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The influence of caffeine administered at 10°C on bone tissue development

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Abstract

Introduction and objective. Caffeine is a natural methylxanthine widespread throughout the food industry. Many research studies have shown that caffeine readily crosses the placenta causing teratogenic and embryotoxic effects. The objective of this study was to assess the influence of caffeine, administered at 10°C, on the development of a rat's bone tissue, 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 10°C) in 30 mg/day doses from the 8th – 21st day of pregnancy. The females in Group C were given water at the same temperature. The foetuses were used to assess the growth and mineralization of the skeleton. 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 47 Group E foetuses were observed. The frequency of the development variants in the Group E rats was statistically higher, compared with Group C.

Conclusions. On the basis of these results, it can be concluded that caffeine in high doses disturbs the development of bone tissue. An additional factor which enhances the adverse effects of this substance on bone tissue is the temperature of the administered solution (10°C). In the Experimental Group, 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, bone development, rat, X-ray microprobe, temperature 10°C

INTRODUCTION

Caffeine is a natural methylxanthine widespread throughout the food industry. Its processed form is a constituent of beverages which improve the associative processes and which abolish the feeling of fatigue and sleepiness. Some of them are routinely consumed chilled (Cola®, Red Bull®).

After consumption, caffeine is metabolised in the liver primarily by cytochrome P450. Its metabolism depends on the gender, age, physiological state of the organism, the use of oral contraceptives and smoking [1, 2, 3]. The pharmacokinetics of caffeine, in addition to the organism's ontogenic characteristics, are also affected by external physical factors, such as temperature and the time of day of its consumption [4, 5]. The relationship between an individual's activity and the time of day the caffeine was administered was observed by Pelissier-Alicot et al [4]. A study on rats found that the effect of caffeine on the heart rate, locomotor activity and daily body temperature cycle is more marked when administered in the morning.

The effect of caffeinated beverages, consumed at temperatures higher than at room temperature, on the activation of physiological processes was demonstrated by Quinlan et al [5]. The study was conducted on healthy, non-smoking men and women who were given hot coffee, tea or water. Next, the systolic and diastolic blood pressures, pulse, skin conductance and temperature, as well as the concentration of cortisol in saliva was measured. On the basis of this research, it was shown that consuming hot drinks (regardless of type) results in a significant acceleration of physiological functions. In addition, stimulation of the autonomic nervous system by the temperature improves mood, regardless of cortisol concentration.

Many research studies have shown that caffeine readily crosses the placenta causing teratogenic and embryotoxic effects [6, 7, 8]. Also shown are the adverse effects of high doses of caffeine ingested during pregnancy, both for the mother and the developing foetus [9–15]. Among the disorders affecting the foetus, the most common are mineralisation abnormalities in the bone tissue of the carpal and metatarsal bones, phalanges, skull, pelvis girdle, sternum and spine [16–20].

The aim of this study was to evaluate the impact of caffeine, administered to pregnant females at a temperature of 10 °C, on the development and mineralisation of foetal bone tissue.

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MATERIALS AND METHOD

The research was based on an animal experimental model, according to international guidelines for the assessment of developmental toxicity on white rats of the Wistar strain CRL:(WI)WUBR. The research was conducted with the consent of the Bioethical Board at the Medical University in Lublin, Poland.

Access to water and feed was freely available to all animals. After a two-week acclimatisation period, the virgin females with a body mass between 220–250g mated overnight with 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 exfoliated vaginal epithelial, in the morning vaginal swab. The fertilised females were randomly divided into two groups of 10 rats, an experimental group (Group E) and a control group (Group C). The day of fertilisation was considered to be the first day of pregnancy.

The study used caffeine (*Caffeine anhydrous powder*, Sigma-Aldrich Chemie GmbH, Germany) with a purity exceeding 99%, administered at a dose of 120 mg/kg body mass, which according to the data in the literature [21] should interfere with the rat's prenatal development.

The substance under investigation was dissolved in sterile distilled water at 10°C and a single daily dose of 2 ml/kg body mass was administered intragastrically to the Group E females from the 8th – 21st day of pregnancy. The Group C females received 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 without damaging the continuity of the external layers. After cutting the covering tissues of the abdominal cavity, the uterus with the foetuses were extracted. Foetuses selected for developmental and skeletal mineralisation assessment were put to sleep with liquid nitrogen vapour. Qualitative analysis of the morphology and mineralisation of bones was based on double staining. The alcian-alizarin method was used where cartilage is dyed alcian blue while the bones are dyed red due to the alizarin (Fig. 1) [20].

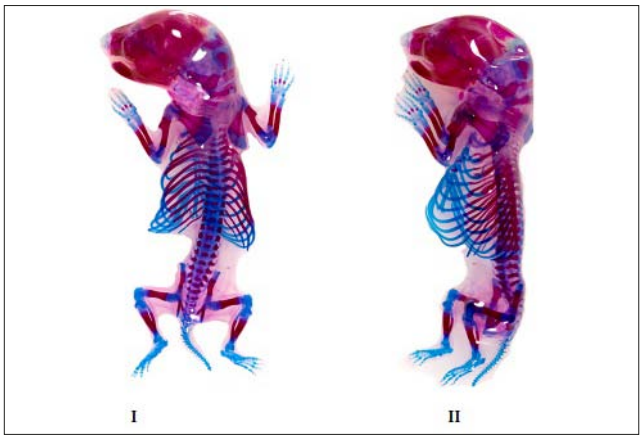

Figure 1. I: Back view skeletal of 21-day fetal rat (Group C). II: side view skeletal of 21-day fetal rat (Group C)

The calcium and potassium content in the foetal femurs was also determined. 10 male foetuses were randomly selected, one from each litter. Femoral bones were extracted from the foetuses and frozen in liquid nitrogen. After thawing, the samples were analysed with a BS340 (TESLA) scanning electron microscope, using the TESCAN system, coupled to an X-ray microprobe. Bone samples were subjected to preliminary treatment (cleaning and drying), and then a layer of gold, 10 nm thick, was vapour-deposited using a Sputron evaporator. An assessment of the calcium and potassium content in the foetal bones was performed by analysing X-ray spectra. Chemical composition analysis was performed using Noran System SIX Model 300 software. The fluorescent peaks corresponding to the calcium (Ca^{2+}) and potassium (K^{+}) ions were identified in the spectra. The sum of the quantities of these elements in the bone was defined as 100%. In order to achieve consistency in the measurement data, images with identical magnification and having identically positioned bone fragments with respect to the EDAX detector were selected for analysis [22].

Statistical analysis. The quantitative characteristics were defined by the arithmetic average and standard deviation. Distribution of the analysed characteristics in the group was obtained using the Kormogolov-Smirnov test. Depending on the distribution of values, the significance of the differences between the groups was evaluated using either the t-Student test or the U test (Mann-Whitney). The significance of the qualitative characteristics was tested using the χ^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 coefficient (p) was less than 0.05.

RESULTS AND DISCUSSION

Skeletal morphology was analysed in 65 Group E foetuses (9 litters) and 61 Group C foetuses (10 litters) (Tab. 1, Tab. 2). Changes in skeletal staining using the alcian-alizarin method was observed in 47 Group E foetuses (72.3%) and in 38 Group C foetuses (62.3%) (Tab. 1). In the examined population, reduced mineralisation was the most common development variant which morphologically corresponds to a reduction in, or a lack of alizarin staining. The frequency of the Group E foetal skeletal development variants, whose mothers received caffeine, was significantly higher compared with Group C, the control group ($p < 0.01$).

Table 1. Total number of foetuses, number of stained preparations and number of foetuses with variations in the groups exposed to caffeine (Group E) and control groups (Group C)

	C		E	
	No. of foetuses	No. of litters	No. of foetuses	No. of litters
Total No. of foetuses[a]	81	10	85	10
No. of stained preparations	61	10	65	9
No. of foetuses with variations	38	6	47	8

In the cranial region, a reduction in, or a lack of staining was observed most frequently in the parietal, interparietal and supraoccipital bones. In the case of parietal bones, mineralisation abnormalities always had a two-sided character. A reduction, as well as a lack of staining, was only observed in foetuses whose mothers were administered

caffeine. Of the examined foetuses, 13.8% had a reduced, while 7.7% had a total lack of staining of the parietal bone. Also, significant statistical differences ($p < 0.01$) were observed in the staining of the interparietal bone between the two groups. In Group E, 10.8% of the foetuses had a reduced, while 63.1% had no staining of this bone. Similar differences ($p < 0.01$) were found with the staining of the supraoccipital bone; in Group E 4.6% of foetuses had reduced while 53.8% had no staining (Tab. 2).

Significant statistical differences between the two groups ($p < 0.01$) were observed in the mineralisation of the individual foetal sternal segments. Lack of staining was only found in the Group E foetuses (60%) whilst in 6.2% a reduction was observed; in 3.1% of foetuses other morphological abnormalities of the sternal segments were of the asymmetric, split and vestigial type (Tab. 2).

In the Group E foetuses, mineralisation abnormalities of the metacarpal, metastarsal and phalangeal bones were also observed more frequently ($p < 0.01$). In Group E, 29 cases (44.6%) showed no metacarpal bone mineralisation, while in Group C there were no such changes. In both groups, there was a lack of ossification in individual metacarpal bones (21 Group C and 33 Group E foetuses) (Tab. 2).

A lack of staining of all distal phalanges in the front paws was significantly more frequent in Group E foetuses than in Group C ($p < 0.01$). In Group E, These changes were observed in 41 (63.1%) Group E and 15 (24.6%) Group C foetuses, respectively (Tab. 2).

Mineralisation abnormalities were also observed in the pelvic girdle and in the bones of the long hind legs. A lack of staining of the pubic and ischium bones, as well as the shortening and widening of the diaphysis in the femur, tibia and fibula occurred in 21 (32.3%) Group E cases.

In both groups of animals, there was no ossification in the different metatarsal bones. However, significantly, the lack of mineralisation was more frequent in Group E foetuses (31 cases, 47.7%) (Tab. 2).

A lack of staining of all distal phalanges in the hind paws was significantly more frequent in Group E ($p < 0.01$). These changes were observed in 35 (53.8%) Group E and 13 (21.3%) Group C foetuses (Tab. 2).

Mineralisation abnormalities of the remaining axial skeletal structure were less frequent and were not statistically significant (Tab. 2).

10 femurs from each group, from male foetuses selected at random from each litter, were selected for calcium and potassium content assessment. Figure 2 shows sample images and X-ray spectra at the proximal end of the primary ossification centre of the femoral diaphysis for each group. In the study, a significant reduction in calcium (47.25%) and an increase in potassium (52.75%) in Group E foetuses ($p < 0.01$) were observed (Fig. 3). The average calcium and potassium content in the bones of Group C foetuses was 61.91% and 38.09%, respectively. In addition, it should be emphasised that the surface distribution of potassium in the test bone samples was uniform, in contrast to the non-uniform distribution of calcium (Fig. 2).

Caffeine consumed by a living organism has the effect of increasing calcium excretion in urine, reducing the levels of total calcium, osteocalcin and alkaline phosphatase in the plasma, as well as inhibiting the proliferation and/or induction of apoptosis in osteoblasts. This uncontrolled lingering pathological process reveals itself through decreased bone mineral density, leading to an increased risk of bone fractures and the development of osteoporosis in the future [23, 24].

Given the fact that caffeine passes through the placental barrier, it may impair the development of many tissues – including bone tissue [16–19, 25, 26].

Research conducted by the authors [22] demonstrated the effect of caffeine on prenatal development in Wistar rats by administering it to pregnant females at a dose of 30 mg/day

Table 2. Incidence of developmental defects and variations in the groups exposed to caffeine (Group E) and control groups (Group C)

Developmental defects and variations in rat foetuses		C		E	
		No. of fetuses	*No. of litters*	*No. of foetuses*	*No. of litters*
parietal bone[b]	reduction of alizarin staining	1	1	9	5
	lack of alizarin staining	-	-	5	5
interparietal bone	reduction in staining	7	4	7	5
	lack of alizarin staining	21	6	41*	7
supraoccipital bone	reduction in staining	-	-	3	3
	lack of alizarin staining	8	2	35*	5
13th rib- wave[b]		-	-	2	2
segments of the sternum	lack of alizarin staining	-	-	39*	6
	reduction of alizarin staining	4	3	6	3
	other varieties[c]	-	-	2	1
metacarpal bone- lack of alizarin staining[b]	all	-	-	29*	5
	four	2	2	3	2
	three	3	2	3	2
	two	15	4	18	4
	one	1	1	9	2
distal phalanges the front paws- lack of alizarin staining[b]	all	15	4	41*	7
	four	-	-	4	2
	three	-	-	6	2
	two	5	3	12	4
	one	-	-	3	2
pubic bone- lack of alizarin staining		-	-	21*	3
ischium- lack of alizarin staining		-	-	21*	3
femur- shortening and extension shaft[b]		-	-	21*	3
tibia- shortening and extension shaft[b]		-	-	21*	3
fibula- shortening and extension shaft[b]		-	-	21*	3
metatarsal- lack of alizarin staining[b]	all	5	3	31*	5
	four	2	2	3	2
	three	2	1	2	2
	two	7	3	8	3
	one	-	-	2	1
distal phalanges the back paws- lack of alizarin staining[b]	all	13	4	35*	6
	four	2	1	3	2
	three	-	-	2	2
	two	2	1	3	2
	one	-	-	7	2

Single foetus may be represented more than once in the above statement
[a] No. of foetuses born alive
[b] Single- or double-sided
[c] Asymmetrical, split and residual
*Significant changes compared to control group

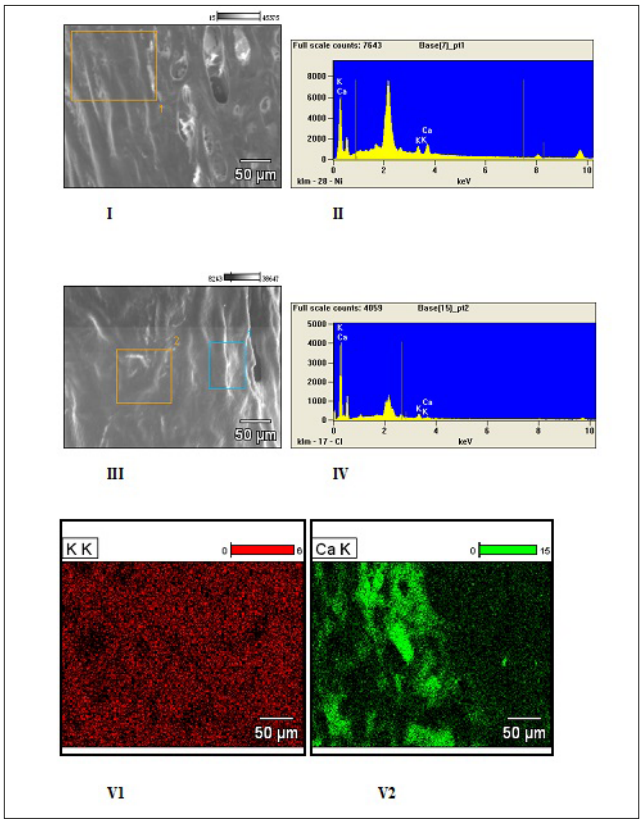

Figure 2. I: The surface of the proximal end of the original nucleus of ossification in the femoral diaphysis of the offspring from the control group (Group C) (SEM, x200). II: X-ray spectrum of the field marked in orange; in the present case the percentage of calcium and potassium on the K energy level is respectively 74,99 i 25,01%. III: The surface of the proximal end of the original nucleus of ossification in the femoral diaphysis of the offspring from the group receiving a solution of caffeine (Group E) (SEM, x200). IV: X-ray spectrum of the field marked in orange; in the present case the percentage of calcium and potassium on the K energy level is respectively 48,71 i 51,29%. V: The surface distribution of potassium (V1) and calcium (V2) in the sample

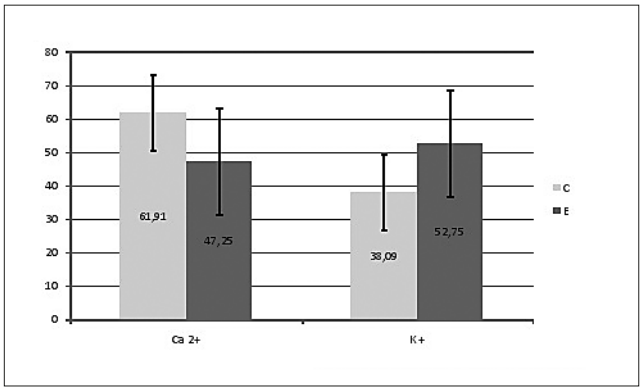

Figure 3. The percentage of the average content of calcium (Ca^{2+}) and potassium (K^{+}), together with a standard deviation in the fetuses femur between groups of animals receiving water (Group C) and caffeine (Group E) ($p<0.05$).

at 25 °C. It was shown that administering caffeine to pregnant females causes foetal bone mineralisation abnormalities. The changes mostly affected the cranial (interparietal bone) and metacarpal bones, the distal phalanges of the front paws and the sternum. An elemental composition analysis of the foetal bones showed that the level of calcium was significantly reduced (53.65%), while that of potassium was raised (46.35%) in the Group E females.

A comparison with the results of the research during which both water and caffeine at 25 °C was administered to pregnant females, in the presented study the following was noted: a reduction in the number of foetuses per litter and an increase in bone malformations (hind paws), both in the experimental group as well as in the control group. It should be emphasised that in the group of foetuses whose mothers received water at 10 °C there was an additional increased incidence of skeletal development variants, compared with the foetuses from mothers who received water at 25 °C [22].

The observed changes in the development of the foetal bone tissue are not only due to the action of the administered caffeine on the pregnant females, but also to the solution's low temperature (thermal stress). In the available literature, only one study was found which researched the effect of internal thermal stress on the metabolism of caffeine [1]. This study was conducted on healthy, non-smoking men and women of similar age and physical fitness. All subjects participated in endurance tests following a previous balanced diet (average daily caffeine intake – 68.9 mg). On the basis of the study, it was found that physical activity does not affect: absorption, distribution, metabolism and elimination of caffeine, with the appropriately maintained environmental and dietary factors.

CONCLUSIONS

On the basis of these results it can be concluded that caffeine in high doses disturbs the development of bone tissue. An additional factor which enhances the adverse effects of this substance on bone tissue is the temperature of the administered solution.

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