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ORIGINAL ARTICLE

Influence of caffeine administered at 45°C on bone tissue development

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Abstract

Introduction and objective. Caffeine is one of the world's most commonly ingested alkaloids which easily permeates the placenta. The teratogenic and embryotoxic influence of large doses of caffeine has been established in many experimental studies on animals. The objective of this work was to assess the influence of caffeine, administered at 45°C, on the development of the bone tissue of rats, with particular reference to elemental bone composition using an X-ray microprobe.

Materials and methods. The research was conducted on white rats of the Wistar strain. The fertilized females were divided into two groups: an Experimental Group (Group E) and a Control Group (Group C). The females in Group E were given caffeine orally (at 45°C) in 30 mg/day doses from the 8th to the 21st day of pregnancy. The females in Group C were given water at the same temperature. The fetuses were used to assess the growth and mineralization of the skeleton. A qualitative analysis of the morphology and mineralization of bones was conducted using the alcian-alizarin method. For calcium and potassium analysis, an X-ray microprobe was used.

Results. By staining the skeleton using the alcian-alizarin method, changes in 52 of Group E fetuses were observed. The frequency of the development variants in the Group E rats was statistically higher, compared with Group C.

Conclusions. Receiving caffeine at a higher temperature may result in different pharmacodynamics and significantly change tolerance to it. In Group E, a significant decrease in the calcium level, as well as an increase in the potassium level, was observed. The X-ray microprobe can be a perfect complement to the methods which enable determination of the mineralization of osseous tissue.

Key words

caffeine, bone tissue, rat, X-ray microprobe, temperature 45°C

INTRODUCTION

Caffeine is widely used in the food industry. In nature, it is found in the seeds of the coffee plant *Coffea Arabia*, or in plants of the *Sterculiaceae* and *Theace* family. Among the crop plants, tea leaves *(Thea Folium)* contain from 1.2 – 4.5% caffeine, coffee seed – from 0.3 – 2.5% and cocoa seeds *(Semen Cackao)* – about 0.3% [1, 2]. Daily caffeine consumption is estimated to be 3–7 mg/kg body weight, (about 200 mg/person/day) and the annual average is about 4.5 kg/person (about 118 tons). Depending on the region of the world, there are differences in the consumption of caffeine. In the Nordic countries, caffeine amounts to 12.2 kg/person/year, and in the USA and the UK – approximately 3 mg/kg and 4 mg/kg [3, 4, 5].

Caffeine is a common ingredient in beverages consumed by the general public. Converted, it can be used as an effective medicine for colds or allergies. It also enhances the analgetic effect of painkillers, especially pyrazolone, p-aminophenol and salicylate derivatives [6, 7].

It should be noted that caffeine passes easily through the placenta, as well as into the milk. The effects of caffeine on embryonic and foetal development (both in experimental animals and humans) have been tested by many researchers. Studies clearly show that consuming caffeine in doses of >300 mg/day increases the risk of premature termination of pregnancy [8], while receiving lower doses (around 150 mg/day) increases the risk of child labour with a low birth weight [9]. Clinical and epidemiological observations led to the US Food and Drug Administration (FDA) issuing a decree for a reduction in caffeine intake by pregnant women (<400 mg/day) [10].

To-date, it is not known whether the consumption temperature of the various food products influences this substance's toxicity [11] on development. This is important, because products containing methylxanthine are consumed at a 4–60°C temperature range. Such a wide temperature range changes the bioavailability of caffeine [12] and may result in different pharmacodynamics, and significantly change tolerance to it.

The objective of the presented study was to assess the influence of caffeine, administrated at 45°C, on the development of bone tissue in rats, with particular reference to elemental bone composition using an X-ray microprobe.

MATERIALS AND METHOD

The research was conducted on white rats of the Wistar strain [CRL:(WI)WUBR] with the consent of the Bioethical Board of the Medical University in Lublin.

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All rats had free access to food and water. After a 2-week acclimatization period, the virgin females with a body mass of (238±24) g mated overnight (20:00 – 08:00) with the males in a ratio of 5:2. Proof of effective copulation was the presence of spermatozoa or a clot containing a mixture of semen and flaked-off vagina epithelium in the morning vaginal swab taken at 08:00. The fertilized females were divided into 2 groups of 10 specimens, an Experimental Group (Group E) and a Control Group (Group C). The day of fertilization was considered to be the first day of pregnancy.

The experiment used 99% pure caffeine (Caffeine anhydrous powder, Sigma-Aldrich Chemie GmbH, Germany) administered in a dose of 30 mg/day.

The substance under investigation was dissolved in sterile distilled water at 45 °C and a single daily 30 mg/day dose was administered to the Group E females, from the 8th to the 21st day of pregnancy, through a tube inserted into the stomach. The Group C females were given the same amount of water at the same temperature to those in Group E. On the 21st day of pregnancy, the pregnant females were killed by decapitation using a specially prepared laboratory guillotine. Death was caused by breaking the continuity of the spinal cord. After cutting the covering tissue of the abdominal cavity, the uterus with the foetus was extracted and killed by freezing in liquid nitrogen (Zakłady Azotowe, Puławy, Poland). They were then used for skeletal growth and mineralization assessments. A qualitative analysis of the morphology and mineralization of bones was conducted using the alcian-alizarin method, where cartilage parts are dyed blue from alcian blue, while the ossified parts are dyed red under the influence of alizarin [6].

In order to identify the presence of calcium and potassium, 10 male fetuses were chosen – one from each litter. Femoral bones were extracted from the fetuses and frozen in liquid nitrogen. After defrosting, the samples were analyzed with an electron scanning microscope (BS340) using the TESCAN system (TESLA, Czech Republic), combined with an X-ray microprobe (Thermo Electron Corporation, USA).

In order to determine the chemical composition, the bone samples first underwent preliminary preparation (cleaning, drying), and then a layer of gold, 10 nm thick, was evaporation-deposited onto the bones using a Sputron evaporator (Thermo Electron Corporation, USA). An assessment of the chemical composition of the fetal bones was conducted using X-ray spectroscopy analysis. The electromagnetic spectra were obtained from the surface of the limb closer to the original point of femoral bone ossification using an X-ray microanalyzer (Thermo Electron Corporation, USA). In order to achieve this, bone samples were 'bombarded' with an electron beam in the scanning chamber of the electron microscope using a 20 kV acceleration voltage. This produced the characteristic X-ray radiation for the atoms in the sample, as a consequence of the electrons dropping from their excited state into their base state. Analysis of the chemical composition, both quantitative and qualitative, was performed using NORAN System SIX Model 300 software.

The spectral fluorescent peaks corresponding to the calcium (Ca^{2+}) and potassium (K^{+}) ions were identified. The sum of the quantities of these elements in the bone was determined to be 100%. In order to achieve unification of the measurement data, identical picture magnifications were selected and the bone fragments were identically positioned with reference to the EDAX detector.

Statistical analysis. The quantitative features were defined by the arithmetic average (Mean) and standard deviation (SD). The distribution of the analyzed features in the group was obtained using the Kormogolov-Smirnov test. Depending on the distribution value, the significance of the differences among the groups was evaluated using either the t-Student test or the U test (Mann-Whitney). The significance of the qualitative features was examined using the X^2 test.

For statistical analysis, the STATISTICA 5.0 (StatSoft Inc, USA) computer programme was used. For all the tests, the statistically significant differences were those where the significance co-efficient (p) was less than 0.05.

RESULTS

Skeleton morphology was analyzed in 70 Group E fetuses (9 litters) and in 72 Group C fetuses (10 litters).

In 52 Group E fetuses (74.3%) and in 9 Group C suckling pups (12.5%) the skeleton was stained differently by the alcian-alizarin method. In the examined population, the development variants with decreased mineralization were dominant, which morphologically corresponds to a reduction in, or a lack of, alizarin staining. The frequency of the development variants in the Group E rats was higher compared with those in Group C ($p<0.05$) (Tab. 1).

In the cranial area, a reduction in, or a lack of staining of the parietal, occipital and interparietal bones was most frequently observed (Fig. 1).

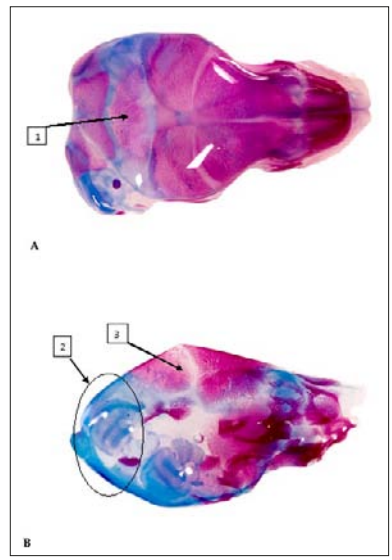

Figure 1. A – Reduced mineralization in intraparietal bone (1) (Group E). **B** – Lack of mineralization in intraparietal and supraoccipital bone (2) and reduced mineralization in parietal bone (3) (Group E)

In the case of parietal bones, mineralization disorders were always characterized as two-sided. A reduction, as well as a lack of staining, was observed in fetuses exposed to caffeine. In Group C, 3 fetuses (4.2%) had a reduced staining of the parietal bone, while 5 fetuses (7.1%) had a reduced or a complete lack of staining of the parietal bone.

In the Group E rats, significant statistical differences in the staining of the interparietal and supraoccipital bones were recorded when compared with those in Group C. In

Table 1. Incidence of developmental defects and variations in the groups exposed to caffeine (Group E) and control group (Group C)

No. of fetuses / No. of litters		C		E	
		No. of fetuses	No. of litters		
total number of fetuses[a]		91	10	95	10
number of stained preparations		72	10	70	9
number of fetuses with variations		9	5	52*	7
parietal bone[b]	reduction of alizarin staining	3	3	5	4
	lack of alizarin staining	-	-	5	4
interparietal bone	reduction in staining	4	3	8	3
	lack of alizarin staining	5	3	36*	7
supraoccipital bone	reduction in staining	4	3	9	4
	lack of alizarin staining	5	3	27*	7
13th rib- wave[b]		4	3	7	5
segments of the sternum	lack of alizarin staining	5	3	19	6
	reduction of alizarin staining	-	-	15	5
	other varieties[c]	-	-	3	1
metacarpal bone, lack of alizarin staining[b]	all	4	3	9	4
	four	2	1	3	2
	three	3	2	15	6
	two	2	1	7	4
	one	1	1	4	3
distal phalanges, front paws lack of alizarin staining[b]	all	5	3	25	7
	four	-	-	3	2
	three	-	-	5	3
	two	1	1	3	2
	one	-	-	2	1
pubic bone - lack of alizarin staining		-	-	4	3
Ischium, lack of alizarin staining		-	-	4	3
Femur, shortening and extension shaft[b]		-	-	5	2
Tibia, shortening and extension shaft[b]		-	-	4	2
Fibula, shortening and extension shaft[b]		-	-	4	2
metatarsal- lack of alizarin staining[b]	all	3	2	15	5
four	2	1	2	1	
three	-	-	5	3	
two	1	1	5	4	
one	-	-	3	2	
distal phalanges, back paws- lack of alizarin staining[b]	all	3	3	25	6
four	-	-	3	3	
three	-	-	3	3	
two	2	1	3	3	
one	-	-	2	1	

single fetus may be represented more than once in the above statement
[a] number of fetuses born alive
[b] single-or double-sided
[c] asymmetrical, split and the residual
*significant changes compared to the control group

Group E, for the interparietal bones, a reduction ($p<0.05$) was observed in 8 suckling pups (11.4%), while a lack of staining in these bones was observed in 36 fetuses (51.4%). For the supraoccipital bones, the figures were 9 suckling pups (12.9%) and 27 fetuses (38.6%), respectively.

In Group E, disturbances in the mineralization of the sternum segments were observed in 15 suckling pups (21.4%)

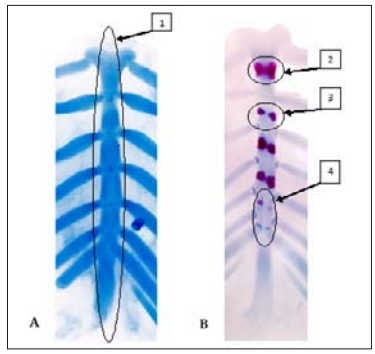

Figure 2. A – Lack of mineralization of the sternum segments (1) (Group E). **B** – Asymmetrical (2), divided (3) and the residual (4) segments of sternum (Group E)

(Fig. 2). A statistically significant increase in the incidence of partial mineralization of particular sternum segments was recorded in Group E ($p<0.05$), while in Group C, anomalies relating to this bone did not occur. In single cases, a complete lack of ossification (27.1%) and other morphology disorders in segments were found to be asymmetrical, divided and in vestigial forms (4.3%) (Fig. 2).

Disturbances in the mineralization of the metacarpal, metatarsus and phalanges bones (front and back paws) were also observed more frequently in Group E (Fig. 3). These disturbances between the 2 groups were not significant statistical differences.

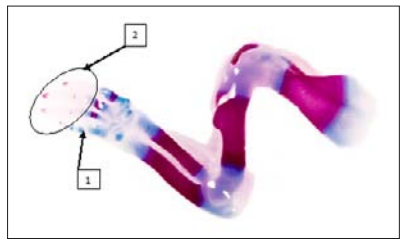

Figure 3. Lack of mineralization in metacarpal bone (1), proximal and middle phalanges (2) (Group E)

Lack of alizarin staining of the pubic and ischium bones, as well as the shortening and widening of the femur, tibia and fibula bones was observed more frequently in Group E than in Group C (Fig. 4). Again, these disturbances between the 2 groups were not significant statistical differences.

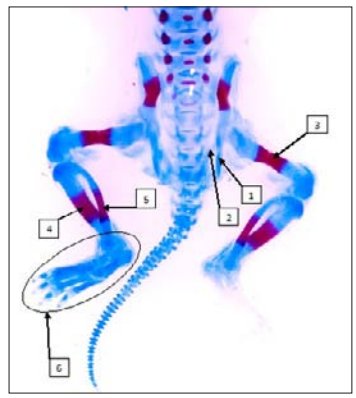

Figure 4. Lack of mineralization of ischial (1) and pubic (2) bone, shortening and broadening of the femoral (3), tibia (4) and fibula (5) shaft, disorders of mineralization of bone posterior feet (6) (Group E)

55 bones from the Group E fetuses and 55 bones from those in Group C were used to obtain specimens for mineral content assessment. The average calcium and potassium content in the rat bones from Group C was 72.08% and 27.92%, respectively. In the experiment, a significant decrease in the level of calcium (54.08%) was observed, as well as an increase in the level of potassium (45.93%) in the group of rats whose mothers were given caffeine during pregnancy ($p<0.05$) (Fig. 5). For 2 randomly selected fetuses, Figures 6 and 7 show example visualizations and X-ray spectra of the bone end, closer to the original ossification centre in the femoral bones.

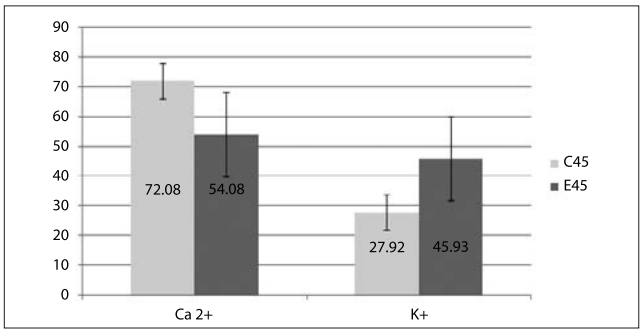

Figure 5. Percentage of average content of calcium (Ca^{2+}) and potassium (K^{+}), together with standard deviation in the fetuses' femur between groups of animals receiving water (Group C) and caffeine (Group E) ($p<0.05$)

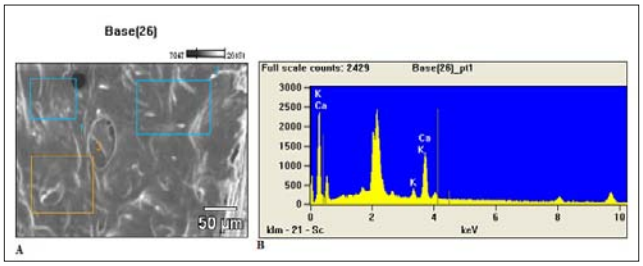

Figure 6. A – Surface of the proximal end of the original nucleus of ossification in the femoral diaphysis of offspring from the control group (Group C) (SEM, x 200). **B** – X-ray spectrum of field marked in orange. In the presented case, the percentage of calcium and potassium on the K energy level is 74.99 and 25.01%, respectively

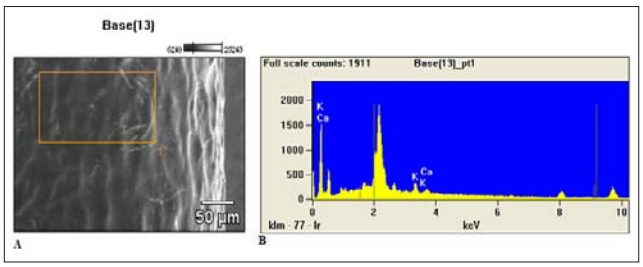

Figure 7. A – Surface of proximal end of the original nucleus of ossification in femoral diaphysis of offspring from the group receiving a solution of caffeine (Group E) (SEM, x 200). **B –** X-ray spectrum of field marked in orange. In the presented case, the percentage of calcium and potassium on the K energy level is 48.71 and 51.29%, respectively

DISCUSSION

Caffeine easily permeates through the placental barrier into the milk of feeding mothers [13; 14]. In many experimental studies on animals, the teratogenic and embryotoxic influence of large caffeine doses has been established. Nishimura and Nakai [8] were the first to discover that caffeine causes abnormalities in the development of offspring. Split palate and deformations in the digits were observed in 43% of the offspring whose mothers were given a caffeine dose of 250 mg/kg gastrointestinally between the 10th and 14th day of pregnancy.

Caffeine during pregnancy can disturb the development of the osseous tissue. In the current experiment, the most common abnormalities related to impaired mineralization of the interparietal and the supraoccipital bones was observed. Additionally, there was a lack of alizarin staining of the pubic and ischium bones, as well as a shortening and widening of the femur, tibia and fibula bones. Similar changes, in the form of delayed mineralization, were observed by Smith et al. [15], Collins et al. [16], and Tomaszewski et al. [17]. Smith et al. [15] supplied caffeine in a dose of 100 mg/kg, and Giannelli et al. [19] showed disturbed mineralization in animals receiving caffeine *in utero* in doses of 24.7–29.0 mg/kg. Abnormalities occurred most commonly in the ribs (supernumerary intrathoracic or lumbar rib, 'floating ribs'), sternum area (incomplete mineralization, complete lack or splitting of the sternum), and the metacarpal and metatarsus bones (impairment of ossification).

Tomaszewski et al. [17] supplied caffeine in a dose of 30 mg/day at 25 °C. The results showed impaired mineralization of the interparietal bone, 2 metacarpal bones, 2 distal phalanges of the front paw, and segments of the sternum. In order to examine mineralization correctly, apart from a morphological examination, it was also necessary to establish the elemental composition of the osseous tissue. The research, performed using an X-ray microprobe, also showed a significant statistical decrease in the calcium content (53.65%) ($p<0.05$) and an increase in the potassium content (46.35%) ($p<0.05$) in the group of rats whose mothers were given a 30 mg/day dose of caffeine during pregnancy. Similar changes, in the balance between calcium and potassium content, were observed in the presented experiment. On the basis of the results obtained, performed using an X-ray microprobe, the figures were 54.08% ($p<0.05$) calcium content and 45.93% ($p<0.05$) potassium content for a 30mg/day dose of caffeine at 45 °C.

Comparing the results of the experiments in which pregnant rat females were subjected to the caffeine and water in the present study, the following data were recorded: the reduction of fetuses per litter (95 fetuses in the group of animals whose mothers received caffeine at 45 °C, 140 fetuses in the group of animals whose mothers received caffeine 25 °C) and increased bone malformations (rear paws), both in the experimental group and in the control. It should be stated that in the group of fetuses of mothers treated with water at 45 °C, an increased incidence of further development of varieties of the skeleton were observed, compared to the sucklings of mothers treated with water at a temperature of 25 °C [17].

The observed changes in the development of foetal bone are caused not only by the action of caffeine administered to pregnant females, but also by the high temperature of the solution (thermal stress). In the available literature there are no reports on the effects of caffeine administered at different temperatures on the bone tissue development, and it is very difficult to compare the current results with the results from other research centres. It is believed that the presented research is pioneering, and the results could become the starting point for further research [17].

The negative influence of caffeine on the elemental composition of the developing osseous tissue has been demonstrated in many experimental works on animals [19, 20, 21, 22, 23]. Nakamoto et al. [19] fed caffeine to female rats from the 8th to the 22nd day of pregnancy in doses of 0.5–2.0 mg/kg orally. In the groups exposed to the highest dose of methylxanthine, the researchers observed a decrease in the amount of calcium, phosphates, magnesium and zinc. Sasahara et al. [22] determined the amount of calcium, magnesium and zinc (spectrophotometrically), and phosphate (calorimetrically) in rat fetuses exposed to caffeine. Pregnant rats were fed with food containing caffeine (20 mg/kg) or caffeine (20 mg/kg) with zinc (0.6 g/kg). They did not determine any significant alterations in the amount of calcium and phosphorus in the femoral bones of the suckling pups from mothers receiving only caffeine, in comparison with the control group animals. However, they observed a significantly higher calcium content in animals with prenatal zinc supplements.

Wink et al. [23], using a scanning and transmission electron microscope, determined the histological structure of bones in quickly developing suckling rat pups exposed to caffeine during pregnancy. Pregnant females were given a 40 mg/kg dose of caffeine orally. With the use of visualizations obtained using a scanning and transmission electron microscope and a fluorescence microscope, it was found that in young animals whose mothers were given caffeine, the number of osteocytes in the femoral bone significantly decreased. It was additionally shown that there were abnormal mitochondria in the osteoblasts and osteocytes, with a decrease in the level of copper and zinc in the cytoplasm of these cells.

Duarte et al. [24] showed correlations between caffeine consumption in the early stages of bone healing and bone density in rats. They found that a high daily caffeine intake (10mg/100g body weight/day) may disturb the early stages of bone healing.

Currently, there is little data on the high-temperature effects of caffeine on bone tissue [11, 12]. Hallström et al. [11] presented a study evaluating the effect of high-temperature caffeine intake on body mass density (BMD). Between 2001 – 2004, a study was conducted on a group of 1,016 residents, in their 70s, living in Uppsala, Sweden. The participants had their routine medical history examined, an assessment of their BMD using Dual-energy X-ray absorptiometry (DXA) was made, and their blood pressure and anthropometry measured, together with blood sampling after an overnight fast. Dietary habits were registered in 850 of the participants. Each participant recorded his/her food consumption over a 7-day period using a pre-coded food diary after receiving instructions from a dietician. Coffee and tea consumption was registered 6 times daily (breakfast, lunch, supper, between meals and in the evening). One cup of filtered coffee (150 mL) was estimated to contain about 100 mg of caffeine, while one cup of tea (200 mL) about 50 mg. Additionally, researchers highlighted the possibility that the participants' genotype for cytochrome P4501A2 (CYP1A2) could modify the relation between coffee consumption and BMD (CYP1A2 is the most important enzyme in the metabolism of caffeine). Two years after the baseline investigation, 898 of the 1,016 participants had their BMD (g/cm^2) assessed again for total proximal femur, femoral neck and trochanteric regions of the proximal femur by DXA. Based on this research, Hallström et al. [11] showed that a high consumption of coffee (4 cups or more per day) seems to contribute to a reduction in the BMD of the proximal femur in elderly men, but not in women. BMD was lower in high consumers of coffee having a rapid caffeine metabolism, suggesting that rapid metabolizers of caffeine may constitute a risk group for bone loss induced by coffee.

CONCLUSIONS

Caffeine is widely distributed in the food industry and it works in many areas of life. On the other hand, the regular intake of this substance, can lead to disturbances of the nervous or cardiovascular system. Additionally caffeine easily crosses the placenta, and into the milk of nursing mothers. According to the FDA (Food and Drug Administration) in the USA, consumption of foodstuffs containing caffeine by pregnant women should be reduced to a minimum [10, 25].

On the basis of the presented results, it should be stated that caffeine given in large doses during pregnancy disturbs the development of the osseous tissue and its mineralization. Additionally, caffeine given at 45 °C increases the frequency of the skeleton development variants. As a result of the current research, caffeine given at a high temperature may result in different pharmacodynamics and significantly change tolerance to it.

It should also be emphasized that the X-ray microprobe can be a perfect complement to the following methods enabling determination of the mineralization of the osseous tissue. These methods include both the traditional (alcian-alizarin dyeing) as well as the more modern ones (scanning and transmission microscope, cremation using spectrophotometry). Its undoubted advantage is a quick qualitative and quantitative analysis of the elemental composition of the examined samples. Employing the new technique may present new capabilities when investigating the essence of the pathologic process.

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