

Selected morphological and mechanical features of the femur of pregnant rats after exposure to caffeine applied at various temperatures

Caffeine in the nutrition of pregnant rats

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Abstract: It was assumed that the temperature at which caffeine is consumed may cause changes in the mechanical and morphological properties of bones and affect the bone metabolism of pregnant female. The aim of the study was to assess the effect of caffeine used at different temperatures on selected morphological and mechanical properties of the femur as well as biochemical indicators of bone formation in female rats in pregnancy. It was used test solution at the temperature 10, 25 and 45°C from 8 to 21 day during pregnancy, once a day. It was found that the temperature of administered caffeine may have an effect on changing the morphometric properties and on the bone metabolism of pregnant female rats. The application of caffeine solution, was administered at 10°C caused the most constrained growth of the femur and weakened resistance to load, and caused increased susceptibility to cracks. Analysis of bone metabolism indicators showed that caffeine administered in the form of a solution at 10°C and 25°C caused the most negativity effects for bone formation and bone turnover indicators. The administration of caffeine at 10°C causes the largest negative changes in bone morphological and strength indicators and hasn't a beneficial effect on the bone metabolism of pregnant female rats.

Key words: 1,3,7-methylxanthine, bone metabolism, bone rigidity.

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Introduction

Caffeine (1, 3, 7-trimethylxanthine) is the most known psychoactive substance in the world. This compound is present in coffee, tea, cola and energy drinks, as well as products containing cocoa. Consumers can eat caffeine can be in different temperatures, which can potentially affect the metabolism of this substance in the body and its influence on bones metabolism. Due to caffeine's various biological effects, it's commonly used to combat infections and alleviate pain [1, 2]. On average, a cup of coffee contains from 80–150 mg of caffeine, in tea is up to 90 mg, but it depend on the type of raw material and the method of its preparation. It is estimated that the average daily intake of this alkaloid per people is 3–7 mg/kg body weight, which corresponds to about 200 mg of pure substance. LD50 for caffeine in humans is 10 g [3]. Factors affecting the pharmacokinetics of caffeine are: age, gender, medications taken, genetic factors, pregnancy and smoking. The large variety of products containing caffeine makes this substance a frequent component of human diet, including pregnant women. Literature data shows that in Europe women consume much more caffeine (3.4–5.8 mg/kg body weight), than in the USA (1 mg/kg body weight) [1]. Pregnant women are recommended not to consume caffeine in excess of 300 mg/day. Bigger doses may lead to fetal cardiovascular dysfunction, damage to the fetal circulation and placenta, disruption of cell respiration in cardiomyocytes and calcium metabolism of the mother and child. Moreover, newborns can be born with low birth weight. This is due to the fact, that caffeine easily penetrates the placenta, inhibits the activity of growth hormone and by negatively effect on cAMP can damage fetal cell development. Caffeine also blocks adenosine receptors and increases the sensitivity of cells to hypoxia. Excessive caffeine consumption increases the level of catecholamines in pregnant women and blocks adenosine receptors, which leads to depleted blood flow through the placenta and makes cells more prone to hypoxia [4]. Caffeine negatively affects the skeletal system through increasing the expression of corticosteroid receptors in osteoblasts that induce a pathological acceleration of osteoporosis process and slower bone tissue repair. The temperature range at which caffeine can be consumed is 4–60°C, therefore it seems reasonable to examine whether temperature can affect the bioavailability, pharmacodynamics and toxicity of this substance. There are few reports in the available literature about the correlation between the temperature of consumed caffeine and its influence on bone metabolism and morphometric bones parameters, characterizing both the mother and the fetus [5].

It was assumed that the temperature at which caffeine is consumed may cause changes in the mechanical and morphological properties of bones and affect the bone metabolism of pregnant female. The aim of the study was to assess the effect of caffeine used at different temperatures on selected morphological and mechanical properties of the femur as well as biochemical indicators of bone formation in female rats in pregnancy.

Materials and Methods

Animals

One hundred and fifty healthy ten-week old female Wistar rats CRL:(WI)WUBR with average BW were randomly divided into six groups. The housing of animals and environmental conditions in animal laboratory were subject to current regulations [6]. After a 2-week acclimatization period, 216 the virgin females with a body mass of 250–270 g mated overnight with the males in a ratio of 6:2. The vivarium room with animals was air-conditioned (temperature $22 \pm 2^{\circ}\text{C}$) with an average humidity between 45% and 47%, and 12/12 h light/dark cycle (light period: 7 a.m.–7 p.m.). The study was conducted in compliance with international guidelines [7–9] with approving by the Local Ethics Committee (2/2007). During the experiment, all animals were kept in an identical environmental conditions with free access to fresh water and a standard diet fed (Agropol S.J., Motycz, Poland) *ad libitum* [10]. The basal diet included crude protein 14.5%, carbohydrates 64%, crude fat 1.5%, crude fiber 5%, ash 10%, calcium 1.10% and inorganic phosphorus 0.70%.

Assessment of fertilization efficiency and distribution of female rats into groups

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 8:00 a.m. The day of fertilization was considered to be the first day of pregnancy. General clinical observation was checked at least three times a day. Fertilized female rats were divided into 6 groups (25 in each). Within the group, rats were allocated to 5 repetitive subgroups (5 in each). Two rats were kept in the one cage. Three control groups — without caffeine (C-10, C-25 and C-45) and three experimental groups — with caffeine (CF-10, CF-25 and CF-45) were created. The differentiating factor was the temperature of the caffeine solution in the experimental groups and Tween 80 in the control groups. The applied solutions had a temperature of 10, 25 and 45°C , respectively.

Preparation, determination of the dosage and application of the test solution

Caffeine of 99% purity (anhydrous powder, Sigma-Aldrich Chemie GmbH, Germany) was used in the experiment. A dose of 120 mg/kg was used, it's according to the literature, disturbs the prenatal development of the rat [11–12]. The experimental factor solution was prepared by triturating caffeine powder with Tween 80 (Sigma-Aldrich Chemie GmbH, Germany) and then suspended in sterile distilled water. Experimental female rats were given intragastrical a suspension of caffeine in an

amount of 2 ml/kg body weight, once a day, from the 8th to the 21st day of pregnancy. In control groups, females were doused with an analogous amount of Tweed 80 solution at the same time as caffeine. The applied dose of caffeine solution was adjusted to the body weight of rats achieved by the animals at the beginning of each week of the experiment.

In vivo animal assessment, feed and water consumption analysis

Female body weight was assessed on the fertilization day and after this parameter was monitored daily, as was feed and water consumption. The amount of caffeine consumed during the experiment by each group was also assessed.

Euthanizing animals and taking samples for analysis

On the 21st day of pregnancy blood was taken for evaluation of bone turnover indicators. Blood samples were taken from caudal vena cava. Then euthanasia was carried out according to the adopted procedure. Female rats were decapitated using a laboratory guillotine. Death occurred by breaking the spinal cord's continuity without compromising the continuity of the outer shells. The right femur bone was posthumously collected for examination. The femurs obtained were cleared of soft tissue. After morphometric measurements, the bones were stored in a frozen state until the morphometric and strength parameters were assessed.

Biochemical evaluation of serum

The bone formation process was assessed by analyzing the serum level of OC, CPX-I and ALP (EC 3.1.3.1) activity. OC concentration was measured using Rat OC/BGP ELISA Kit (Elabscience, Biotechnology Co., Ltd, Houston, Texas) and CPX-1 was analyzed using Rat C-telopeptide of type I collagen (Kamiya, Biomedical Co, Seattle, USA), using immunoassay tests Elisa. Serum ALP activity was determined using kits from Cormay (Poland). As part of the analysis of bone resorption markers in the blood, Ca was determined by colorimetric method (Cormay, Poland) and hormones determining its level in blood by Elisa immunoassay. PTH levels were assessed using Rat I-PTH ELISA Kit and Ct using Rat Ct ELISA Kit (Elabscience, Biotechnology Co., Ltd, Houston, Texas).

Morphometric and mechanical measurements of the femur

The evaluation of morphometric parameters of the femur included their mass, width, length and relative femur mass. The femur length was evaluated by measuring the furthest extremities of the bone. The width of the femur was measured at half of the

bone core. Measurements were made using a vernier caliper. Relative femur weight was measured as the femur to rat mass ratio multiplied by 100%. To determine a femur mass was used a laboratory balance. The maximum load (F max) on the femur was tested using a three-point bending flexural test. Tissue properties were analysed on the basis of a modulus of longitudinal elasticity (E-Young's modulus) [13]. Bones for strength testing were stored frozen at -15°C . After defrosting, each bone was placed on a special holder to apply perpendicular loads at a speed of 2 mm per minute, until it broke. The machine was equipped with a 2500 N load cell (accuracy 0.01%). This test is based on the assumption that the femur supported at both ends and loaded in the middle part of the stem bents within a specified range of deformation and broken. Femur resilience (L), modulus of rupture were also evaluated (MR) have been measured. All linear measurements were made using Lloyd LRX, a materials testing machine manufactured by Lloyd Instruments. Parameters describing the mechanical properties of the femur were calculated in the Nexygen program.

Statistical analysis

The obtained data was verified by one-way ANOVA assay, and significant differences between groups were determined by Tukey's multiple range test. Data variability was expressed as the pooled standard error of the mean (SEM). The differences were considered significant at $P \leq 0.05$. The Statistica software package version 10 (StatSoft Inc., 2011) was used for statistical calculations.

Results

No clinical symptoms of caffeine toxicity were observed among the examined animals. The initial body weight of pregnant females used in the experiment ranged from $286.56 \text{ g} \pm 41.38$ for the CF-25 group to $322.17 \text{ g} \pm 28.23$ for the CF-10 group. The final body weight analysis of the CF-10 rats showed that after 2 weeks of caffeine consumption, this trait was significantly lower ($P = 0.04$) than in the females from the C-10 control group. At the same time, the body weight gain of females in this group (CF-10) was significantly smaller ($P = 0.047$) than in the control (C-10). Analysis of caffeine intake (Table 1) showed that during the 2-week application of experimental factor, pregnant rat females from the CF-25 group and CF-45 have received the higher ($P = 0.44$) dose of caffeine compared to CF-10.

It was calculated the highest dose of caffeine per 1 kg of body weight was received by pregnant female rats treated with a solution of this xenobiotic at 25°C (CF-25), the strongest most negative changes were seen in rats receiving a solution of 10°C (CF-10) caffeine. Body weight analysis of pregnant females from the CF-25 and CF-45 groups did not differ significantly from the results of the control groups (C-25 and C-45,

Table 1. Calculated total caffeine intake during experiment (mg), per 1 kg of body weight.

Item	Groups			P-value
	CF-10 n = 25	CF-25 n = 25	CF-45 n = 25	
Total caffeine intake (mg/head/week)				
— first week of experiment, without application of caffeine	—	—	—	—
— second week of experiment	264.84 ^b	270.33 ^{ab}	275.66 ^a	0.049
— third week of experiment	268.02 ^b	286.06 ^a	279.34 ^{ab}	0.047
Total intake of caffeine during experiment (mg/head)	532.86 ^b	556.39 ^a	555.0 ^a	0.044

^{a, b} — mean values that do not share common superscript letter differ significantly for $P \leq 0.05$.

respectively, all $P > 0.05$). Weight gain during the 21 days of the experiment was definitely lower ($P = 0.027$) in females from the CF-25 group than in the control (C-25). Administration of a 45°C caffeine solution for 21 days of the experiment resulted in significantly less weight gain ($P = 0.039$) in rat females in the CF-45 group than in control (C-45). Analysis of the blood of pregnant females showed that the application of caffeine solution at 25°C resulted in a decrease in the OC level ($P = 0.045$), an increase in CTX-I ($p = 0.035$) and bone resorption markers: Ca ($P = 0.026$), PTH ($P = 0.041$) and Ct ($P = 0.038$), compared to the results of the control group (C-25). An increase in ALP activity ($P = 0.047$) relative to the control group was also noted (Table 2).

The consumption of 45°C caffeine solution by pregnant female rats caused a significant decrease in the concentration of biochemical markers of bone resorption: Ca ($P = 0.047$), PTH ($P = 0.028$), Ct ($P = 0.037$), compared to the results of the control group (C-45) receiving only Tween 80. The results of the analysis of individual characteristics and mechanical properties of the femur are presented in Table 3.

Obtained data showed no significant difference from the results obtained by Chovancova [14]. However, intragastric administration of caffeine at 10°C (CF-10) for 2 weeks resulted in a decrease ($P = 0.03$) in femur length and Young's modulus ($p = 0.017$) compared to the control group (C-10). The analysis of the selected parameters used to describe the mechanical properties of the femur based on the maximum load to determine the breaking point of the femur F max. ($P = 0.04$) showed significantly lower values for the resilience and the modulus of rupture MR ($P = 0.04$) in the group of CF-10 administered caffeine at 10°C. Higher solution temperatures (CF-25 and CF-45, respectively) didn't affect significant the described parameters relative to the control group (C-25 and C-45; all $P > 0.05$).

Table 2. Biochemical indices in the rats' female blood receiving caffeine at 10, 25 and 45°C.

Item	C-10 n = 25	CF-10 n = 25	P-value	C-25 n = 25	CF-25 n = 25	P-value	C-45 n = 25	CF-45 n = 25	P-value
Ca mmol/L	3.76 ^a ± 0.66	1.52 ^b ± 0.56	0.006	3.12 ^a ± 0.35	0.35 ± 0.41	0.026	3.98 ^a ± 0.23	1.67 ^b ± 0.35	0.047
ALP U/L	148.0 ^b ± 2.34	174.0 ^a ± 1.87	0.031	144.0 ^b ± 2.12	161.0 ^a ± 2.65	0.047	145.0 ± 2.54	142.0 ± 3.1	0.052
OC ng/mL	3.79 ^a ± 0.21	3.05 ^b ± 0.44	0.047	3.77 ^a ± 0.87	3.15 ^b ± 0.47	0.045	3.58 ± 0.47	3.54 ± 0.24	0.52
CTX-I ng/mL	0.041 ^b ± 0.001	0.059 ^a ± 0.002	0.027	0.039 ^b ± 0.002	0.054 ^a ± 0.001	0.035	0.041 ± 0.002	0.044 ± 0.001	0.58
Ct ng/mL	2.77 ^b ± 0.16	3.33 ^a ± 0.33	0.042	3.00 ^a ± 0.96	2.01 ^b ± 0.21	0.038	3.46 ^a ± 0.48	2.83 ^b ± 0.87	0.037
PTH ng/mL	3.61 ^b ± 0.26	4.06 ^a ± 0.07	0.036	3.95 ^b ± 0.43	5.68 ^a ± 0.45	0.041	4.16 ^b ± 0.17	5.88 ± 0.14	0.028

^{a, b} — mean values that do not share common superscript letter differ significantly for P ≤ 0.05.

Table 3. Selected individual characteristics and mechanical properties of the femur in pregnant female rats exposed to 10, 25 and 45°C caffeine solutions.

Item	C-10 n = 25	CF-10 n = 25	p-value	C-25 n = 25	CF-25 n = 25	p-value	C-45 n = 25	CF-45 n = 25	p-value
Femur mass [g]	1.07 ± 0.14	1.04 ± 0.16	0.64	0.97 ± 0.15	0.88 ± 0.22	0.94	0.99 ± 0.14	0.87 ± 0.27	0.33
Relativ femur mass [%]	0.34 ± 0.05	0.3 ± 0.06	0.16	0.2 ± 0.04	0.2 ± 0.07	0.4	0.3 ± 0.05	0.26 ± 0.08	0.2
Femur length [mm]	33.3 ^a ± 4.18	30.5 ^b ± 3.24	0.03	29.8 ± 1.72	30.0 ± 2.59	0.84	29.36 ± 2.8	27.99 ± 4.1	0.54
Femur width [mm]	3.26 ± 0.36	3.1 ± 0.27	0.59	3.3 ± 0.34	3.1 ± 0.28	0.16	3.28 ± 0.19	3.17 ± 0.23	0.49
F max [N]	121.1 ^a ± 15.21	108.2 ^b ± 16.05	0.04	112.0 ± 7.19	117.0 ± 15.13	0.4	100.2 ± 11.9	104.7 ± 16.18	0.51
E [MPa]	2023.1 ^a ± 211.6	1897.5 ^b ± 251.5	0.02	2000.6 ± 285.7	2060.6 ± 335.9	0.8	2045.2 ± 341.5	1928.7 ± 255.7	0.28
L [J]	0.006 ± 0.002	0.005 ± 0.002	0.59	0.005 ± 0.002	0.006 ± 0.002	0.9	0.005 ± 0.003	0.004 ± 0.002	0.75
MR [MPa]	7.71 ^a ± 0.968	6.888 ^b ± 1.022	0.04	7.14 ± 0.46	7.45 ± 0.96	0.4	6.42 ± 0.76	6.67 ± 1.03	0.51

^{a, b} — mean values that do not share common superscript letter differ significantly for P ≤ 0.05.

Discussion

The skeletal system in a pregnant female has to be prepared for increased mechanical loads due to the increased weight of the female and the weight of the fetus. The literature describes many environmental and mother-specific factors that affect the adaptation of the skeletal [15, 16]. These include, for instance, the mother's levels of hormones and diet. There are no unambiguous reports on the impact of pregnancy on the appearance of bone tissue disorders, including osteoporosis, later in life. Many authors [17, 18] would argue that pregnancy has no impact on future osteoporotic fractures. According to Chen *et al.* [19] maternal caffeine intake during pregnancy is associated with risk of low birth weight of the child. Our results shown that biochemical bone metabolism markers in blood and morphometrical properties of femur bones after application of caffeine were significantly changed. In the present study, in the group of pregnant female rats fed with solution of caffeine at temperature 10°C observed the smallest length of the femur connect with a reduction of F max and MR. This could suggest that it is this temperature range that has the strongest impact on bone morphology. According to Nakamoto *et al.* [20] and Schneider *et al.* [21] caffeine in doses 10 mg/kg body weight interferes with fetal rat skeletal development. In turn the organic phase of bones is responsible for bone elastic properties, it contributes to overall bone integrity and provides structural scaffolding to the inorganic matrix [22]. The studies of Heaney [23] shown that caffeine and the other methyl xanthines can negatively act on a different tissues, generally by interfering with the action of phosphodiesterase and thereby potentiating the activity of agonists acting through the adenylate cyclase-cAMP pathway. The latest studies show that caffeine may disturb maternal physiology and also development of the fetuses [23]. Tomaszewski *et al.* [24] tested three caffeine solutions (30 mg/day) in different temperatures: 10, 25 and 45°C. They demonstrated a significant relationship between the solution's temperature and caffeine's developmental toxicity administered to pregnant female rats. The results of its adverse effect were developmental abnormalities such as hematomas and saturated bleeding in the internal organs, which occurred in fetuses of female rats that received caffeine at 10°C or 45°C [24]. In turn Adams *et al.* [25] estimated that the consumption of less than 180 mg of caffeine after the 16th week of pregnancy of human has no impact on the offspring's weight [25]. According to this author the intake of 300 mg of caffeine has no significant effects on the birth weight compared to the control group. Research Gronbaek *et al.* [26] and Turan *et al.* [27] showed that for the reduction of femur length and stiffness (modulus of elasticity determined in the tensile test) of females of rats can be caused by reduced production of IGF-1 (somatomedin C) and growth retardation. Young's modulus is considered an indicator of material index of bone rigidity. In the presented studies, its value was significantly different in the bones of female rats receiving caffeine at 10°C, than in the

other groups. It can therefore be assumed that caffeine administered at 10°C may be a factor predisposing bones to deformation under the influence of external forces. In addition, this effect may be associated with a decrease in the level of calcium and osteocalcin in the blood, which may indicate changes in the mineral phase or the structure of the organic matrix of bones [28]. Huang *et al.* [16] have shown that there is no evidence to support the claim that caffeine disrupts bone homeostasis provided that sufficient calcium is available in diet. In turn Tsuang *et al.* [29] confirmed the negative effects of caffeine on development and functioning osteoblasts in rats. They demonstrated that caffeine does significantly reduce the lifespan, and stimulates the apoptosis of osteoblasts. According to Ribeiro-Alves *et al.* [30] use of hormone oral contraceptives blunts the acute increase in urinary excretion of calcium induced by caffeine in adult women. The oral contraceptives were considered to influence the metabolism of caffeine by reducing paraxanthine urinary excretion and therefore stimulating calcium excretion, possibly limiting physiological effects of calcium. These findings are particularly relevant for women with low calcium intake who need to preserve minerals essential for bone health [24]. Caffeine has been shown to have a negative effect on the excretion of calcium and magnesium in urine was demonstrated in people with insufficient dietary calcium intake [18]. In middle-aged population (35–49 years) Meyer *et al.* [18] was found an increased risk of fracture was found in women who drank more than 9 cups of coffee per day. At the same time they were people in the blood whose reduced calcium level was found and low level of estrogen [17]. Large doses of caffeine could be increased risk of fracture, especially in people with low calcium intake [22, 25, 31]. Vajda *et al.* [32] were observed changes in bone structure and mass (cortical and cancellous) with mechanical properties during and after their first reproductive cycle in rats [32]. During the final stage of pregnancy they observed increased periosteal formation of cortical bone and its increased volume and suppressed of synthesis of cancellers part processes. At the same time the mechanical properties of bones, both cortical and cancellers, did not change. The literature [32–34] has reports of osteoporosis during pregnancy and post-childbirth osteoporotic fractures. It is assumed that pregnancy might reveal, rather than cause, low bone mass.

Pregnancy leads to postural changes (hyperlordosis), which, combined with small but chronic loss of bone mass, can damage the weakened bones. The pregnancy has effects on the bone and the effect is particularly clearly marked for modulus of rupture. According to Currey *et al.* [35] the modulus of rupture is not an exact measurement of tensile strength when a material undergoes plastic deformation. It is most likely that the thinning of the walls may in itself have an effect in reducing the calculated modulus of rupture, apart from any changes in the material properties. According to Alghadir *et al.* [36] physiological pregnancy (including a healthy mother) does not significantly affect the mechanical (length and mass) properties of the child's femur [36]. Osteoporosis is often diagnosed during pregnancy on the

occasion of back pain, but such pain can be caused by ligament lengthening or hyperlordosis [34]. Philips *et al.* [33] analyzed the cause of back pain in pregnant women to examine women diagnosed with osteoporosis during pregnancy [33]. Women had back pain and vertebral compression fractures, while and they were complained about hip pain. The densitometry tests were performed up to 8 years following pregnancy. Body weight of women after the childbirth (up to 6 months) was low but it increased gradually to reach the lower limit of the norm. This reversible loss of bone mass was caused by pregnancy itself, but pre-existing osteopenia was also possible. What have fundamental importance for the evaluation of the mechanical properties of bones are the parameters related to loads that cause elastic deformations. During standard physical activity bone tissue is under stress that is below the yield point. Such elastic deformations can be reversible provided that the external forces that cause the deformation no longer apply. The mechanical properties of the bone at yield point are crucial as beyond that point the bone is permanently deformed, which can lead to microdamage. Osteocalcin is considered as a bone formation marker [37]. The caffeine solution administered at 10°C was the most significant affect to reducing the level of this non-collagen protein and the penetration of C-terminal telopeptides of type I collagen, what causes a bone damage. Due to this, it can be assumed that therefore, they caffeine theoretically exerts effects directly on the cellular apparatus controlling bone remodeling. According Li *et al.* [38] bone mineral phase, in forms of hydroxyapatite crystals, contributes to bone fracture strength and stiffness. It is important to note that in the study the femur was exposed to forces greater than 100 N, which can be reasonably expected to never apply to animals under normal conditions. The utilized statistical model, too, does not fully account for the forces that apply to a specimen (including the point of application, torsion forces, and bone joints).

According to the experiment scenario, we evaluated whether caffeine affected the mechanical properties of the femur (bone tissue), including in particular parameters at yield point in pregnant female rats, by modulating metabolism. During lactation, cortical and cancellous bone strength decreased, and bone volume was reduced. There was also an increase in bone turnover in cancellous bone and on the internal surfaces of the cortical bone. Following lactation, the mechanical properties of bone tissue either completely or partially returned to normal. In study by Zeni *et al.* [39] was observed that pregnancy have little impact on morphological and mechanical properties female rats bones, while lactation caused a significant loss of bone tissue and in trabecular part in particular. According this authors [39] during pregnancy bone mineral content and bone skeleton size were increased but bone mineral density did not change. In turn completely different developments were observed during the 23rd day of lactation. No changes were found in skeleton size, only a small decrease in bone mineral content, and a significant decrease in bone mineral density. Even though bone mineral content and bone mineral density were similar on the date of birth, at the end of lactation bone mineral content was

12% lower and bone mineral density 4.9% lower in the latter group. During lactation the greatest negative changes were found in trabecular areas of bone, while only insignificant changes were observed in the cortical part of bone. According to Dew *et al.* [40] among women however, in whom calcium balance performance is impaired, high caffeine intake may predispose to cortical bone loss from the proximal femur. Heaney [23] believes there is no evidence that caffeine has any harmful effect on bone status or on the calcium economy in individuals who ingest the currently recommended daily allowances of calcium. According to Heaney [23] in the intestinal mucosa, caffeine may cause absorption interference associated with weakening of the calcium transport system. In addition, it can also destroy the surface of enterocytes, which, according to this author, can explain the increase in skeletal fragility, in addition to affecting the calcium metabolism itself.

Scientific studies show that frequent consumption of caffeine plays a role in the development of diabetes [41]. Folwarczna *et al.* [42] verified these hypothesis in her research. They founded that diabetes influences osteoporosis and consuming caffeine is one of the factors that accelerate the process. Effects of orally administered caffeine (20 mg/kg daily for four weeks) were examined in three-month-old diabetes female Wistar rats. Two weeks before the start of caffeine administration rats received streptozotocin (60 mg/kg, intraperitoneally) alone or streptozotocin after nicotinamide (230 mg/kg, intraperitoneally). In this study bone turnover markers, mass, mineral density, histomorphometric parameters, and mechanical properties were examined. Results of research showed caffeine at a dose 20 mg/kg daily for four weeks did not exert a damaging effect on the skeletal system of diabetic rats [42].

Conclusions

The temperature of administrated caffeine may have an effect on changing the morphometric properties and on the bone metabolism of pregnant female rats. The administration of caffeine at 10°C causes the largest negative changes in bone morphological and strength indicators and hasn't a beneficial effect on the bone metabolism of pregnant female rats.

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Conflicts of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Abbreviations

ALP	— alkaline phosphates
C-10	— control group receiving a Tween 80 solution at 10°C
C-25	— control group receiving a Tween 80 solution at 25°C
C-45	— control group receiving a Tween 80 solution at 45°C
Ca	— calcium
cAMP	— cyclic adenosine monophosphate
CF-10	— group receiving a caffeine solution at 10°C
CF-25	— group receiving a caffeine solution at 25°C
CF-45	— group receiving a caffeine solution at 45°C
CPX-I	— collagen degradation products C-terminal telopeptides of type I collagen
CRL:(WI)WUBR	— the strain of albino rats of Wistar
Ct	— calcitonin
I-PTH	— intact parathormone
OC	— osteocalcin
PTH	— parathormone

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