J Forensic Sci, November 2006, Vol. 51, No. 6
doi:10.1111/j.1556-4029.2006.00260.x
Available online at: www.blackwell-synergy.com

Monika Martiniaková,[1] *Ph.D.; Birgit Grosskopf,*[2] *Ph.D.; Radoslav Omelka,*[3] *Ph.D.; Mária Vondráková,*[1] *Ph.D.; and Mária Bauerová,*[3] *Ph.D.*

Differences Among Species in Compact Bone Tissue Microstructure of Mammalian Skeleton: Use of a Discriminant Function Analysis for Species Identification

ABSTRACT: In order to develop an identification key for distinguishing between several mammalian species, bone structure of their compact bone tissue was analyzed using qualitative and quantitative characteristics. Ninety femora of adult male humans, pigs, cows, sheep, rabbits, and rats were studied. The average area, perimeter, minimum, and maximum diameter of 1863 Haversian canals and 1863 secondary osteons were measured using a digital image device. The observed data were first used to evaluate inter- and intraspecies diversity. After that, we applied a discriminant function analysis for differentiation of the species by these variables. Classification functions for investigated species give cross-validated correct classification rates for 76.17% of cases. This percentage value can be increased by integrating conclusions from the qualitative analysis.

KEYWORDS: forensic science, forensic anthropology, bone tissue, human versus nonhuman origin, histomorphometry, discriminant function analysis

Forensic scientists are frequently required to confirm or exclude the human origin of skeletal remains. In situations where badly degraded or charred fragments of bone are found, this may be impossible by gross morphology alone, and histological or biomolecular methods have to be used (1). However, histological investigation of the microscopic structure of mammalian skeleton for species identification is not at the center of scientific attention. Biomolecular methods are in general widely used for this aim. On the other hand, histological analysis of the found bones is a suitable method for identification in cases of bone remnants where genetic information (DNA) is not sufficiently present (2). Although bones of various mammalian species are composed of the same skeletal elements, there are among-species differences identified in the microscopic structure of compact bone. The differences are in general caused by the different structure and pattern of the osteons and/or the Haversian canals (qualitative characteristics) as well as by their quantitative conditions (quantitative characteristics).

Histological differences in mammalian bones were partially discovered at the beginning of the 20th century. Kenyeres and Hegyi (3) reported that thin sections of compact bone in the diaphysis of long bones can be used to determine the human origin of bone fragments, as the average diameter of the Haversian canals in human bones is significantly greater than that of other animal bone tissues. This fact was later confirmed by other authors (4–8). Sauer and Lackey (9) stated that Haversian canals with diameters less than 50 μm indicate a nonhuman origin. However, this assertion is still controversial. According to Demeter and Mátyás (10), individual species of mammals differ on the basis of qualitative characteristics of the bone tissue microstructure too. However, the authors suspected the existence of a "relationship" among some species. More recently, plexiform bone has been considered to be a general determinant of nonhuman bone tissue (11). Nowadays, a small number of articles with a focus on human versus nonhuman identification have been published using histomorphometric characteristics of the compact bone tissue (1,8). In 2003, Dittmann's results (7) showed that it is also possible to use histomorphometry to identify various species of mammals from metacarpi or radii bone microstructures. On the other hand, the combination of qualitative and quantitative characteristics to identify animal species is absent.

The main aim of this study was to present a detailed analysis of the compact bone tissue microstructure in selected mammalian species with a focus on finding an adequate identification key. Microscopic structure of the compact bone tissue was evaluated both qualitatively and quantitatively.

Materials and Methods

Our research used femora from the following: 15 adult male (*Homo sapiens*), 15 adult pigs (*Sus scrofa domestica*), 15 adult cows (*Bos taurus*), 15 of adult sheep (*Ovis aries*), 15 adult rabbits (*Oryctolagus cuniculus*), and 15 from adult rats (*Rattus norvegicus*). Human femora were obtained from Slovak cadavers dissected in our University Hospital in 2002 and 2003. The ages of the males ranged from 36 to 51 years. All studied animals were obtained from an experimental farm of the Research Institute of Animal Production in Nitra (Slovakia). In our study, we used animals with the age at death 10–14 months (pigs), 25–30 months (cows), 12–15 months (sheep), 5–7 months (rabbits), and 4–6 months (rats). Each of the bones was sectioned at the mid-shaft of its diaphysis, where the compact bone is thick and provides a large

[1]Department of Zoology and Anthropology, Constantine the Philosopher University, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic.
[2]Institute of Zoology and Anthropology, Georg-August University, Bürgerstrasse 50, 37 073 Göttingen, Germany.
[3]Department of Botany and Genetics, Constantine the Philosopher University, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic.

Received 04 June 2005; and in revised form 30 Dec. 2005, 13 April 2006, 26 May 2006; accepted 11 June 2006; published 30 Oct. 2006.

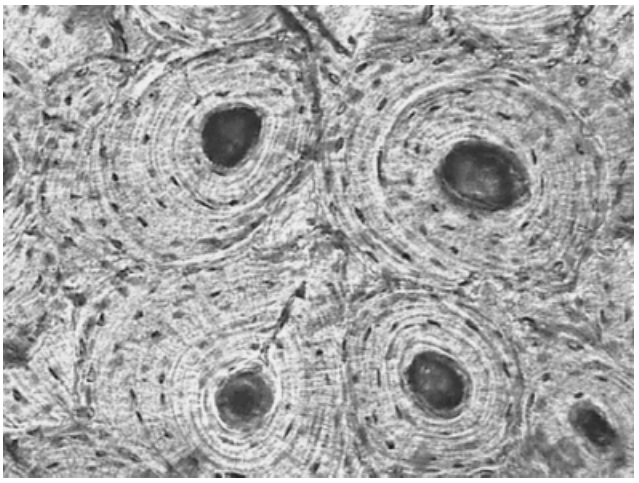

FIG. 1—*Human's dense Haversian bone tissue (magnification ×200).*

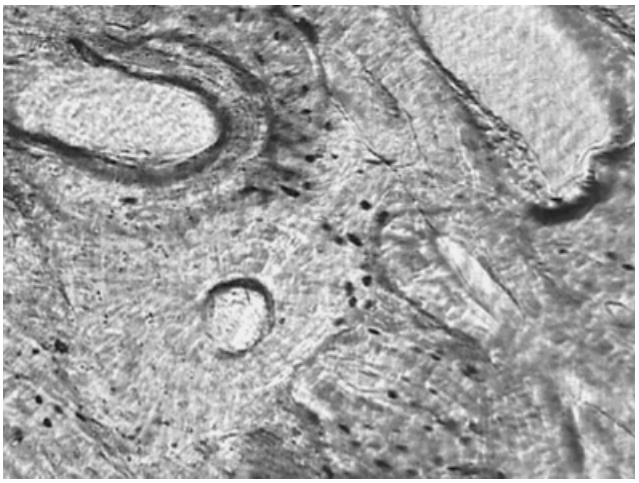

FIG. 3—*Resorption lacunae around secondary osteon in pig (magnification ×200).*

area for study of the bone tissue microstructure. In total, 90 transversal sections of the femur diaphysis were cut. The bone rings were washed in running water for 5 days to remove soft tissues in the bone marrow. The bones were then defatted in a mixed solution of chloroform and methanol for 7 days and bleached in 2% H_2O_2 solution for 1 day (12). After dehydrating through graded alcohols, material were embedded in epoxy resin Biodur (Günter von Hagens, Heidelberg, Germany). Transverse thin sections (70–100 μm) were prepared with a sawing microtome (Leitz 1600), mounted on glass slides with Eukitt (Merck, Darmstadt, Germany), and examined by light microscopy at ×200.

The qualitative characteristics of analyzed microstructure were determined according to Enlow and Brown's (13) classification system in anterior, posterior, medial, and lateral views of thin sections; the quantitative ones were assessed using the software Scion Image (Scion Corporation, Frederick, MD). Measurements were taken on all mature osteons present in the views described previously, which were not in a resorption phase and that could be clearly outlined, using the computer software Scion Image on a microphotograph at a magnification of ×200. Areas, perimeters, and minimum and maximum diameters of 1863 secondary osteons and 1863 Haversian canals were measured as independent variables. Quantitative differences were reported separately for the secondary osteons and the Haversian canals in the investigated species. Analysis of variance and Tukey's test were used for species determination. We used a stepwise discriminant function analysis to determine the origin (i.e., the species) of each of the samples (Statistica 4.3, 1993).

Results

We found that every species differed qualitatively from each of the others either in the type of bone tissue or in the combination of types of bone tissue. The diaphysis of a human femur consisted entirely of dense Haversian bone tissue with several secondary osteons (Fig. 1). No primary osteons were identified. The basic structural pattern of a pig's, cow's, and sheep's femur was primary vascular plexiform (Fig. 2). In addition, dense Haversian bone tissue could also be found in these species (in the middle parts of substantia compacta, mainly in anterior views). There was a nonvascular bone tissue identified at the endosteal border (in anterior and posterior views) as well as at the periosteal border (in lateral views) in cow. Many resorption lacunae were found between the Haversian systems at the antero-medial views in pigs (Fig. 3). We found irregular Haversian bone tissue with scattered, isolated, and relatively few Haversian systems (Fig. 4) at the periosteal border

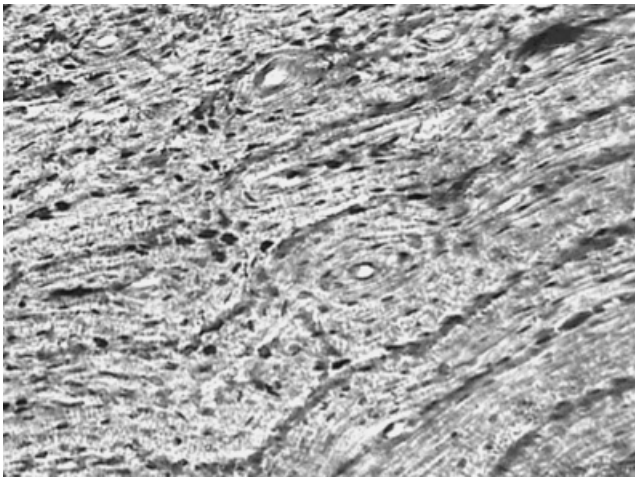

FIG. 2—*Primary vascular plexiform bone tissue of cow (magnification ×200).*

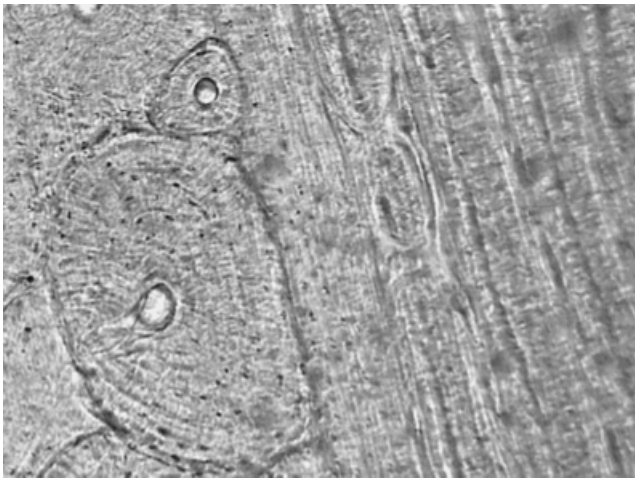

FIG. 4—*Irregular Haversian bone tissue of sheep (magnification ×200).*

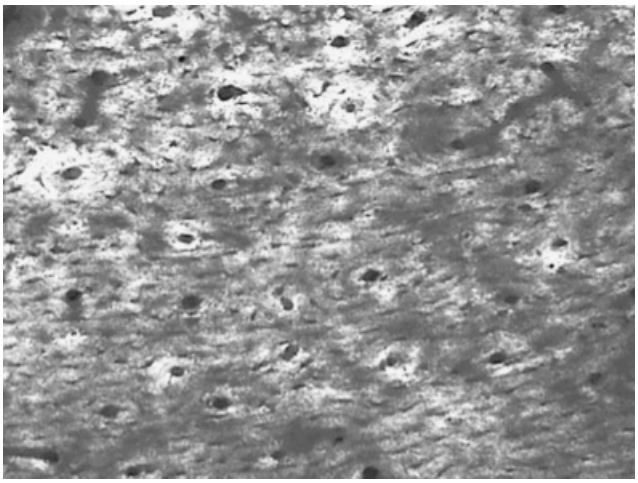

FIG. 5—*Rabbit's primary vascular longitudinal bone tissue (magnification ×200).*

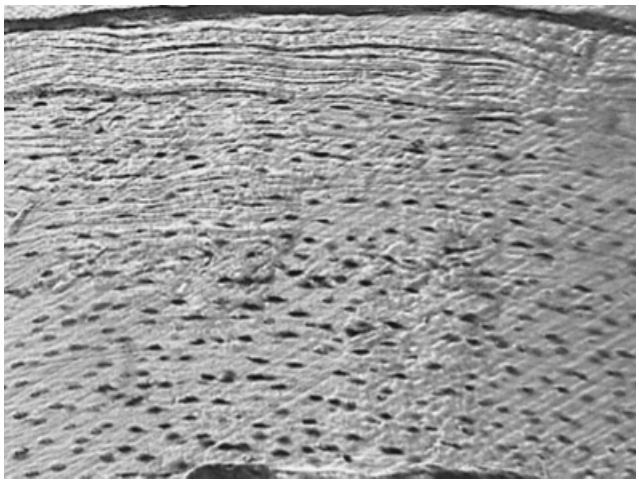

FIG. 6—*Nonvascular bone tissue from rat (magnification ×200).*

(mainly in antero-lateral views) in sheep. The femur diaphysis of rabbit was mainly composed of primary vascular longitudinal bone tissue (Fig. 5). Furthermore, primary vascular radial and/or irregular Haversian and/or dense Haversian bone tissue can be seen in the middle part of substantia compacta in rabbit. The microscopic structure of a rat's femur diaphysis comprised nonvascular bone tissue (Fig. 6); secondary osteons were not formed.

Measurements of the variables (area, perimeter, maximum and minimum diameter) of the Haversian canals and the secondary osteons showed great variability in quantitative characteristics for each species, as well. However, variability between individuals from one taxonomic group was not statistically significant in most cases. The values of investigated variables are shown as mean ± SD. The values of all measured variables of the Haversian canals observed in compact bone tissue decreased from human across cow, pig, and sheep to rabbit (Table 1). Using the Tukey's test, we found numerous statistically significant differences in the variables between investigated species. No significant difference was identified for minimum diameter of Haversian canals between cow and pig. Similar results were discovered with measurements of secondary osteons' variables between the species. Humans had the highest values for area, perimeter, and minimum diameter of canals, followed by cow, pig, sheep, and rabbit, respectively (Table 2). Testing of the secondary osteons' variables indicated that there are no significant differences between pig and sheep and/or between human and cow in maximum diameter of the osteons. Our study appears to be the first to statistically identify differences between sheep and rabbit osteohistology.

The quantitative differences in compact bone tissue microstructure were used to formulate equations for species determination. By means of a discriminant function analysis, we established an identification key. It is not possible to use it for distinguishing rat femora because secondary osteons and Haversian canals are absent in the microstructure. In general, discriminant analysis evaluates $n-1$ discriminant functions for n groups (in our case, consistently four functions). For the four discriminant functions, the analysis showed their significance ($p<0.001$; Wilk's $\lambda = 0.023$; F test = 439.72). The variable maximum diameter of secondary osteons was excluded because of not showing a significant influence on the species differentiation. Discriminant analysis automatically computes the classification functions. These are not to be confused with the dicriminant functions. Our classification functions give cross-validated correct classification rates for 76.17% of cases (Table 3). The accuracy of identification was 100% for human, 78.54% for cow, 42.67% for pig, 57.33% for sheep, and 86.47% for rabbit.

Discussion

Our results from the qualitative analysis correspond to those reported by other researchers (14,15). Plexiform bone tissue was typical for long bones of the large- and medium-sized mammals (cow, pig, and sheep) while this tissue was not found in small mammals (rabbit and rat) and humans. In addition, Zoetis et al. (16) note that plexiform bone is only rarely seen in humans, occasionally when a child is going through a very rapid growth spurt. Dense Haversian bone tissue represented the basic structural pattern in

TABLE 1—*Results of the Haversian canals' variables between investigated species.*

Species	n	Area (μm^2)	Perimeter (μm)	Maximum Diameter (μm)	Minimum Diameter (μm)
(1) Cow	15	1224.71 ± 653.33	99.72 ± 26.49	48.76 ± 15.59	15.58 ± 4.32
(2) Pig	15	1015.21 ± 539.63	87.40 ± 25.04	40.60 ± 14.55	15.61 ± 5.18
(3) Sheep	15	609.23 ± 234.15	69.60 ± 14.12	33.63 ± 8.65	11.46 ± 3.07
(4) Rabbit	15	384.01 ± 227.45	55.23 ± 19.74	26.85 ± 11.97	8.96 ± 2.99
(5) Human	15	2164.15 ± 1096.98	127.09 ± 35.84	59.99 ± 21.59	32.26 ± 7.23
Tukey's test		1:2, 3, 4, 5^{+++}	1:2, 3, 4, 5^{+++}	1:2, 3, 4, 5^{+++}	1:3, 4, 5^{+++}
		2:3, 4, 5^{+++}	2:3, 4, 5^{+++}	2:3, 4, 5^{+++}	2:3, 4, 5^{+++}
		3:4, 5^{+++}	3:4, 5^{+++}	3:4, 5^{+++}	3:4, 5^{+++}
		4:5^{+++}	4:5^{+++}	4:5^{+++}	4:5^{+++}

$^{+++}p<0.001$.

TABLE 2—*Results of the secondary osteons' variables between investigated species.*

Species	*n*	Area (μm^2)	Perimeter (μm)	Maximum Diameter (μm)	Minimum Diameter (μm)
(1) Cow	15	32664.97 ± 11110.13	533.61 ± 107.31	269.63 ± 69.15	76.22 ± 14.63
(2) Pig	15	28031.80 ± 10004.39	459.27 ± 97.53	211.07 ± 55.42	83.15 ± 17.24
(3) Sheep	15	21034.67 ± 8425.89	419.82 ± 94.62	206.27 ± 66.87	65.11 ± 17.31
(4) Rabbit	15	8631.22 ± 3455.78	265.96 ± 51.58	130.81 ± 29.28	41.81 ± 12.98
(5) Human	15	37762.06 ± 12860.20	550.85 ± 102.48	263.76 ± 60.08	90.20 ± 19.19
Tukey's test		1:2, 3, 4, 5^{+++} 2:3, 4, 5^{+++} 3:4, 5^{+++} 4:5^{+++}	1:2, 3, 4^{+++}, 5^{+} 2:3, 4, 5^{+++} 3:4, 5^{+++} 4:5^{+++}	1:2, 3, 4^{+++} 2:3, 4, 5^{+++} 3:5^{+++} 4:5^{+++}	1:2, 3, 4, 5^{+++} 2:3, 4, 5^{+++} 3:4, 5^{+++} 4:5^{+++}

$^{+}p<0.05$.
$^{+++}p<0.001$.

human skeletal material. This latter tissue type can also be found in younger tissue areas in the remaining animals (excepting rat). However, human osteons were rounder, less "plexiform" in shape, and they overlap one another in a seemingly random manner. Thus, they can be distinguishable from other mammals.

We found human Haversian canals' and secondary osteons' area to be 2164.15 ± 1096.98 and 37762.06 ± 12860.20 μm^2, respectively. These values were lower than the values reported by Watanabe et al. (17) and Urbanová and Novotný (8). However, Watanabe et al. (17) used for measurement right femora from humans with ages ranging from 43 days to 92 years, and Urbanová and Novotný (8) analyzed femur and also tibia bones. Therefore, similar discrepancies with the latter work were seen for the other variables for the human Haversian canals and the secondary osteons. In the other direction, the mean diameter of human Haversian canals (46.13 ± 14.41 μm) was comparable with the ones obtained by Singh and Gunberg (18) and Günter (19). The values of rabbit and sheep mean diameter of Haversian canals (17.91 ± 7.49 and 22.54 ± 5.86 μm, respectively) were higher than the ones from a study by Müller and Demarez (4). However, Haversian canals' diameter was measured from various bones in their study. This apparently led to lower values of the variable being observed in pig and cow in our study. Comparison of values of all measured Haversian canals' variables in cow, pig, and sheep with the ones found by Urbanová and Novotný (8) indicated that area and maximum diameter of the canals were higher in all species in our study. In the case of distinguishing secondary osteons' variables, we found out that cow and sheep had lower values of their maximum diameter in the study by Urbanová and Novotný (8).

Biostatistical methods addressing problems of quantitative evaluation of bone structure have not been a common part of histological examinations dealing with the taxonomic origin of osteological samples. Cattaneo et al. (1) formulated one discriminant canonical equation for the separation of human and nonhuman animals. But it should not be considered as a practical guide for evaluating the taxonomic classification of osteological remains because the authors did not provide specific guidelines for the measurement of bone microstructure. Urbanová and Novotný (8) published four classification equations for distinguishing human from nonhuman bone samples. In addition to micrometric variables of Haversian canals and secondary osteons, the authors used cortical thickness of the midshaft of femur as a very strong discriminative parameter. However, classification functions for all species studied were absent in their study. Dittmann (7) noted a correct classification of 83.3% of cases for bones of cattle, pigs, sheep, and goats using Haversian canals' and secondary osteons' variables. However, the classification functions are also absent in her study. In contrast, our paper provides classification functions for all investigated species (except rat) that give cross-validated correct classification rates of 76.17% of cases. For identification of new bone samples, the *y*-index with the highest classification score reflects the taxonomic group of individual. According to Harsányi (6), it is necessary to measure variables of 50–100 Haversian canals and secondary osteons per species. After that, the average values should be put into equations instead of variables x_{1-7}.

However, the measured values of basic structural units in compact bone tissue change with the age of the individual and vary with the skeletal part studied. On the other hand, these values are relatively constant between adult individuals of the same species for the same skeletal element (20,21). In addition, Rajtová and Globočník (22) mention some changes in the qualitative histological characteristics for adult individuals. Therefore, we suppose that a combination of qualitative and quantitative characteristics to identify animal species seems to be the best method for their determination. This provides more accurate results than either the qualitative or the quantitative analysis separately. In fact, the accuracy of identification can be increased when conclusions from the qualitative analysis are also taken into consideration. Human bones can be distinguished from animal bones on the basis of dense Haversian bone tissue identified in all views of thin sections. For animal species from Artiodactyla order (cow, pig, sheep in our study), the basic structural pattern of the bone is primary vascular plexiform. However, the presence of nonvascular bone tissue indicates that the investigated bone belongs to the cow. Many resorption lacunae between secondary osteons suggest that the bone comes from the pig. The finding of irregular Haversian bone tissue at the periosteal and endosteal borders means that the bone belongs to the sheep. For rabbit, the basic structural pattern

TABLE 3—*Classification functions for identification of the species.*

	Regression Coefficients				
	Human	Pig	Cow	Sheep	Rabbit
x_1	− 0.056	− 0.040	− 0.040	− 0.036	− 0.031
x_2	0.822	0.588	0.635	0.479	0.414
x_3	2.842	0.364	0.334	0.391	0.3339
x_4	7.175	2.665	2.611	2.396	2.079
x_5	− 0.006	− 0.006	− 0.006	− 0.006	− 0.005
x_6	0.545	0.544	0.556	0.544	0.434
x_7	1.473	1.557	1.498	1.487	1.179
Constant	− 190.21	− 137.44	− 140.21	− 124.90	− 81.64

x_1, area of Haversian canals; x_2, perimeter of Haversian canals; x_3, max. diameter of Haversian canals; x_4, min. diameter of Haversian canals; x_5, area of secondary osteons; x_6, perimeter of secondary osteons; x_7, min. diameter of secondary osteons.

of the bone is primary vascular longitudinal. Finally, nonvascular bone tissue identified in all views of thin sections is typical for rat.

The combination of qualitative and quantitative histological characteristics to identify various species of mammals could be of great importance in forensic analyses, especially in cases when only small bone fragments are available. If our results are verified and expanded to other species (mainly dogs and horses), this approach can be perspective applied in archeozoology, forensic anthropology, and/or criminological practice.

In conclusion, the measured variables of the Haversian canals and the secondary osteons (area, perimeter, minimum and maximum diameter) may not always be completely independent variables. Nevertheless, we are convinced from our results that the proposed identification key is an adequate and valuable tool.

Acknowledgments

The authors thank Kim Dammers, M.S. (Department of EFL, Konyang University, Republic of Korea) for reading the English language text. We would like to acknowledge the thoughts and comments provided by anonymous reviewers. This study has been supported by the grant UKF: CGA VI/6/2004.

References

1. Cattaneo C, Dimartino S, Scali S, Craig OE, Grandi M, Sokol RJ. Determining the human origin of fragments of burnt bone: a comparative study of histological, immunological and DNA techniques. Forensic Sci Int 1999;102:181–91.
2. Martiniaková M. Differences among species in microstructure of mammalian skeleton [dissertation]. Nitra: Constantine the Philospher University, 2003.
3. Kenyeres B, Hegyi M. Unterscheidung des menschlichen und tierischen Knochengewebes. Vierteljahresschr Gerichtsmed 1903;26:225–7.
4. Müller M, Demarez R. Le diagnostic différentiel de l'os de singe et de l'os humain. Med Leg 1934;14:498–560.
5. Paaver K. Izmenčivost'osteonnoj organizacii mlekopitajušich. Tallin: Valgus, 1973.
6. Harsányi L. Differential diagnosis of human and animal bone. In: Grupe G, Garland N, editors. Histology of ancient human bone: methods and diagnosis. Göttingen: Springer Verlag, 1993:82–91.
7. Dittmann K. Histomorphometrische Untersuchung der Knochenmikrostruktur von Primaten und Haustieren mit dem Ziel der Speciesidentifikation unter Berücksichtigung von Domestikationseffekten. Anthrop Anz 2003;61:175–88.
8. Urbanová P, Novotný V. Distinguishing between human and non–human bones: histometric method for forensic anthropology. Anthropology 2005;43:77–85.
9. Sauer NJ, Lackey WL. Anthropology: skeletal analysis. In: Siegel J, Knupfer G, Sauko P, editors. Encyclopedia of forensic sciences. London: Academic Press, 2000:261–70.
10. Demeter G, Mátyás J. Mikroskopisch vergleichend-anatomische Studien an Rohrenknochen mit besonderer Rucksicht auf die Unterscheidung menschlicher und tierischer Knochen. Zschr Anat Entw 1928;87:45–99.
11. Owsley DW, Mires AM, Keith MS. Case involving differentiation of deer and human bone fragments. J Forensic Sci 1985;28:572–8.
12. Martiniaková M, Vondráková M, Fabiš M. Investigation of the microscopic structure of rabbit compact bone tissue. Scripta medica 2003;76: 215–20.
13. Enlow DH, Brown SO. A comparative histological study of fossil and recent bone tissues. Part I. Texas J Sci 1956;8:405–12.
14. Enlow DH, Brown SO. A comparative histological study of fossil and recent bone tissues. Part III. Texas J Sci 1958;10:212–7.
15. Mulhern DM, Ubelaker DH. Differences in osteon banding between human and nonhuman bone. J Forensic Sci 2001;46:220–2.
16. Zoetis T, Tassinari MS, Bagi C, Walthall K, Hurtt ME. Species comparison of postnatal bone growth and development. Birth Def Res (Part B) 2003;68:86–110.
17. Watanabe Y, Konishi M, Shimada M, Tsuji H, Nishio H, Suzuki K, et al. Estimation of age from the femur of Japanese cadavers. Acta Anat Nippon 1998;73:33–41.
18. Singh IJ, Gunberg DL. Estimation of age at death in human males from quantitative histology of bone fragments. Am J Phys Anthropol 1970;33:373–82.
19. Günter A. Struktur und Alterveränderungen der Osteone des Femurs [dissertation]. Hamburg: University of Hamburg, 1993.
20. Burr DB. Estimated intracortical bone turnover in the femur of growing macaques: implication for their use as models in skeletal pathology. Anat Rec 1992;232:180–9.
21. Martiniaková M, Omelka R, Chrenek P, Vondráková M, Bauerová M. Age-related changes in histological structure of the femur in juvenile and adult rabbits. Bull Vet Inst Pulawy 2005;49:227–30.
22. Rajtová V, Globočník E. Histologisches Studium der Alterveränderungen in der Femurcompacta bei Labor-und Hausmäusen. Gegenbaurs Morphol Jahb 1978;124:649–62.

Additional information and reprint requests:
Monika Martiniaková, Ph.D.
Department of Zoology and Anthropology
Constantine the Philosopher University
Nábrežie mládeže 91
949 74 Nitra
Slovak Republic
E-mail: mmartiniakova@pobox.sk