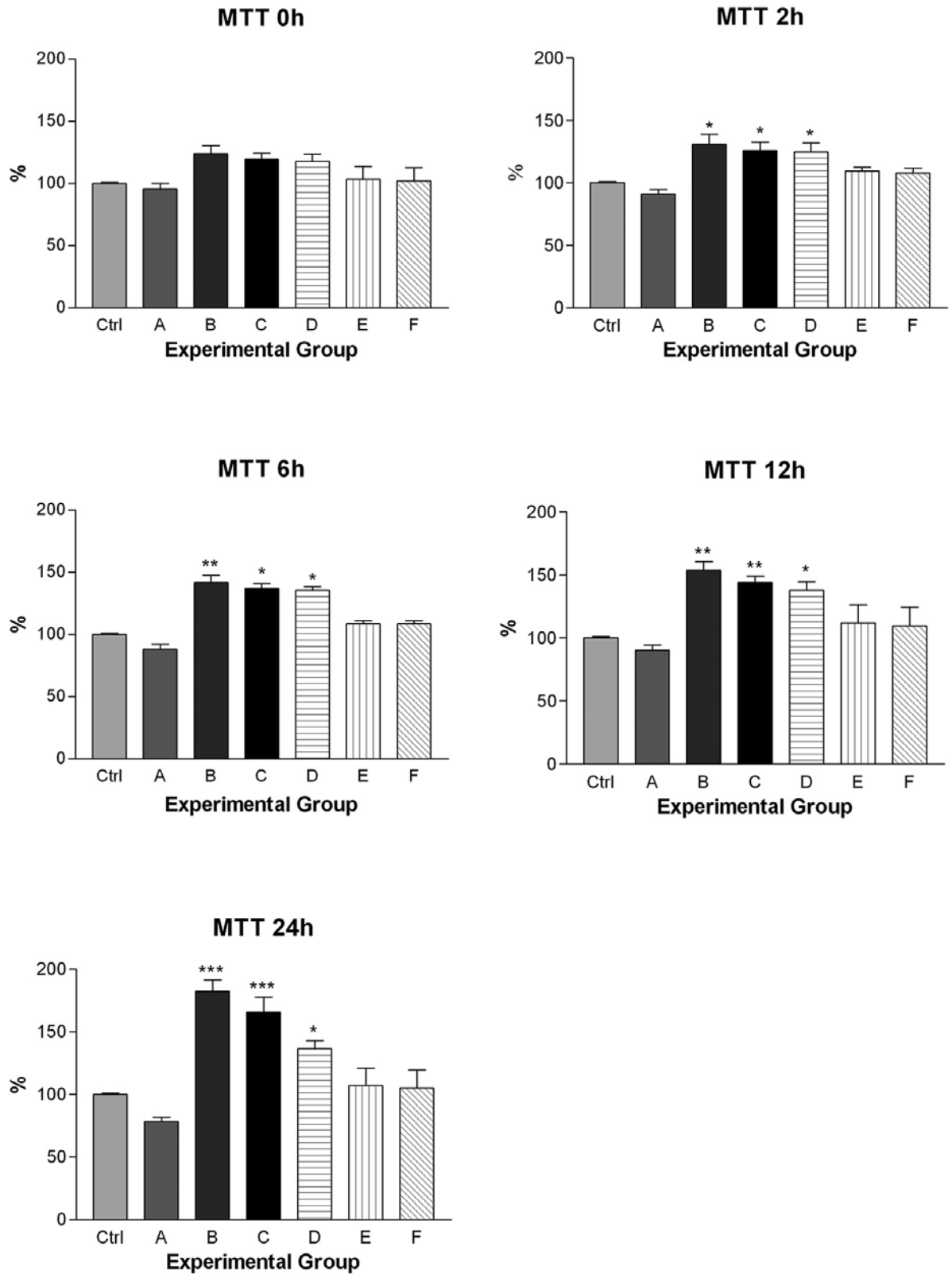

Fig. 1. The effect of various doses of quercetin on the viability of bovine spermatozoa (n = 20) at 0 h, 2 h, 6 h, 12 h and 24 h. Each bar represents mean (± SEM) optical density as the percentage of controls, which symbolize 100 %. The level of significance was set at * — P < 0.001; ** — P < 0.01; * — P < 0.05. The comparison was carried out between the Control and Experimental groups within a specific timeframe. Ctrl — 0; A — 200; B — 100; C — 50; D — 10; E — 5; F — 1 µmol.l^{-1} QUE**

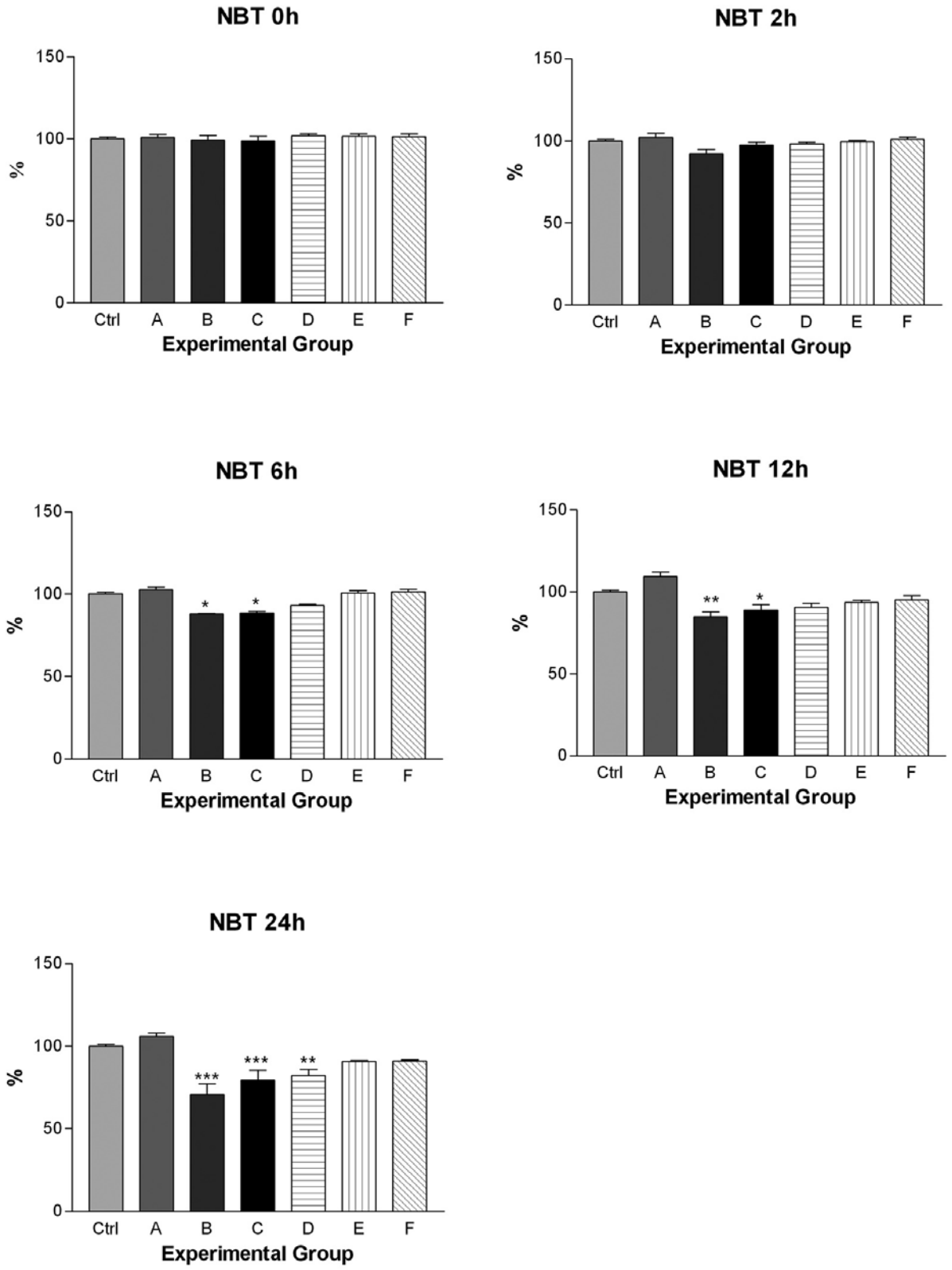

Fig. 2. The effect of various doses of quercetin on the spermatozoa (n = 20) superoxide production at 0 h, 2 h, 6 h, 12 h and 24 h. Each bar represents the mean (± SEM) optical density as the percentage of controls, which symbolize 100 %. The level of significance was set at * — P < 0.001; ** — P < 0.01; * — P < 0.05. The comparison was carried out between the Control and Experimental groups within a specific timeframe. Ctrl — 0; A — 200; B — 100; C — 50; D — 10; E — 5; F — 1 μmol.l^{-1} QUE**

100 µmol.l^{-1} (groups B, C and D), exhibited a long-term and statistically significant antioxidant protection of the male germ cells accompanied by a prevention of the escalating intracellular superoxide production, considered to be the first step in the creation of oxidative stress ($P < 0.01$ in group B and $P < 0.05$ in case of group C, Time 12 h; $P < 0.001$ with respect to the groups B and C, $P < 0.01$ in the experimental group D, Time 24 h). On the other hand, high (group A) as well as low (groups E and F) concentrations of QUE had no significant effect on the superoxide generation within the spermatozoon neither during short-term or during long-term timeframes of the *in vitro* culture (Figure 2).

DISCUSSION

The mammalian spermatozoon has been repeatedly reported as a cell exceptionally sensitive to OS, due to its specific structural and biochemical properties, as well as a high risk of exposure to a variety of environments [4], [7]. Therefore, antioxidants have been proposed as suitable supplements to media used for sperm processing and *in vitro* culture [19]. In order to shed more light on the *in vitro* mechanisms of action of quercetin on the spermatozoon, this study was designed to investigate if quercetin would provide stimulation and protection to bovine spermatozoa exposed to an *in vitro* environment.

Interpreting our results, specific QUE concentrations supplemented to physiological solution may serve as effective motion promoting and mitochondrial protecting agents, as well as an effective superoxide scavengers, significantly improving sperm motility, viability, as well as oxidative balance.

Improved spermatozoa vitality after QUE administration recorded in this study disagrees with the results of a previous *in vitro* report [10], according to which human spermatozoa motility (at 5—200 µmol.l^{-1}) and viability (50—100 µmol.l^{-1}) decreased, being accompanied by a downregulation of the Ca^{2+}-ATPase, a key enzyme involved in the control of sperm motility. Moreover QUE has been identified as a possible specific inhibitor of plasma membrane calcium-ATPase, inducing an increase of intracellular calcium, thus exhibiting modulatory effects on sperm capacitation [10]. In addition, this polyphenol has been showed to possess inhibitory effects on the hyaluronidase and penetration activity of non-capacitated, capacitated or acrosome-reacted *Cynomolgus* monkey spermatozoa in a dose-dependent manner [21]. Talking in favor of the current data, Boo-Keun et al. [8] did suggest a positive impact of QUE on boar sperm *in vitro* characteristics, although it must be agreed on other reports, that QUE may act dose-dependently as either a stimulant at low doses or as an inhibitor at high doses [29]. This hypothesis was fortified *in vivo* [5] as treatment with a higher QUE dose (300 mg per kg body weight) reduced the fertility rate of male rats during the first two matings. Conversely, the Thai study [29] showed that the injection of 270 mg QUE.kg body weight^{-1}.day^{-1} led to an increase in the weight of the testes, epididymis and *vas deferens*. Furthermore, administration of 90 and 270 mg QUE.kg body weight^{-1}.day^{-1} led to significant improvements in semen quality.

With respect to the effects of QUE on sperm quality in the presence of FR, a double faced action was recorded by Italian colleagues [23] comparing the potential of QUE to resveratrol, a different antioxidant substance, on human spermatozoa incubated with tert-butylhydroperoxide (TBHP). On one hand, QUE showed a higher toxicity on the sperm activity; on the other hand, its antioxidant activity was stronger compared to resveratrol, as evidenced by fluorescence and microscopic analyses. At the same time, QUE showed protective and Reactive Oxygen Species (ROS) scavenging actions on spermatozoa, which are in accordance with the NBT results. A different scientific report [18], showed beneficial effects of QUE in maintaining healthy male reproduction and oxidative balance in chemically-induced diabetic rats. Studies focused on the effects of QUE on oxidative damage in cultured chicken spermatogonial cells showed that at 1 µg.ml^{-1}, it increased the cell number and reduced OS-associated insults in the testes [24]. In recent experiments testing the effect of QUE on ram and human spermatozoa during cryopreservation, it was shown that this polyphenol was able to reduce the oxidative damage to mitochondrial membranes and DNA [28], [33], complementing our NBT data. The mitochondrial system is the main source of intracellular ROS, with the respiratory chain acting through an autoxidation process, whereas QUE plays important roles in the absorption and neutralization of FR, because of its stabilizing properties towards NADPH oxidase and NADH-dependent oxidoreductase, localized in the sperm plasma membrane and mitochondria [30].

Our results, even though preliminary, support the evidence for the dose-dependent *in vitro* activity and the scavenger potential of quercetin concentrations ranging between 10 and 100 µmol.l^{-1} in bovine spermatozoa. The development of new spermatozoa culture media providing a better protecttion to spermatozoa against oxidative damage. as well as improvementof their energy requirements is of great interest. Quercetin, in specific amounts, could be used as a FR scavenging and a metabolism-promoting supplement, especially in routine andrology techniques, including *in vitro* fertilization, artificial insemination and spermatozoa cryopreservation. The current results obviously cannot foresee an *in vivo* outcome, since a direct and more specific impact of quercetin supplementation on male fertility preservation needs to be explored further. To translate these finding into a practical reality, comprehensive studies on the possible toxicity, pharmacokinetics and bioavailability of quercetin in the organism will be needed.

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SOME METHODS FOR ESTIMATING THE TIME OF DEATH IN ANIMALS (A REVIEW)

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ABSTRACT

This review discusses some of the different methods used to determine the time of death (TOD) in animals. Knowing the TOD is highly important in cases dealing with cruelty to animals, in poaching and other violations of certain local, state and national laws. The determination of the TOD in animals, for the needs of law enforcement agencies particularly, has grown in the past twenty years and it is a priority in confirming or excluding an alibi of perpetrators of criminal acts. The need for the knowledge of the TOD in animals is of increasing interest for companion animals, as well as for farm livestock. The TOD, together with other *post mortem* changes, is critical in establishing the cause of death.

Key words: animals; death; law; law enforcement officer; legislation

INTRODUCTION

The establishment of the time since death is a daily task in human forensic casework [10] and also for the estimation of the *post mortem* interval, i. e., the period between death and the eventual medical examination. This information may assist investigators in narrowing the "window of opportunity" necessary in order to eliminate specific events and suspects. Because of the importance of this task, much time and energy has been invested in researching an almost bewildering variety of methods for determining the actual time of death. Veterinarians may be involved in widely different incidents, including the out-of-season shooting of game animals, poaching, death of livestock during transportation, and cases of neglect or deliberate injury to companion animals [17]. The establishment of the approximate time of death of a legally protected wildlife species, can serve the same purpose of determining alibis or opportunities, just as for human and companion animal deaths. Another example of breaking rules is the action of irresponsible freewheeling animal breeders. The puppy mills produce puppies and trade them over 1000 km in appalling conditions without the correct treatments or vaccinations [26]. The illegal dog trade regularly demonstrates multiple offences and illegal practices including: offences on transport, animal welfare and health violations, registration and payment of taxes [27].

ESTIMATION OF THE TIME OF DEATH

Although different methods used for the estimation of the time of death in human forensics have been well documented, there exists a paucity of information in the field of veterinary medicine. The current state of knowledge should allow the experienced pathologist, in the normal course of events, to estimate the *post mortem* period in blocks of time, such as: less than 24 hours, 1—3 days, 3—7 days, 7—21 days, weeks, month or years [16]. Conservation officers frequently need to know the time since death of an animal they

suspect that was taken illegally. This need commonly occurs when a carcass is checked shortly after the opening of the legal shooting hours or when a warm carcass is inspected, sometime after the close of the legal shooting time [13].

THE METHODS FOR ESTABLISHING THE TIME OF DEATH

Table 1 presents a review of methods used for the estimation of the *post mortem* period.

Countless crime novels and TV murder dramas have reinforced the popular misconception that a single simple measurement of the rectal temperature provides a reliable estimate of the *post mortem* interval. In reality, cooling of the body after death is affected by many factors including: insulation, ambient temperature, movement of air, and immersion in water [16]. It is very important to remember the factors affecting the rate of the cooling process, such as the difference in temperature between the body and the environment, and the physical build of the cadaver (the rate of heat loss is proportional to the weight of the body to its surface area). Thus, children and older people cool more rapidly than others. The physique of the cadaver and body condition is another factor; fat is a poor conductor of heat and a fat body goes through *algor mortis* slowly and lean bodies more rapidly.

A body kept in a well ventilated room will cool more rapidly than one in a closed room. Cooling in water is rapid because water is a better conductor of heat. Cooling in still water is about twice as fast as in air, and in flowing water, it is about three times as fast. Bodies cool more slowly in water containing sewage effluent or other putrefying organic material than in fresh water or sea water [28].

There are two basic approaches to estimate the time of death: (1) measurement of changes that takes place at a known rate (e. g. *rigor mortis*, cooling of the body and putrefaction), and (2) comparison of the occurrence of the events known to have taken place at a specific time with the time of death (e. g. extent of digestion of last meal). The sources of evidence that are relied upon when attempting to estimate the time of death in people include: corporeal evidence and anamnestic evidence based on a deceased's ordinary habits, movements and day to day activities [16]. This information is important in animal cases as well.

Table 1. Methods for estimating the *post mortem* interval

Temperature based methods
Post-mortem chemistry
Electrical stimulation of muscles and nerves
Gross appearance of the body
Rigor mortis Eye shape, colour and luminosity, etc.
Decomposition
Histopathology and electron microscopy
Radiology
DNA and RNA analyses
Entomology
Environmental and associated evidence

Source:
Munro & Munro: Techniques for estimation of the time of death [17]

According to Merck [15], the rule of thumb says: that a warm body that is flexible, (not stiff) has been dead for less than three hours; if the body is warm and stiff, the death has been between three to eight hours; if the body is cold and stiff, the death has occurred between eight to thirty six hours and finally; and if the body is cold and flexible, the death most likely occurred more than thirty six hours before.

During life, a body loses heat by radiation, convection, evaporation (if the body is wet) and also, conduction is an important factor after death as it may lose considerable heat if the body is lying on a cold surface. For estimating the time of death, a loose formula may be given, such as: Heat conduction equation, plus Newton's Law of Cooling. Newton's law of cooling states, that the rate of cooling of a body is determined by the difference between the temperature of the body and that of its immediate environment. Consequently, a plot of the temperature against the time gives a curve which is exponential. Newton's law applies to small inorganic bodies and does not accurately describe the cooling of the human body, which has a large mass, irregular shape and is composed of tissues of different physical properties. Practical observations indicate that the cooling of the human/animal body is best represented by a sigmoid curve with temperatures plotted against time. In some situations, it appears that the body temperature at the time of death may be normal, but in some individual cases, it may be sub-normal or markedly raised. Also, in deaths from hypothermia, the body temperature at death may be subnormal in cases of congestive cardiac failure, massive haemorrhage or shock. The body temperature may be raised at the time of death in: heat stroke, some infections and pontine haemorrhage with an initial temperature at death of 42.8 °C. In another situation, a temperature of 37.4 °C was found after about three hours after death in the case of manual strangulation [29].

In the case of death due to a fulminating infection (septicaemia), the body temperature may continue to rise for some hours after death [29]. The linear rate of *post mortem* cooling is affected by environmental factors and cadaveric factors other than the environmental temperature and the body temperature at the time of death. These factors depend on the size of the body; the greater the surface area of the body relative to its mass, the more rapid will be its cooling. However, the use of a simple formula only, such as the Newton's Law of Cooling, for estimating the time of death is now regarded as naive. The best researched and documented method for assessing the time of death from body temperature is that of Henssge [29]. Henssge's nomogram was based upon a formula which approximates the sigmoid shaped cooling curve. This formula has two exponential terms within it. The first constant describes the *post mortem* plateau and the second constant expresses the exponential drop of the temperature after the plateau according to Newton's Law of Cooling. Henssge involved two nomograms, one for the ambient temperature above 23 °C, and the second, for the ambient temperature below 23 °C, and there is a different allowance for the effect of

the environmental temperatures on the rate of cooling, as well as an allowance for the effects of body weight [29].

TEMPERATURE BASED METHODS

Merck [15] noted that the rectal temperature may be taken hourly over a three to six hour period to establish the rate of cooling taking into consideration different environmental conditions. Human studies show that the temperature cooling follows a sigmoid shape with a plateau at the beginning and at the end of the process. Under the average environmental conditions, human bodies cool at the rate of 2—2.5 °F per hour (1.1—1.3 °C) for the first few hours and then slow to an average of 1.5—2 °F (0.8—1.1 °C) during the first twelve hours and 1 °F (0.6 °C) for the next twelve to eighteen hours. The initial plateau is attributed to heat generated by residual metabolic processes of the tissues and the metabolic activity of intestinal bacteria; it rarely lasts longer than three to four hours [19]. Therefore, a plateau is more likely to occur if the environmental temperature is relatively high, there is body and/or surface insulation (which slows down the conduction and convection heat loss), and/or the body is large with a greater amount of external body fat [9].

In humans, the average rate of heat loss is 1.5 °F (0.8 °C) loss per hour under 70—75 °F (21—24 °C) environmental temperatures.

The equation for establishing the time of death at a crime scene

$$\text{Time since death (hours)} = \frac{[\text{normal body temperature °F (°C)} - \text{rectal temperature °F (°C)}]}{1.5\ \text{°F}\ (0.8\ \text{°C})}$$

A study was conducted on dogs indoors with still room air and an average ambient temperature about 70.7 °F (21.5 °C). The *post mortem* rate of cooling (in an animal was 0.9 °F) (0.5 °C), which is similar to what has been reported in humans. The study found that sex, body mass and hair density had no effect on the rate of the body temperature reduction [21].

For explanation:

If 0 °C = 32 °F then 32.5 °F = 32.9 °F; it means that 0.5 °C corresponds to 0.9 °F cooling rate.

Decrease from 102.2 °F (39 °C) by 0.9 °F to 101.3 °F means 38.5 °C, i. e. drop by 0.5 °C.

Establishment of reliable evidence to support prosecution for out-of-season killing of deer, was the primary aim of a number of studies conducted in North America. The widespread practice of "field dressing" culled deer, whereby he abdominal cavity is opened and the gastrointestinal tract removed soon after the deer has been shot, renders rectal temperature measurement impossible or meaningless in these animals; thigh and/or nasopharyngeal temperatures in culled deer were recorded by Neubrech [18], Gil and O'Meara [5], Pex et al. [20], Wolf et al. [24], Kienzler et al. [14], Cox et al. [3] and Hadley et al. [7].

These studies allowed the construction of tables, temperature charts and field manuals [14] to assist rangers and law enforcement agencies to estimate the time since death, taking into account body weight and the prevailing ambient temperatures. The development of computers software programmes by Kienzler [14] and Cox [3] made this task easier [18]. Experiments on the cooling rates of pig cadavers were conducted by Kalizsan et al. [12]. This study aimed to assess the practical value of the two-exponential model for the time of death estimation in comparison with a single exponential model. The single exponential model applied to eyeball cooling allowed very precise estimation of the time of death up to 13 h *post mortem*. Thereafter, a better time of death estimation was obtained from muscle or rectal probes [12].

Woolf and Gremillion-Smith [25] claimed in their study that the vitreous humour potassium increased after death and might be useful to determine the *post mortem* intervals. Vitreous humour samples were taken from 197 deer immediately after death, then again at 3, 6, 9, 12 hours *post mortem*. Samples from deer were obtained over a 10 hour *post mortem* interval (PMI), except for 6—8 hour PMI. The mean K levels increased, but because the standard deviation was large, there was no correlation between the PMI and vitreous humour K levels.

Proctor et al. [21] mentioned in their research that 36.1 percent of U. S. households have dogs. The time of death (TOD) of dogs at a crime scene can be useful to forensic investigations. This usefulness usually extended to cases where a human and animal deaths were coincident, but can also include cases involving: the violation of animal and cruelty laws, the death of an animal when the killing of an animal is itself a crime, and the investigation of crimes against protected wildlife. This study was conducted indoors in still air at normal room temperature; the *post mortem* reduction in rectal, liver, brain and aural temperatures in 16 dogs were monitored for 32 h after death. They found that: sex, body mass and hair coat density did not affect the rate of the body temperature reduction, but increased body weight and volume may slow it. Researchers assumed also, that if a dog was found dead in still air at normal room temperature with rectal temperature of 34.5 °C, the mean time of death determined from their data would be 3.2 h. The range within 1 Standard Deviation (SD) (68 % of the observations) would be 2.1—4.7 h and for 2 SD (95 % of the observations) 1.0—6.2 h, assuming an underlying normal distribution.

Erlandsson and Munro [4] studied dogs (laboratory beagles "retired breeders") from a large breeding facility which failed to meet the requirements for the use of these dogs in regulatory scientific procedures. The dogs were of similar weights and held under identical conditions. It was shown that a combination of rectal temperature, environmental temperature, gross pathology and histological changes can provide a scientifically based estimate of the *post mortem* interval. The ambient temperature in the storage room varied from a minimum of 10.9 °C to a maximum of 16.8 °C, with the humidity range from 34 % to 63 %. The rectal temperature of all ten dogs approximated the ambient temperature between 24—48 h after death.

Abdulazeez and Noordin [1] found that the cooling under tropical conditions were less consistent than in temperate climates. When the body temperature was close to ambient temperature, the cooling curve showed peaks and short plateau, which lasted for an average of 70 min. Under tropical conditions, the body temperature reached the ambient temperature in 26 ± 8 hours. The determination of the elapsed time since death in small cetaceans can be important for understanding of the nature of their interactions with fishing operation.

Hood et al. [11] conducted a pilot study entitled, "*Post mortem* ocular fluid and core temperature analysis", in order to deter-

mine the potential diagnostic usefulness of ocular fluid and core body temperatures to estimate the *post mortem* interval in harbour porpoises (*Phocoenaphocoena*). The core temperature and vitreous humour conditions were determined in 24 incidentally caught harbour porpoise. The recorded parameters were compared with the published values for the rectal temperature and serum concentrations of several selected elements in the live harbour porpoises. The potassium and magnesium levels in the vitreous humour increased following death and the data suggested that most of the analysed animals had been dead for several hours. The mean *post mortem* core temperature of these 24 animals was 14.6 to 5.2 °C, with a range of 8—30 °C [12].

Henríquez et al. [8] assumed that the time elapsed from death until the moment the corpse's temperature equals its ambient temperature is known as, "early *post mortem* interval" and during this period, the decrease in body temperature (*algor mortis)* is a useful routine measure to estimate the PMI in human forensics. Early PMI for humans is 24 hours, but there is a lack of information regarding the use of *algor mortis* in animals as an estimator of early PMI. In their study, the core body and ambient temperatures were measured continuously in the deaths of 50 laboratory rats. Their results showed that the early *post mortem* interval (PMI) was 11 h 14 min, ranging from 8 to 17 hours. It was the first experimental study aimed to obtain parameters to estimate the PMI from *algor mortis* in animals.

OTHER POSSIBILITIES FOR THE ESTIMATION OF THE TIME OF DEATH

Gonder [6] analysed wildlife animals in various decomposition stages to make guideline for law enforcement agencies that investigating poaching of wildlife species. The results of this study are of value following the discovery of carcasses found in advanced stages of decomposition with little information about their time of death. Documenting decomposition changes over time can provide markers for wildlife law enforcement officers to utilize for the time of death (TOD) estimates. For this study, decomposition data had been collected from eight grey wolves, four mountain lions, two black bears and one white-tail deer. The practical research in this study provided baseline data on long term decomposition in order to develop standards for use in the field by wildlife law enforcement officers. The TOD determination is also appropriate for livestock depredation claim investigations. It may be critical to establish the TOD of predatory carnivores, in order to assure that it was legally lethally removed by a livestock manager at the time when the prey livestock were in the area.

Akihiko et al. [2] considered that the biological clock may stop at the time of death in the dead body. Therefore, the biological clock seems useful for estimating the time of death. They argued that the accumulated evidence indicates that the biological clock system works in most cells in most tissues (peripheral clock) in addition to the core clock in the hypothalamic suprachiasmatic nucleus (SCN), and several genes have been identified as associated with the biological clock, as well. These findings indicate that gene expression analyses of the biological clock could be powerful methods for estimation of the time of death. The circadian rhythm in biological, physiological and behavioural processes has been found in organisms from cyanobacteria to humans and can be helpful in establishing the time of death. Reading the biological clock presented in the concurrence method is quite difference from conventional methods based on *post mortem* changes, but may be helpful in forensic practice.

Rajagopal et al. [22] from the Medical Entomology Unit for Medical Research in Malaysia has shown that forensic entomologist can provide invaluable aid in death determinations when the dead body is colonized by insects. The forensic entomologist is responsible for determining the period of insect activity according to all the variables affecting insect invasion of remains. Three species of cyclorrphagic flies were identified as *Chrysomyarufifacies Macquarat* (the hairy maggot blowfly), *C. megacephala Fabricius* (the oriental latrine fly) and *Hermetiaillucen Linnaeatus* (the black soldier fly). These flies were the most common species found in cadavers in Malaysia. The methods used by the entomologist and pathologist for estimating the *post mortem* interval (PMI) demonstrated a significant correlation at the 0.01 level, and the researchers concluded that the techniques are useful forensic tools for estimating the PMI.

Romanelli et al. [23] suggested a new tool for estimating the time of death. Nasal ciliary motility has been occasionally observed in *post mortem* periods. Specimens of ciliated epithelium from 100 consecutive cadavers were obtained by scraping the nasal mucosa at three *post mortem* intervals. *Post mortem* nasal ciliary movements and statistically significant relationships between decreasing ciliary movements and an increasing *post mortem* interval were detected even in the presence of putrefactive changes of the nasal ultrastructural integrity.

CONCLUSION

Acts of cruelty to animals are not easily defined or addressed. Similarly, determination of the time of death may be crucial where several animals are found and the question arises of whether this was a single episode or an ongoing problem [17]. This article provides information about methods to help law enforcement officers to determine the time of death. Temperature methods that are using post mortal cooling (as an indicator for establishing the death time) have certain restriction, which is the time from 24 h to 48 h. Other problems involve climate conditions, such as a fall in temperature after a death in tropical conditions. We will use the above mentioned methods in experiments to establish the time of death, mainly in dogs and cats. The results should be useful for authorities in collecting evidence in cases related to animal protection.

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CONTENTS, 58, 2015

No. 1

No. 2

No. 3

No. 4

Suppl. No. 1

Suppl. **No. 2**

FOLIA VETERINARIA, 58, 4: 241—244, 2014

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Types of Papers. Please state clearly which category of paper is being submitted. (If an author believes that his/her article or short communication is of outstanding topicality and importance, he/she should indicate this in a covering letter. It may merit fast-track publications.)

Standard Full Length Papers. Full papers should be concise. They should not exceed 12 pages (A4) including tables, graphs, illustrations, photographs, and references.

The Title Page. The papers should be headed with the full title, which should accurately and concisely describe the topic in no more than two lines. The surname(s) and initials of the author(s) and the name and place(s) of their employment should follow this. (If the work was carried out in an institution other than the place of employment, this should be noted in the body of the text.) Acknowledgements (see above) should be typed in a separate section, separated by a reasonable space. A short title (the running head) of no more than forty characters (counting letters and spaces) should be included at the foot of the page. Each manuscript should be thematically complete: serialization is discouraged.

The Abstract. The second page should carry an abstract, which should be self-contained and not exceed 250 words. It should briefly incorporate the purpose and relevance to veterinary science of the work, basic procedures, the main findings, and principal conclusions. It should emphasize new and important aspects of the study or observations.

Key words. Key words should be listed below the abstract, from which they are separated by a one-line space. They should consist of three to ten words in alphabetical order, written in lower case and separated by semi-colons.

The Introduction. State the purpose of the article and summarize the rationale for the study or observation. Give only strictly pertinent references and do not include data or conclusions from the work being reported.

Material and Methods. Describe your selection of observational or experimental subjects (including controls) clearly. Identify the age, sex, state of health, and other important characteristics of the subjects.

Identify the methods, apparatus (with the manufacturer's name and address in parentheses), and procedures in sufficient detail for other workers to reproduce the experiment. Quote established methods, including statistical methods; provide references and brief descriptions for methods that have been published but are not well known; describe new or substantially modified methods in full; give reasons for using them, and evaluate their limitations. Precisely identify all drugs and chemicals used, including generic name, dose, and route of administration.

Results. These should be as succinct as possible and presented in a logical sequence in the text, with graphs and tables. Emphasize or summarize only the important observations in the text. Do not duplicate in the text all the data in the graphs and tables.

Discussion. Emphasize the new and important aspects of the study and the conclusions that follow from them. Do not repeat in detail data or other material given in the Introduction or the Results sections. Include in the Discussion section the implications of the findings and the limitations, together with their significance for future research. Relate the observations to other relevant studies.

Link the conclusions with the aims of the study, but avoid unqualified statements and conclusions not completely supported by the data. Avoid claiming priority and alluding to work that has not been completed. Recommendations, when appropriate, may be included.

Notes and Short Communications. Such manuscripts should have the same form as full papers, but are much shorter. Separate headings are needed only for the Acknowledgements, Key Words, Abstract, Main Text, and References. These scripts fall under the following main headings and should be marked accordingly.

Technical Notes. Such notes should record a new method, technique, or procedure of interest to veterinary scientists. They should include the reason(s) for the new procedure, a comparison of results obtained by the new method with those from other methods, together with a discussion of the advantages and disadvantages of the new technique. A technical note should not exceed six printed pages, including figures and tables.

Research Communications. These are short articles, no more than four printed pages, which should introduce novel and significant findings to the commonwealth of veterinarians.

Observations. Research of this kind contributes to knowledge, but not to the advancement of ideas or the development of concepts. In some cases, these papers underpin what may seem obvious, with statistical data. Such communications should not exceed four type-set pages.

Current Issues. Papers that deal with issues of topical interest to veterinary scientists will be considered. Issues may include items on environmental concerns, legislative proposals, etc.

Review Articles. These should provide a substantial survey, with an appropriate historical perspective, of the literature on some aspect of veterinary medicine. Alternatively, such articles may review a topic of veterinary interest, which may not come within the normal purview of many veterinarians (e.g. Asefa Asmare, A., 2000: The Camel,…, *Folia Veterinaria*, 44, 4, 215—221). Authors submitting review manuscripts should include a section describing the methods used for locating, selecting, extracting, and synthesizing data. These methods should be summarized in the abstract.

Book. Reviews may be submitted. They should bring a new text to the readership and evaluate it.

Letters to the Editor. These are items of scientific correspondence, designed to offer readers the chance to discuss or comment on published material and for authors to advance new ideas. Should a letter be polemical, a reply or replies for simultaneous publication may be sought from interested parties.

Editorial Board