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Concentrations of manganese, iron, and strontium in bones of the domestic dog (*Canis lupus familiaris*)

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Abstract: The aim of this study was to determine concentrations of manganese (Mn), iron (Fe), and strontium (Sr) in the cartilage, compact bone, spongy bone, and cartilage with adjacent compact bone of the domestic dog (*Canis lupus familiaris*). The experiment was carried out on bones from the hip joints of 24 dogs (dogs of <8 years, dogs of >8 years) from northwestern Poland. The concentrations of Mn, Fe, and Sr were assessed by atomic absorption spectrophotometry. Fe concentration was the highest in the cartilage and compact bone. Mn concentration was the lowest in all three types of bone material and had a significantly lower concentration in spongy bone than in the other bone materials. Sr concentration was the highest in the spongy bone. Age-dependent comparison showed that both Mn and Fe concentration in dogs of <8 years was higher in cartilage. The strongest correlations (rs > 0.70) were found between Mn and Fe and between Mn and Sr concentrations in cartilage, and between Sr concentrations in compact bone and cartilage with adjacent compact bone. In most of the studied dogs, bone Mn, Fe, and Sr concentrations were similar to those found in other canines and different from ungulates and micromammals.

Key words: Iron, manganese, strontium, dog, Poland

1. Introduction

Elements such as manganese (Mn) and iron (Fe) that are essential for life and those with a likely positive impact on the body (such as strontium) can have favorable or, depending on the level, toxic effects on mammalian organisms. Fe is an essential trace element for the proper functioning of the body, although little is known about its role in the formation of bone and cartilage. Fe has been shown to be necessary for the proper functioning of the osteoblasts responsible for bone formation processes (1). Fe-deficient rats have been shown to have reduced bone mechanical strength compared to healthy animals with similar bone mass density and a group supplemented with calcium (Ca) (2). In addition, in female rats, Fe deficiency results in reduced weight and volume of the femur, and also affected the growth of the animals (3). In humans and animals an excess of Fe may result in osteoblast dysfunction and metabolic bone disorders, including osteopenia, osteoporosis, and osteomalacia (4). Mn is an essential element for the proper functioning of warm-blooded vertebrates. It is a component of many enzymes involved in reproduction, growth, metabolism of carbohydrates and fats, functioning of the immune system, and formation of cartilage and bone (5). Mn deficiency in animals can cause deformation of bones, stunted growth, and impaired motor coordination (6). Moreover, the complete absence of Mn in the diet causes a decrease in bone density (7). The effects of excess Mn in animals, including in bones, have been relatively well documented in laboratory animals. As a result of Mn intoxication in mice, fetuses were observed to have poor growth of ribs and a reduction or complete absence of ossification, e.g., in segments of the sternum and the parietal and occipital bones (8). In addition, Doyle and Kapron (9) showed the negative impact of excessive Mn concentrations on the process of calcification in the growth plate in cultures of murine chondrocytes. Strontium (Sr) belongs to the group of alkali metals and has similar physical and chemical properties to Ca. The majority of absorbed Sr, i.e. 99%, is deposited in bones and teeth (10). Small doses of Sr reduce bone resorption and stimulate bone formation, causing an increase in their weight (11). The presence of Sr is closely linked to Ca metabolism in osteocytes where it facilitates the differentiation of osteoblasts while inhibiting the differentiation of osteoclasts and reducing osteoclastic functions (12). Sr deficiency in the diet can

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cause stunted growth and calcification of bones and a greater susceptibility to tooth decay (13). An excess results in abnormal Ca metabolism and phosphate, which can result in abnormal bone mineralization, which in turn may lead to skeletal deformities (13). Research on the effects of environmental factors on the human body and the surroundings have concerned various warm-blooded animal species including the domestic dog (*Canis lupus familiaris*), not only because of the long history of strong relations between man and this mammal and the shared environment, but above all due to the many similarities between humans and dogs (14). The similarities are reflected, for example, in the genetic proximity of certain diseases such as tumors, cardiomyopathy, epilepsy, and hip dysplasia (15). Over the last decades, the average age of domestic dogs has considerably lengthened, which, similar to humans, has resulted in dogs developing a variety of disorders associated with aging. Hip osteopathy usually occurs in old dogs and the osteoporotic changes are similar to those observed in humans. It can be assumed that changes in the mineral composition of hip bones will be similar in humans and dogs. The domestic dog is one such long-lived mammal that is used as a model in orthopedic and dental studies (16). Changes in the skeletal system of canines occur both in young and old dogs, and osteoporotic changes or osteoarthritis are similar in nature to those observed in elderly humans (17). Mn and Fe belong to the group of essential elements. Mn plays an important role in many physiological processes including bone formation. However, information about Mn concentrations in bone in domesticated animals is very scarce. Given the significant analogy between the transformations in bones of both dogs and humans, it is justifiable to gather comparative data with regard to diverse bone materials (cartilage, compact bone, spongy bone, and cartilage with adjacent compact bone).

The aim of this study was to determine Mn, Fe, and Sr concentrations in the cartilage, compact bone, spongy bone, and cartilage with adjacent compact bone of the domestic dog (*Canis lupus familiaris*), and also relations between Mn, Fe, and Sr concentrations in the bone materials and the age of the dog. Studies using the dog as an animal model may contribute to a better understanding of the roles of Mn, Fe, and Sr, which are increasingly often recommended as a supplement in the treatment of osteoporosis. The results of this study may also provide a basis for further analysis in the indirect evaluation of environmental threats to human health.

2. Materials and methods

2.1. Material

The study was performed on hip joint bone elements from dogs using samples of articular cartilage, compact bone, spongy bone, and cartilage with adjacent compact bone from 24 domestic dogs from northwestern Poland. The dogs came from veterinary practices where they had been put down due to respiratory deficiencies or tumors (they were not terminated specifically for this study). This research was approved by the Local Veterinary Office in Szczecin and the Local Animal Research Ethics Committee (Resolution No. 4/2009). The ages of the dogs were determined by the veterinary surgeons based on information in medical documentation and from the owners of the animals. The dogs were divided into two age categories: CF1 (<8 years, n = 7) and CF2 (>8 years, n = 17).

2.2. Preparation of material for analysis of bone samples

At necropsy, we carefully took samples of dog legs and stored them at –20 °C until laboratory analysis. As described in earlier publications (18), the femur head (from the left leg) was cleaned of soft tissue; a titanium tool was used to cut and scrub samples. Cartilage, compact bone, and spongy bone were macroscopically isolated. Bone samples were put in crystallizing dishes and dried to a constant weight at 105 °C for 3 weeks in a laboratory drier. This procedure was used to determine water content (gravimetric method). Dried samples were ground in an agate mortar and put in plastic test tubes (18).

2.3. Determination of Mn, Fe, and Sr

The crushed material was divided into samples of 0.5–1.0 g. The weighed samples were mineralized in a mixture of 65% nitric acid (HNO_3) and 70% perchloric acid ($HClO_4$) (Suprapur Merck) using a Velp Scientifica mineralizer (Italy). Mineralization was carried out in a four-step heating regime to a maximum temperature of 200 °C. The concentrations of Mn, Fe, and Sr were assessed by atomic absorption spectrophotometry emission in inductively coupled argon plasma (ICP AES) with a Perkin-Elmer Optima 2000 DV. The limits of detection of the apparatus were 0.1 mg/L for Mn and Fe and 0.05 mg/L for Sr. Results were converted into dry weight (dw).

2.4. Validation of analytical proceedings

The accuracy of the analytical procedure was monitored by determination of the studied elements in two types of reference materials with known concentrations of Mn, Fe, and Sr: NIST SRM 1486 Bone Meal (National Institute of Standards and Technology) and IAEA-407 Trace Elements and Methylmercury in Fish Tissue (International Atomic Energy Agency). The concentration values of the reference materials given by the manufacturers and our determinations are shown in Table 1. In order to determine the possible loss of analyte during the chemical process or the impact of other factors on the results of research, we conducted recovery testing.

Table 1. Concentrations of Mn, Fe, and Sr in certified reference materials in mg/kg dry weight (RV, reference value; OD, own determination).

Metal	Bone Meal SRM NIST 1486		OD/RV (%)	Fish Tissue IAEA-407		OD/RV (%)
	RV	OD (n = 7)		RV	OD (n = 8)	
Mn	1.00*	0.90 ± 0.04	90.00	3.52 ± 0.32	3.33 ± 0.31	94.6
Fe	99.00 ± 8.00	106.00 ± 17.00	107.00	146.00 ± 14.00	141.25 ± 16.00	96.7
Sr	264.00 ± 7.00	226.00 ± 8.00	85.60	130.00 ± 11.00	115.76 ± 26.00	89.0

*Estimated value.

2.5. Statistical analysis

Statistical studies were performed using StatSoft Statistica 9.0 and Microsoft Excel 2007. We calculated the arithmetic mean (AM), standard deviation of the AM (SD), median (Med), and lower (Q_L) and upper quartile (Q_u). In order to determine the conformity of the distribution of results with an expected normal distribution, we used a Kolmogorov–Smirnov test with Lilliefors correction ($P < 0.05$). When the distribution of empirical data conformed to the expected normal distribution, an ANOVA test was used, and in the case of statistically significant differences, a Student t-test was used. In the absence of conformity between an expected normal distribution and the empirical data, we used a nonparametric Kruskal–Wallis test, and the significance of differences was confirmed by the Mann–Whitney U test. Finally, a Spearman rank correlation coefficient was calculated between trace elements occurring in the material, as well as between the different metals studied in different parts of the hip joint.

3. Results

Of the three studied elements, the highest overall median in the tested bone materials was that of Sr, with the lowest being Mn (Table 2). The Mn concentrations in the tested bones of the dog were the lowest in spongy bone and the highest in compact bone. The Fe concentrations ranged from 82.2 mg/kg dw in spongy bone to 91.7 mg/kg dw in compact bone. The lowest median of Sr was observed in compact bone and the largest in spongy bone. In cartilage, cartilage with adjacent compact bone, and compact bone,

Table 2. Concentration of Mn, Fe, and Sr (mg/kg dry weight, dw) in three types of bone material from dogs from northwestern Poland (Med, median; Q_L, lower quartile; Q_u, upper quartile; AM, arithmetic mean; SD, standard deviation; F, ANOVA test; P, level of significance; NS, nonsignificant).

Metal	Parameter	Cartilage	Compact bone	Cartilage with compact bone	Spongy bone	ANOVA test
Mn	Med	0.49	0.58	42.2	93.1	F = 3.08 P < 0.05
	Q_L–Q_U	0.25–0.93	0.43–0.74	0.40–0.80	0.23–0.48	
	AM ± SD	0.60 ± 0.46	0.63 ± 0.40	0.62 ± 0.26	0.45 ± 0.42	
	Min–max	0.05–1.58	0.06–1.50	0.27–1.24	0.02–2.19	
Fe	Med	70.6	66.9	79.2	57.3	NS
	Q_L-Q_U	21.2–112.7	45.2–140.0	44.5–122.7	22.5–120.3	
	AM ± SD	83.9 ± 74.6	96.4 ± 81.0	90.1 ± 56.4	84.2 ± 81.5	
	Min–max	0.11–233.9	2.30–316.2	18.0–244.3	2.69–298.2	
Sr	Med	60.0	43.2	49.4	86.8	F = 4.22 P < 0.01
	Q_L-Q_U	35.6–71.5	32.8–75.0	39.3–62.3	32.8–115.2	
	AM ± SD	52.1 ± 31.4	49.0 ± 25.8	50.6 ± 19.5	81.2 ± 60.5	
	Min–max	0.96–118.9	0.02–97.1	12.2–100.7	4.40–221.0	

metal concentrations could be arranged in the descending order of Fe > Sr > Mn, while in spongy bone the descending order was Sr > Fe > Mn.

Testing of the compatibility of the data distribution with an expected normal distribution was performed using a Kolmogorov–Smirnov test with Lilliefors correction. In most cases there was no evidence to reject the null hypothesis assuming that the distribution of the empirical data was consistent with the expected normal distribution. Only the distributions of Mn concentration in the cartilage with adjacent compact bone, Fe in spongy bone, and Sr in compact bone differed from a normal distribution. In view of the fact that in most cases the data distribution was consistent with an expected normal distribution, in comparisons of median levels between the several types of bone material obtained from dogs ANOVA analysis of variance was applied. This analysis revealed statistically significant differences for Sr ($F = 3.08$, $P < 0.05$ and $F = 4.22$, $P < 0.01$). When comparing the levels of metals between types of bone material, a Student t-test was used. The Mn concentration was significantly higher in compact bone (52%) than in spongy bone of the dogs. In addition, a statistically significant difference was observed in Mn concentrations between the cartilage and the spongy bone, and between the cartilage with adjacent compact bone and the spongy bone (Table 3).

There were no statistically significant correlations between metal concentrations in the corresponding bone samples and the sexes of the dogs.

We observed statistically significant differences in comparisons of metal concentrations between the two age groups of the dogs, i.e. between those younger than 8 years (CF1) and those older than 8 years (CF2). In the younger CF1 individuals, concentrations of all three metals were greater in cartilage, cartilage with adjacent compact bone, and spongy bone compared to the older group of CF2 dogs (Table 4), with concentrations lower only in compact bone. The Mn concentration was nearly three times higher in the cartilage in younger dogs, with differences confirmed statistically.

Table 3. The conformity of distributions of Mn, Fe, and Sr concentrations in the bone material from dogs with the expected normal distribution.

Material	Parameters	Test and its significance for the conformity of distributions		
		Mn	Fe	Sr
Cartilage	K-S	0.167	0.167	0.177
	P	NS	NS	NS
	Lil. corr., P	<0.10	<0.15	<0.10
	Distribution	Normal	Normal	Normal
Compact bone	K-S	0.173	0.211	0.482
	P	NS	<0.20	<0.01
	Lil. corr., P	<0.10	<0.01	<0.01
	Distribution	Normal	Nonnormal	Nonnormal
Cartilage with compact bone	K-S	0.214	0.126	0.083
	P	<0.20	NS	NS
	Lil. corr., P	<0.01	NS	NS
	Distribution	Nonnormal	Normal	Normal
Spongy bone	K-S	0.090	0.220	0.137
	P	NS	<0.15	NS
	Lil. corr., P	NS	<0.01	NS
	Distribution	Normal	Nonnormal	Normal

K-S, Kolmogorov–Smirnov test; Lil. corr., Lilliefors corrected Kolmogorov–Smirnov test; P, level of significance; NS, difference nonsignificant.

Table 4. Significance of differences between Mn, Fe, and Sr concentrations in different dog bone materials.

Metal	Parameter	C vs. CB	C vs. SB	CB vs. SB	C + CB vs. SB
Mn	t	NS	2.42	−2.81	3.85
	P		0.02	0.01	0.001
Fe	t	NS	NS	NS	NS
	P				
Sr	t	NS	−2.03	−2.48	NS
	P		0.05	0.02	

C, Cartilage; CB, compact bone; C + CB, cartilage with compact bone; SB, spongy bone; t, Student t-test; P, level of significance; NS, difference nonsignificant.

Statistically confirmed differences between metal concentrations in the bone material of younger and older dogs concerned also Mn in the cartilage with adjacent compact bone and Fe in the cartilage, in which they were 70% and four times higher in the younger group of dogs, respectively. There were correlations between Mn, Fe, and Sr concentrations in the same types of bone material. Relations were synergistic, and in one case antagonistic. The strongest relation existed between Mn and Fe in the cartilage. A weaker correlation between these metals was found in the cartilage with adjacent compact bone. The smallest significant correlation was observed between Mn and Sr in compact bone. The negative correlation was observed only between Mn in compact bone and Sr in cartilage (Table 4).

We separately analyzed correlations between the same metal concentrations present in the different types of bone material obtained from the dogs (Table 5). We found positive correlations between Mn concentration in the cartilage and in the cartilage with adjacent compact bone ($r_s = 0.592$) and a negative correlation between Mn in the cartilage and the compact bone ($r_s = -0.439$). Significant correlations also existed between Fe concentrations in the cartilage and in the cartilage with adjacent compact bone ($r_s = 0.560$) and between Fe in the compact bone and the cartilage with adjacent compact bone ($r_s = 0.661$). Only one correlation, but with the greatest Spearman correlation coefficient value, was observed for Sr in the compact bone and in the cartilage with adjacent compact bone ($r_s = 0.896$).

4. Discussion

In scientific papers metal concentrations in bone are presented in wet and dry weight, and in ash. In order to be able to make appropriate calculations, based on our own and other studies we determined the average water content in dry weight as 12% in the spongy bone of dogs and 20% in the compact bone (18,19).

4.1. Manganese

In the literature, Mn concentrations in dry matter obtained from different types of animal bones (most often analysis not taking into account the division into cartilage, compact bone, and spongy bone) range from 1.0 to 3.0 mg/kg dw. The highest concentrations (≥5 mg/kg dw) in wild animals were found in a small insectivorous mammal, the greater white-toothed shrew, and in the herbivorous roe deer, while among domestic animals in cattle (20–22). Very low Mn concentrations (<0.10 mg/kg dw) have been reported in laboratory mice (23).

In this study, Mn concentrations in dogs from northwestern Poland, depending on the type of bone material, ranged from 0.4 to 0.6 mg/kg dw. Similar Mn concentrations were found by Schor et al. (24) in rib cartilage from dachshund and beagle at 0.62 and 0.77 mg/kg dw, respectively. Slightly higher Mn concentrations (from 0.7 to 1.1 mg/kg dw) were found in the bones of fox from northwestern Poland (25). Mertin et al. (26) found a much higher Mn concentration than that presented in this paper in the bones of farm-bred raccoon dogs from Poland (2 mg/kg dw), due probably to the high Mn content in the diet of these animals, where farmers used Mn supplementation to improve the quality of the fur.

In laboratory tests on mice, mean Mn concentrations in the parietal bone were highest in mice aged 5 to 43 days old (0.61 mg/kg dw) and smallest in newborn infants aged 2 days old (0.09 mg/kg dw) (23). In this study, in the bones of domestic dogs from northwestern Poland an inverse relationship was observed, with younger dogs having higher Mn concentrations in cartilage and in cartilage with adjacent compact bone than older dogs. No age-related differences in Mn concentrations in bones between immature and mature individuals were reported in fox from northwestern Poland (25). Based on the work of Mertin et al. (25) it can be concluded that supplementation of Mn greatly increases its

Table 5. Comparison of metal concentrations in analogous dog bone materials between two age groups.

Material	Parameter	Mn	Fe	Sr
CF1 = 7				
C	Med	1.10	162.4	62.6
	Q_L–Q_U	0.53–1.58	24.4–224.6	37.5–67.1
	AM ± SD	1.03 ± 0.48	134.6 ± 90.6	56.7 ± 14.6
	Min–max	0.31–1.58	10.0–233.4	35.6–73.1
CB	Med	0.52	64.0	57.5
	Q_L–Q_U	0.17–0.90	45.2–140.0	38.3–81.0
	AM ± SD	0.52 ± 0.35	93.9 ± 79.2	60.6 ± 19.6
	Min–max	0.06–1.00	35.2–255.3	34.3–81.6
C + CB	Med	0.80	98.8	62.3
	Q_L–Q_U	0.55–0.96	76.6–134.9	45.8–69.7
	AM ± SD	0.78 ± 0.28	114.3 ± 65.2	58.6 ± 13.6
	Min–max	0.42–1.24	40.9–244.3	35.9–72.1
SB	Med	0.36	38.6	39.7
	Q_L–Q_U	0.34–0.43	2.7–19.1	18.1–158.0
	AM ± SD	0.4 ± 0.09	66.7 ± 103.2	77.6 ± 70.8
	Min–max	0.33–0.58	2.69–298.2	6.72–182.7
CF2 = 17				
C	Med	0.37	43.5	60.3
	Q_L–Q_U	0.21–0.56	16.6–92.2	39.9–74.6
	AM ± SD	0.42 ± 0.35	64.2 ± 63.7	56.1 ± 33.5
	Min–max	0.05–1.40	0.11–233.9	0.96–118.9
CB	Med	0.59	54.9	37.6
	Q_L–Q_U	0.44-0.66	41.6-107.5	32.0-64.0
	AM ± SD	0.61 ± 0.38	90.7 ± 85.0	46.4 ± 28.0
	Min–max	0.09–1.50	2.30–316.2	0.02–97.1
C + CB	Med	0.47	64.7	48.3
	Q_L–Q_U	0.37–0.50	41.3–104.8	42.4–60.0
	AM ± SD	0.52 ± 0.2	77.5 ± 53.1	51.3 ± 18.6
	Min–max	0.27–1.0	18.0–214.0	16.5–100.7
SB	Med	0.26	81.3	95.0
	Q_L–Q_U	0.20–0.47	24.5–120.3	32.8–115.2
	AM ± SD	0.33 ± 0.20	88.6 ± 78.3	87.4 ± 59.9
	Min–max	0.02–0.69	5.47–289.6	4.4–221.0
CF1 vs. CF2				
C	t	3.421	2.175	NS
	P	0.002	0.04	
CB	t	NS	NS	NS
	P			
C + CB	t	2.572	NS	NS
	P	0.02		
SB	t	NS	NS	NS
	P			

C, Cartilage; CB, compact bone; C + CB, cartilage with compact bone; SB, spongy bone; AM, arithmetic mean; SD, standard deviation; CV, coefficient of variation in percent; Med, median; Q_L, lower quartile; Q_U, upper quartile; CF1, <8 years; CF2, >8 years; t, Student t-test; P, level of significance; NS, statistically nonsignificant difference.

concentration in the bones of animals. In wild mammals, such as fox, accumulation of Mn in hard tissues may indicate the excess of this metal in their environment. Dogs as companions of humans are subject to the same environmental influences; an increase in Mn pollution, especially in cities in the form of vehicle exhaust, as well as the increasingly frequent use of ready-made feeds rich in macro- and micronutrients (including Mn), significantly increase the accumulation of this metal in the bones of these animals.

4.2. Iron

The bone material of different animal species accumulate from several milligrams to several hundred milligrams of Fe in dry weight. The largest Fe concentrations in wild animals (200 mg/kg dw) were found among representatives of small mammals, Micromammalia, and for warm-blooded vertebrates in domesticated cattle (21,26,27).

The lowest Fe concentration (<15 mg/kg dw) has been reported in free-living elk and domesticated sheep (28). Fe concentrations in animal bone usually range from 30 to 150 mg/kg dw. However, in the bones of the farmed raccoon dog from Poland Fe concentrations are lower, at about 60 mg/kg dw (26). In the domestic dog from West Pomerania, Fe concentrations in the bones were about 80 to 90 mg/kg dw. In fox in the same study area, Fe levels ranged from 80 to 195 mg/kg dw (25).

4.3. Strontium

Sr concentrations in the bones of mammals depend on the trophic group (29). Carnivorous animals accumulate the least Sr in bones (114 to 331 mg/kg dw), omnivores from 170 to 455 mg/kg dw, and herbivores the most at 455 to 570 mg/kg dw.

In a study on dogs in Denmark, Raffalt et al. (30) determined Sr concentrations in compact bone after supplementing feed with different amounts of Sr-malonate (from 300 to 3000 mg kg^{-1} day^{-1}). They found two orders of magnitude greater Sr concentrations in bone material from the animals fed with Sr supplementation (from 7200 to 9800 mg/kg) compared to the control group receiving feed without the additive (76 mg/kg dw).

Sr concentration in the same material in dogs from northwestern Poland examined in this study amounted to about 51 mg/kg dw. However, in foxes in the same area Sr concentration in the bones amount to about 69 mg/kg dw (26). These concentrations can be considered similar to those reported by the Danish researchers (30) in dogs not supplemented with Sr.

Based on the above it can be concluded that in most cases Mn, Fe, and Sr concentrations in the bones of West Pomeranian dogs are similar to those found in other canines. There are differences in the concentrations of these metals in bone material from these carnivores compared to ungulates and micromammals. Concentrations of various trace elements in bone samples were strongly correlated with environmental conditions, diet, and the health status of the populations of people and animals. Thanks to its properties, bone tissue may reflect chronic exposure and may be the basis of indirect assessment of environmental exposure, including human habitats. The results of this study may serve as the basis for further analyses in the indirect assessment of environmental threats to humans.

In addition, due to the rather low number of publications in this field, it seems justified to examine more animals from various taxa in different environments and also take into account corresponding bone and/or structural components in comparative studies.

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