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EFFECTS OF CAFFEINE INTAKE IN MOTHERS ON MATERNAL CORTISOL LEVELS AND OFFSPRING ENDOCHONDRAL OSSIFICATION

Amanda Maria Sena Reis, Ana Cláudia Moura Batista, Natália de Melo Ocarino, Rogéria Serakides*
Universidade Federal de Minas Gerais. Brasil

Summary

The objective of the present study was to assess the effects of caffeine intake in mothers on maternal cortisol levels and on endochondral ossification of the offspring after birth. A total of 38 Wistar rats were analyzed, including a control group and three treated with caffeine at doses of 25, 50 and 100 mg/kg throughout pregnancy and lactation. Maternal cortisol levels were measured by chemiluminescence in the morning period. Endochondral ossification of the offspring was assessed at 3 and 21 days of age by histomorphometry. Only the mothers in the group treated with 100 mg/kg of caffeine exhibited significantly higher cortisol levels in comparison to the control group. The offspring of mothers treated with 50 and 100 mg/kg doses of caffeine showed significant reduction in the length of their limbs and vertebral bodies. Bone histomorphometry at 3 days of age showed abnormalities at all doses of caffeine. The 21-day-old offspring of mothers treated with caffeine remained significantly lower in weight and long bone length compared at 3 days of age.

We conclude that caffeine does not stop endochondral ossification, but rather inhibits. In addition, the effects of caffeine were more detrimental to the cartilage growth of 3-day-old pups, and the frequency and intensity of the observed changes were dose-dependent.
Keywords: cartilaginous tissue, growth, caffeine, rats

Resumen

EFECTOS DE LA INGESTA DE CAFEÍNA MATERNA EN LOS NIVELES DE CORTISOL MATERNO Y EN LA OSIFICACIÓN ENDOCONDRAL DE LA PROLE

El objetivo del presente estudio fue evaluar los efectos de ingesta de cafeína sobre los niveles de cortisol materno y sobre la osificación endocondral de las crías. Se analizaron 38 ratas Wistar, incluyendo un grupo control y tres tra-

** Dirección Postal: Escola de Veterinária da UFMG. Av. Antônio Carlos 6627, Caixa Postal 567, campus Pampulha da UFMG, CEP 30161-970. Belo Horizonte, MG, Brasil.*
Correo electrónico: serakidesufmg@gmail.com (R. Serakides)

tados con cafeína 25, 50 y 100 mg/kg durante la gestación y lactancia. Los niveles de cortisol materno se midieron por quimioluminiscencia por la mañana. La osificación endocondral de las crías a los 3 y 21 días de edad fue evaluada por histomorfometría. Sólo las madres en el grupo 100 mg/kg de cafeína mostraron niveles de cortisol significativamente mayores en comparación con el control. Las crías de madres tratadas con cafeína 50 y 100 mg/kg mostraron una reducción significativa en la longitud de sus extremidades y cuerpos vertebrales. La histomorfometria ósea a los 3 días de edad mostró alteraciones en todas las dosis de cafeína. A los 21 días de edad, las crías presentan peso y longitud de huesos largos significativamente inferiores en comparación a los 3 días de edad. Se concluye que la cafeína no detiene osificación endocondral, sino que la inhibe. Además, los efectos de la cafeína fueron más perjudiciales en las crías de 3 días de edad, y la frecuencia y la intensidad de los cambios fueron dependientes de la dosis.

Palabras clave: tejido cartilaginoso, crecimiento, cafeína.

Introduction

Caffeine (1,3,7-trimethylxanthine) is a pharmacologically active xanthine present in coffee, tea, sodas, energy drinks, chocolates, cocoa based foods and drugs; thus, it is widely consumed in the human diet [1,2,3,4].

In pregnant women, caffeine crosses the placenta and is also conveyed through breast milk. Therefore, the consumption of this substance by the mother may pose a potential risk to the health of the fetus [5,6].

Although there are some beneficial effects of caffeine, there have been several reports of adverse effects on organisms [4,7,8,9,10].

Previous studies have shown that caffeine causes changes in the bone structure of fetuses and younger rats. In rat fetuses exposed to high doses of caffeine, teratogenic changes such as palatoschisis, malformations of the limbs, ectrodactyly[11,12] and reduced bone mineral content have been observed[7]. However, studies conclude that the dietary exposures of caffeine are not teratogenic or are directly responsible for an increased risk of spontaneous abortion or fetal growth retardation[13].

Demineralized bone fragments implanted into the subcutaneous tissue of growing rats treated with caffeine resulted in the inhibition of chondrogenesis and a reduction of the expression of collagen II, histone and TGF beta[14]. Thus, it has been postulated that high doses of caffeine can inhibit endochondral ossification in young rats[15].

Recently, the effects of prenatal exposure to caffeine at a dose of 120 mg/kg were investigated. The results demonstrated that caffeine inhibits bone growth, and this effect is associated with maternal exposure to glucocorticoids and the inhibition of IGF-1 expression in the epiphyseal plates[16].

Studies have shown that young, rapidly growing rats fed a diet supplemented with 20-40mg/kg caffeine exhibited abnormalities in bone tissue structures that were similar to those occurring with age, such as a smaller cross-sectional bone area with fewer osteocytes present; however, these studies failed to examine the effects of caffeine on cartilaginous tissue[8,17].

The objective of this study was to assess the effects of caffeine intake (different doses) in mothers on endochondral ossification after birth by histomorphometric analysis of the epiphyseal cartilage of 3-day-old rats and the articular cartilage and epiphyseal plates of various bones in 21-day-old rats. The histomorphometry studies were subsequently correlated with maternal serum cortisol levels.

Materials and methods

All experimental procedures were approved by the Institutional Ethics Committee in Animal Experimentation at *Universidade Federal de Minas Gerais* (UFMG) (protocol no. 177/2010).

Thirty-eight adult female Wistar rats were

used in this study. Animals from the same experimental group were housed at a density of six rats per cage on a 12-h light–dark cycle. The rats were fed commercial rat chow containing 22% crude protein, 1.4% calcium and 0.6% phosphorus. Food and water were provided ad libitum to all of the animals. After a 30-day adaptation period, the rats were randomly divided into four groups, three of which were treated with different doses of caffeine and a control group. The treated groups (10 animals/group) received daily caffeine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 5 mL of distilled water at doses of 25, 50 or 100 mg/kg. Doses were administered by an oral-gastric probe at the same time each day over the course of the experimental period. As a placebo, the control group (8 animals) received 5 mL of distilled water administered daily by oral-gastric probe. Rats from all groups were subjected to vaginal cytology to monitor the estrous cycle. Rats in estrus were kept in plastic cages with adult male rats for 12 h. After this period, vaginal smears were obtained daily to detect spermatozoa. Copulation was confirmed by the presence of spermatozoa in the vaginal cytology samples, and this day was considered Day 0 of gestation. After copulation, the females were kept individually in plastic cages. Animals in the treated and control groups continued to receive caffeine and water, respectively, by an oral-gastric probe throughout the duration of the experimental period (pregnancy and lactation).

Measurement of maternal levels of serum cortisol

After breastfeeding elapsed (21 days), the rats were anesthetized in the morning with ketamine (40 mg/kg) and xylazine (10 mg/kg), and blood was collected by intracardiac puncture. The blood was collected in tubes and serum was obtained and stored at -20° C for subsequent measurement of cortisol levels. The cortisol measurements were performed by chemiluminescence (Access Immunoassay System, Sanofi Diagnostics Pasteur, Inc., Chaska, MN, USA) using a fully automated system according to the manufacturer's recommendations and the kit instructions.

Necropsy and histological processing

Three days after birth, three pups from each litter were euthanized and both the right hind limb and the lumbar spine were collected for histopathological analysis. At weaning, three 21-day-old pups from each litter were euthanized; the left humerus, femur, and tibia, and the thoracic and lumbar vertebrae were collected for histomorphometric analysis. The bones were fixed in 10% formalin neutral buffer for 24 hours, decalcified in 21% formic acid for a period of 20 days with changes of the solution occurring every three days. Subsequently, the bones were processed using a routine paraffin inclusion technique. Histological sections (4 µm) were stained with hematoxylin–eosin (HE) before histological evaluation.

Weight and size of offspring

Weight measurements were recorded throughout the duration of the experimental period from birth to the end of weaning. The size of 3-day-old pups was measured as the distance from the neck to the base of the tail, and the length of the pelvic and thoracic members were also measured with a caliper. In 21-day-old pups, the length and width of the long bones (femur, tibia and humerus) were measured with a caliper[18].

Histomorphometric analysis of the bones of 3-day-old rats

The trabecular bone percentage was determined using histological sections of vertebrae and long bones of 3-day-old rats. The percentage of trabecular bone was determined in the bones located directly below the epiphyseal plate (i.e., the primary spongy bone) of two vertebral bodies in a random field of the lumbar spine and in a random field of the tibia

per animal. This analysis was performed with the aid of a 121-point graticule attached to the microscope underneath a 40x objective.

The thickness of the epiphyseal plates of two vertebrae/animal from the 3-day-old rats was determined at three equidistant points with the aid of an ocular micrometer on a 40x objective. The length of the vertebral bodies was measured for two vertebrae per animal with a 10x objective. The values were obtained using an eyepiece micrometer and were subsequently converted to mm using a micrometric slide.

In 3-day-old rats, the percentage of nuclei, the percentage of chondroblast gaps and the percentage of cartilaginous tissue matrix in the cartilaginous epiphysis of the distal femur and of the proximal tibia were determined with the aid of a 121-point graticule attached to the microscope under the 40x objective.

Histomorphometric analysis of the bones of 21-day-old rats

The percentage of trabecular bone was determined using histological sections of vertebrae and long bones from 21-day-old rats. The percentage of trabecular bone was determined in six random fields of primary spongy bone, in three vertebral bodies per animal in the thoracic spine 1-7, in the thoracic spine 8-13, in the lumbar spine 1-3, in the lumbar spine 4-6, and in primary spongy bone of the femur, tibia, and humerus. This analysis was performed with the aid of a 121-point graticule attached to the microscope under the 40x objective.

The thickness of the epiphyseal plates of three vertebrae bodies per animal from 21-day-old rats was determined at three equidistant points with the aid of a micrometer ocular on a 10x objective. In articular cartilage and the epiphyseal plates of femurs, tibias and humeri, the mean thickness was measured at 15 points distributed throughout their length with the aid of an eyepiece micrometer and a 10x objective. The length of the vertebral bodies was measured in three vertebrae per animal with a 4x objective. Values were obtained using an eyepiece micrometer and were subsequently converted to mm using a micrometric slide.

Statistical analysis.

Delineation was performed in a random manner and the mean and standard deviation were determined for each variable. We performed an ANOVA and compared the means using a Student-Newman-Keuls test. Differences were considered to be significant if $p<0.05$ [19]. For data analysis the GraphPad InStat software version 3.05 was used.

Results

Measurement of maternal levels of serum cortisol

The group control exhibited mean and standard deviation of serum cortisol of 0.486±0.089. The group treated with dose of caffeine 25 mg/kg exhibited mean and standard deviation of serum cortisol of 0.4625±0.0744. The group treated with dose of caffeine 50 mg/kg exhibited mean and standard deviation of serum cortisol of 0.555±0.1740. The group treated with dose of caffeine 100 mg/kg exhibited mean and standard deviation of serum cortisol of 0.7625±0.1847.

The groups treated with lower doses of caffeine (25 and 50 mg/kg) exhibited serum cortisol levels similar to the control group ($p\geq0.05$). However, concentrations of cortisol were significantly higher in the mothers treated with 100 mg/kg caffeine than in the control group.

Weight, size of offspring and histomorphometric analysis of the bones of 3-day-old rats

The weights of the offspring of rats treated with 50 and 100 mg/kg of caffeine were significantly lower than those of the control group throughout the experimental period. However, the weight of the offspring of rats treated

with 25 mg/kg caffeine was not different from those of the offspring of control rats (Table 1).

Similar to previously described weight results, the distance measured from the neck to the base of the tail and the length of the hind limbs of the 3-day-old offspring of the rats treated with 50 and 100 mg/kg of caffeine were significantly lower compared to the control group. No significant difference was observed between the 25 mg/kg caffeine-treated group and the control group (Table 2); however, it is interesting that the length of the forelimbs of 3-day-old rats was significantly lower in all the caffeine-treated groups when compared to the forelimb lengths of the control group (Table 2). The length of the spine of the 3-day-old offspring of the 100 mg/kg caffeine-treated rats was significantly lower when compared to the control group and the other caffeine-treated groups. However, no significant differences were observed among the 25 mg/kg caffeine-treated group, the 50 mg/kg caffeine-treated group and the control group (Table 2).

Epiphyseal plate and primary spongy bone of the tibia and vertebrae

The epiphyseal plate of long bones and vertebrae of 3-day-old offspring of rats treated with caffeine was poorly differentiated and different areas could not be distinguished from each other. In contrast, it was possible to visualize a well-differentiated hypertrophic columnar zone in the control group. In the offspring of the 100 mg/kg caffeine-treated rats, the epiphyseal plate cells were quite disorganized and were undifferentiated in all of the areas examined. The thickness of the epiphyseal plate of the 3-day-old offspring of the 50 and 100 mg/kg caffeine-treated rats

Table 1. Pup weight (g) of rats treated with different doses of caffeine and control rats from birth to weaning (mean±SD).

Days	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine100mg/Kg
Day 1	7,23 ± 0,62 A	7,38 ± 0,66 AB	6,53 ± 0,65 B	5,87 ± 0,96 B
Day 3	9,55 ± 0,77 A	9,19 ± 1,11 A	8,17 ± 1,75 A	6,84 ± 1,13 B
Day 5	12,65 ± 1,48 A	11,67 ± 1,46 A	9,85 ± 1,30 B	9,12 ± 1,07 B
Day 7	16,50 ± 2,05 A	14,63 ± 2,37 A	12,19 ± 2,04 B	11,06 ± 1,59 B
Day 9	18,89 ± 2,71 A	17,72 ± 2,87 A	14,58 ± 3,27 B	13,43 ± 1,35 B
Day 11	23,59 ± 3,08 A	21,50 ± 3,67 A	18,44 ± 1,83 B	16,26 ± 1,59 B
Day 13	26,46 ± 3,67 A	25,19 ± 4,91 A	21,61 ± 2,09 B	19,42 ± 2,46 B
Day 15	29,38 ± 4,80 A	28,01 ± 5,25 A	24,86 ± 2,44 AB	22,79 ± 2,68 B
Day 17	33,47 ± 5,64 A	31,08 ± 5,64 AB	27,55 ± 2,91 B	25,21 ± 2,70 B
Day 19	39,30 ± 7,00 A	36,16 ± 6,06 AB	31,91 ± 3,58 B	28,58 ± 2,93 B
Day 21	44,66 ± 9,30 A	44,32 ± 6,26 A	38,82 ± 4,70 AB	34,96 ± 3,82 B

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).
Day 1 = 24 hours after parturition.

was significantly lower than that of the control group. Empty chondroblast lacunae were also observed in the offspring of caffeine-treated rats (Table 2).

The primary spongy bone of the tibia of 3-day-old offspring of rats treated with 50 and 100 mg/kg of caffeine displayed thin trabecular bones with a significant reduction in the percentage of trabecular bone. The group treated with 25 mg/kg of caffeine exhibited characteristics of the primary spongy bone of the tibia that were similar to those of the control group. The percentage of primary spongy bone in the lumbar vertebrae of all groups treated with caffeine was significantly lower when compared to the control group (Table 3, Figure 1). Interestingly, regardless of which bone was examined, all caffeine-treated groups displayed trabeculae lined by active cuboidal osteoblasts, as observed in the control group (Figure 1).

Cartilaginous epiphysis of the femur and tibia of 3-day-old rats

In all groups of offspring from caffeine-treated mothers, pyknotic chondroblasts and a large number of empty lacunae were observed, both of which are characteristic of tissue degeneration and necrosis. The groups treated with 50 or 100 mg/kg of caffeine, the

Table 2. Body length measured from the neck to the base of the tail, the length (mm) of the members, the length (mm) of the vertebral body, and vertebral epiphyseal plate width (mm) of three-day-old offspring of untreated control rats and those treated with different doses of caffeine (mean±SD).

Variable (mm)	Control	Caffeine 25 mg/Kg	Caffeine 50 mg/Kg	Caffeine 100mg/Kg
Body length	38,200 ± 3,084 A	38,125 ± 1,246 A	33,330 ± 2,550 B	31,937 ± 2,367 B
Pelvic member	3,160 ± 0,150 A	2,675 ± 0,088 A	2,060 ± 0,084 B	1,980 ± 0,063 B
Thoracic member	3,270 ± 0,231 A	3,087 ± 0,064 B	2,550 ± 0,143 C	2,230 ± 0,200 C
Length of the vertebral body	1,505 ± 0,126 A	1,490 ± 0,136 A	1,300 ± 0,107 A	1,125 ± 0,143 B
Epiphyseal plate width	0,398 ± 0,015 A	0,375 ± 0,043 AB	0,322 ± 0,055 BC	0,305 ± 0,037 C

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).

Table 3. Percentage of trabecular bone in the primary spongy bone of the proximal tibia and lumbar vertebrae of 3-day-old offspring of control rats and those treated with different doses of caffeine (mean±SD).

Bone site	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine 100mg/Kg
Tíbia	39,812 ± 8,965 A	39,498 ± 14,138 A	22,968 ± 9,644 B	19,502 ± 1,902 B
Lumbar vertebrae	43,796 ± 14,080 A	28,342 ± 7,773 B	31,566 ± 8,067 B	21,402 ± 2,436 B

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).

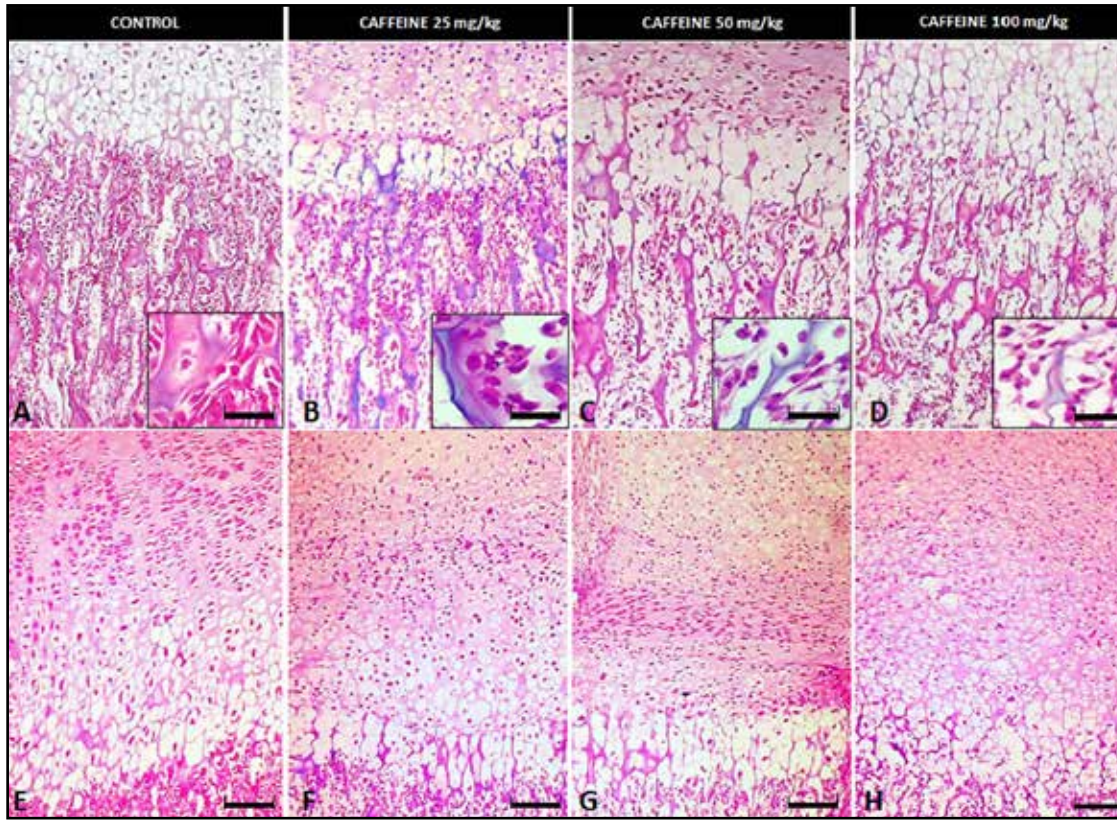

Figure 1. Three-day-old rat. HE, bar = 94.59 µm. A, B, C and D) The primary spongy bone of the tibia of mice of control groups and groups treated with 25, 50 and 100 mg/kg of caffeine, respectively. The amount of trabecular bone in the groups treated with caffeine at doses of 50 and 100 mg/kg (C, D) was smaller when compared to the control group (A) and the group treated with 25 mg/kg caffeine (B). E, F, G and H) The primary spongy bone of the vertebrae of the rats of the control group and groups treated with caffeine at doses of 25, 50 and 100 mg/kg, respectively. The amount of trabecular bone in the groups treated with caffeine at doses of 25 mg/kg (B), 50 mg/kg (C), and 100 mg/kg (D) was lower when compared to the control group (A).

percentages of pyknotic chondroblasts and empty lacunae were significantly higher in the cartilaginous epiphysis of the femur and the tibia when compared to the control group (Figure 2, Table 4).

Weight, size of offspring and histomorphometric analysis of the bones of 21-day-old rats

The weights of the offspring of rats treated with 50 and 100 mg/kg of caffeine were significantly lower than those of the control group throughout the experimental period. However, the weight of the offspring of rats treated with 25 mg/kg caffeine was not different from those of the control rats.

The length of the long bones (femur, tibia and humerus) of 21-day-old pups of rats treated with all doses of caffeine was significantly lower when compared to the control group (Table 5).

With regard to the width of the long bones, only the femur and tibia widths of the offspring of rats treated with 50 and 100 mg/kg of caffeine were significantly lower when compared to the control group. The width of the humerus did

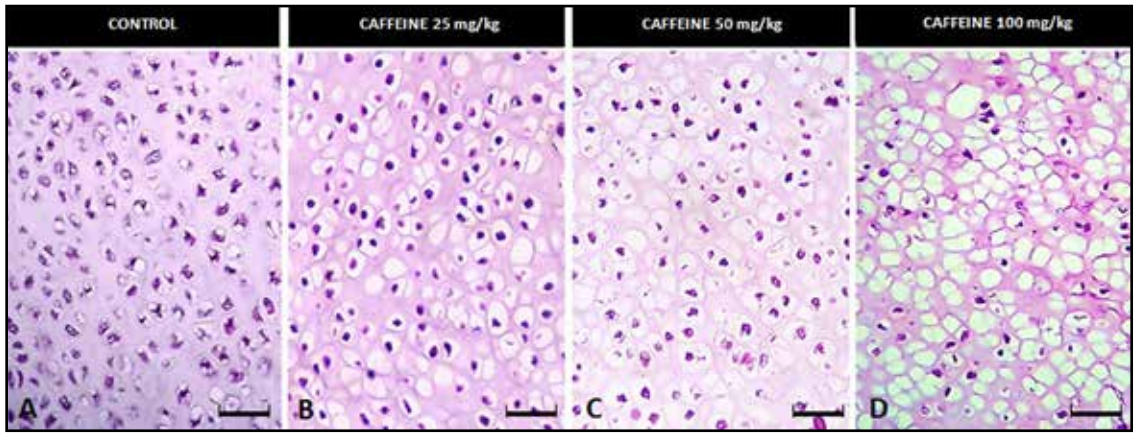

Figure 2. Cartilaginous epiphysis of the tibia of rats (three days old). HE, bar = 23.64 µm. A, B, C and D) Control groups and groups treated with caffeine at doses of 25, 50 and 100 mg/kg, respectively. Empty chondroblast gaps were present in all groups treated with caffeine. Groups treated with 50 or 100 mg/kg of caffeine had the largest number of pyknotic nuclei and empty gaps of chondroblasts when compared to the control group.

Table 4. Percentage of empty lacunae in 100 cells in the cartilaginous epiphysis of the femur and tibia of 3- day-old offspring of control rats and those treated with different doses of caffeine (mean±SD).

Bone site	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine 100mg/Kg
Femur	7,00 ± 2,23 C	19,00 ± 11,11 B	29,60 ± 11,52 BC	55,00 ± 20,00 A
Tíbia	4,40 ± 2,40 C	21,80 ± 8,46 B	32,00 ± 7,03 B	58,25 ± 19,38 A

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).

not differ significantly among the groups treated with caffeine and the control group (Table 5). The length of the vertebral bodies of the entire column was significantly smaller in 21-day-old offspring of the rats treated with 100 mg/kg of caffeine when compared to the control group. At a dose of 50 mg/kg of caffeine, the length of the vertebral bodies was significantly lower when compared to the control group but only in the segment of the fourth to sixth lumbar vertebrae. The vertebral length of the 25 mg/kg caffeine-treated group did not differ significantly from that of the control group (Table 5).

In the control group, the articular cartilage of the long bones showed a well-differentiated layer of chondroblasts and each was morphologically distinct. The chondroblasts of the surface zone displayed a lower nuclear volume and a smaller gap in comparison with those found in the mid and deep layers, where the chondrocytes contained a large nucleus with relaxed chromatin and were housed in large gaps. In all of the caffeine-treated groups, chondrocytes showed more intense and diffuse nuclear pyknosis than was observed in the control group. Furthermore, at a dose of

Table 5. Length and width (cm) of the femur (F), tibia (T) and humerus (H) and thoracic (VT) and lumbar (VL) vertebral length (mm) of 21-day-old offspring of control rats and those treated with different doses of caffeine (mean±SD).

Variable	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine 100mg/Kg
length F	1,804 ± 0,046 A	1,730 ± 0,068 B	1,653 ± 0,057 C	1,586 ± 0,059 D
length T	2,344 ± 0,081 A	2,156 ± 0,088 B	2,127 ± 0,061 B	1,977 ± 0,079 C
length H	1,569 ± 0,037 A	1,514 ± 0,045 B	1,454 ± 0,047 C	1,399 ± 0,049 D
width F	0,207 ± 0,011 A	0,201 ± 0,003 AB	0,196 ± 0,007 B	0,195 ± 0,014 B
width T	0,153 ± 0,005 A	0,150 ± 0,0003 A	0,138 ± 0,010 B	0,114 ± 0,013 C
width H	0,169 ± 0,015 A	0,161 ± 0,018 A	0,171 ± 0,016 A	0,257 ± 0,232 A
VL 1-3	2,962 ± 0,269 A	2,687 ± 0,470 A	2,533 ± 0,265 AB	2,256 ± 0,380 B
VL 4-6	3,102 ± 0,174 A	2,946 ± 0,400 AB	2,699 ± 0,210 B	2,513 ± 0,311 B
VT1-7	1,907 ± 0,139 A	1,789 ± 0,210 A	1,758 ± 0,204 A	1,519 ± 0,208 B
VT 8-13	2,148 ± 0,459 A	1,944 ± 0,295 AB	1,915 ± 0,205 AB	1,669 ± 0,152 B

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).

100 mg/kg, pups still displayed a large quantity of empty chondrocytes lacunae. The thickness of the articular cartilage was significantly greater in the humerus of the offspring of rats treated with 100 mg/kg of caffeine (Table 6).

The thickness of the epiphyseal plates of the long bones and vertebrae of 21-day-old offspring of rats treated with caffeine did not differ significantly when compared to the control group (Data not shown). However, several changes were observed in the morphology of the epiphyseal plate of bones, especially in the areas of proliferation and differentiation, or the columnar zone. In the control group, the epiphyseal plate in all of the bones examined appeared well-differentiated and the cellular morphology of each of the zones was distinctly different. In the zone of proliferation, the chondroblasts presented with voluminous nuclei and gaps, sometimes with prominent nucleoli. Chondroblasts in this area in the group treated with 25 mg/kg of caffeine exhibited some nuclear pyknosis. However, the changes were more intense in the groups treated with 50 and 100 mg/kg of caffeine: the chondroblasts showed intense nuclear pyknosis (Figure 3) that presented with either a multifocal or diffuse distribution. Foci of empty lacunae of chondroblasts were present. For the caffeine-treated groups, chondrocytes in the zone of differentiation were small and displayed multifocal nuclear pyknosis diffusely distributed throughout that zone. The zone of differentiation was also less organized in the caffeine-treated groups; it lacked a columnar pattern as well-defined as the control group. All of these features were more pronounced in the

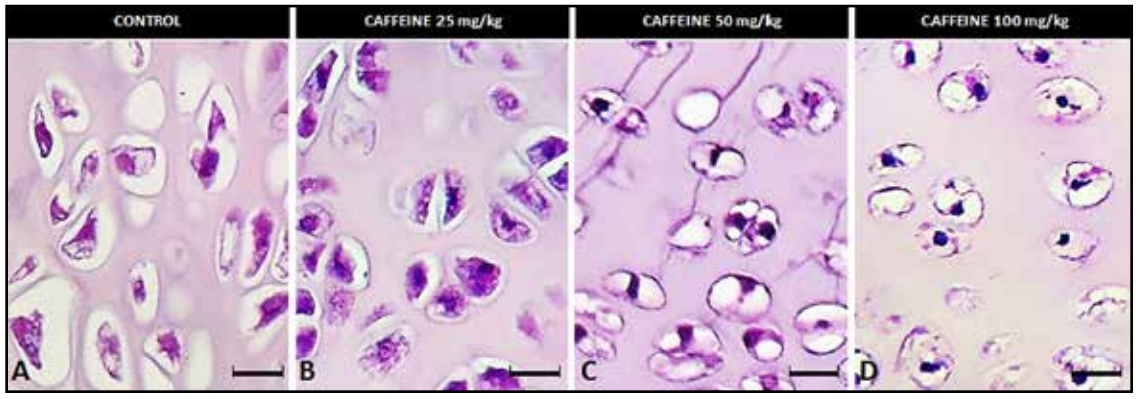

Figure 3. Proliferation Zone of the growth plate of the femur of rats at 21 days of age. HE, bar = 9.45 µm. A, B, C and D) control groups and groups treated with caffeine at doses of 25, 50 and 100 mg/kg, respectively. A) Chondrocytes with numerous nuclei and loose chromatin. B) Chondrocytes with numerous nuclei with slightly condensed chromatin. C) Chondrocytes with pyknotic nuclei and some empty gaps. D) Chondrocytes with intensely pyknotic nuclei and with some empty gaps.

Table 6. Thickness (mm) of the articular cartilage of the long bones (femur, tibia, and humerus) of 21-day- old offspring of control rats and those treated with different doses of caffeine (mean±SD).

Bone site	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine 100mg/Kg
Femur	0,493 ± 0,074 A	0,523 ± 0,045 A	0,530 ± 0,089 A	0,565 ± 0,045 A
Humerus	0,379 ± 0,040 B	0,408 ± 0,037 B	0,385 ± 0,053 B	0,488 ± 0,094 A
Tíbia	0,474 ± 0,053 A	0,445 ± 0,800 A	0,472 ± 0,058 A	0,497 ± 0,075 A

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).

groups treated with 50 and 100 mg/kg of caffeine (Figure 4).

The hypertrophic zone of the epiphyseal plate showed similar morphology among all the groups, including the control group. Chondrocytes showed large gaps and cores that were sometimes bulky and sometimes pyknotic with vascular invasion and a small amount of extracellular matrix. No formation of a terminal distal bone plate was observed in any of the animals.

The percentage of trabecular bone in the epiphysis, the primary spongy bone of the femur, tibia, and humerus, the thoracic vertebrae (8-13) and the lumbar vertebrae (1-6) did not differ significantly among any of the groups (Table 7). However, the percentage of trabecular bone in the thoracic vertebrae segment 1-7 obtained from the offspring of rats treated with 50 and 100 mg/kg of caffeine was significantly lower when compared to the control group (Table 7, Figure 5). In these caffeine-treated groups, the trabeculae were present in fewer numbers and were less interconnected. However, the morphology of the osteoblasts and osteocytes was similar among

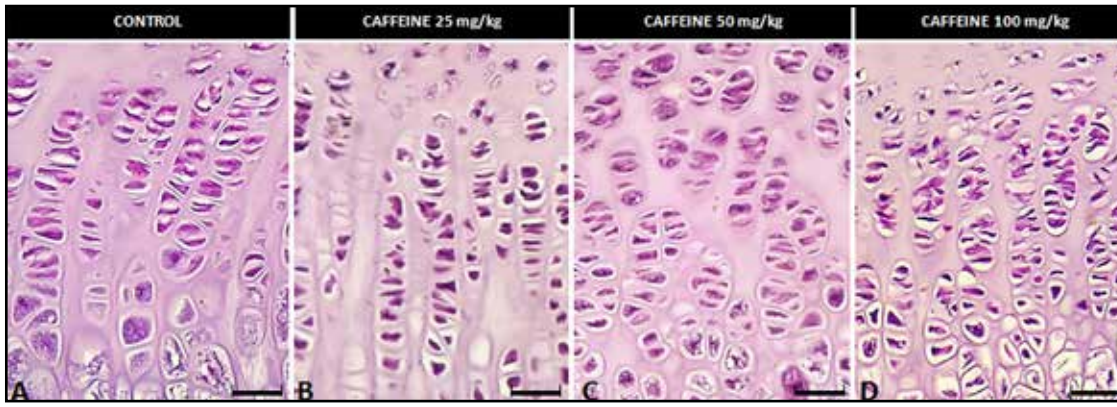

Figure 4. Columnar zone of the epiphyseal plate of the femur of rats at 21 days of age. HE, bar = 9.45 µm. A, B, C and D) Control group and groups treated with caffeine 25, 50 and 100 mg/kg, respectively. A) Chondrocytes arranged in columns with large nuclei and loose chromatin. B) Chondrocytes arranged in columns with smaller nuclei compared to the control with slightly condensed chromatin. C) Chondrocytes poorly organized in columns with small nuclei and discretely condensed chromatin. D) Chondrocytes with large amounts of pyknotic nuclei and flattening of the columnar zone.

the groups. The osteocytes were voluminous, were sometimes housed in enlarged gaps, and sometimes exhibited small nuclei within narrow gaps. The osteoblasts were always voluminous and were arranged in one or more layers coating the surface of the trabeculae (Figure 5).

Discussion

The plasma concentrations of cortisol in mothers at 21 days of lactation were higher only in the group treated with caffeine at a dose of 100 mg/kg when compared to the control group. The elevation of cortisol can be explained by the psychoactive effects of caffeine on the nervous system[20], which induce a release of dopamine, and consequently increase the levels of glucocorticoids[21]. Caffeine can also increase cortisol secretion by acting directly on the hypothalamus or by acting indirectly through stimulation of the release of epinephrine by the adrenal gland. These mechanisms of action have important implications for the ability of caffeine to affect behavioral stress[21]. Cortisol in pregnant or lactating females can also pass through the placenta and the milk[22,23]. Thus, one cannot rule out the possibility that cortisol is a contributing factor in the changes found during the growth of offspring whose mothers received caffeine at a dose of 100 mg/kg during pregnancy and lactation. The administration of caffeine at a dose of 120 mg/kg during the middle third to the end of gestation also resulted in high levels of maternal cortisol [16]; however, it is important to note that this would not be the main mechanism by which caffeine affects endochondral growth because the 25 and 50 mg/kg doses were able to alter bone growth despite levels of maternal cortisol that were statistically similar to the control group.

Chronic use of high doses of caffeine (50 and 100 mg/kg) by the rat during pregnancy and lactation also affected the weight of their offspring. Research has shown that both low doses (4 mg/day) and high doses of caffeine (120 mg/kg) in mice and doses above 300 mg/day in humans can also reduce the body weight of offspring [16,24,25]. It is likely that caffeine interferes with the intrinsic fetal-maternal-placental balance, affecting the formation and growth of the fetus, and consequently

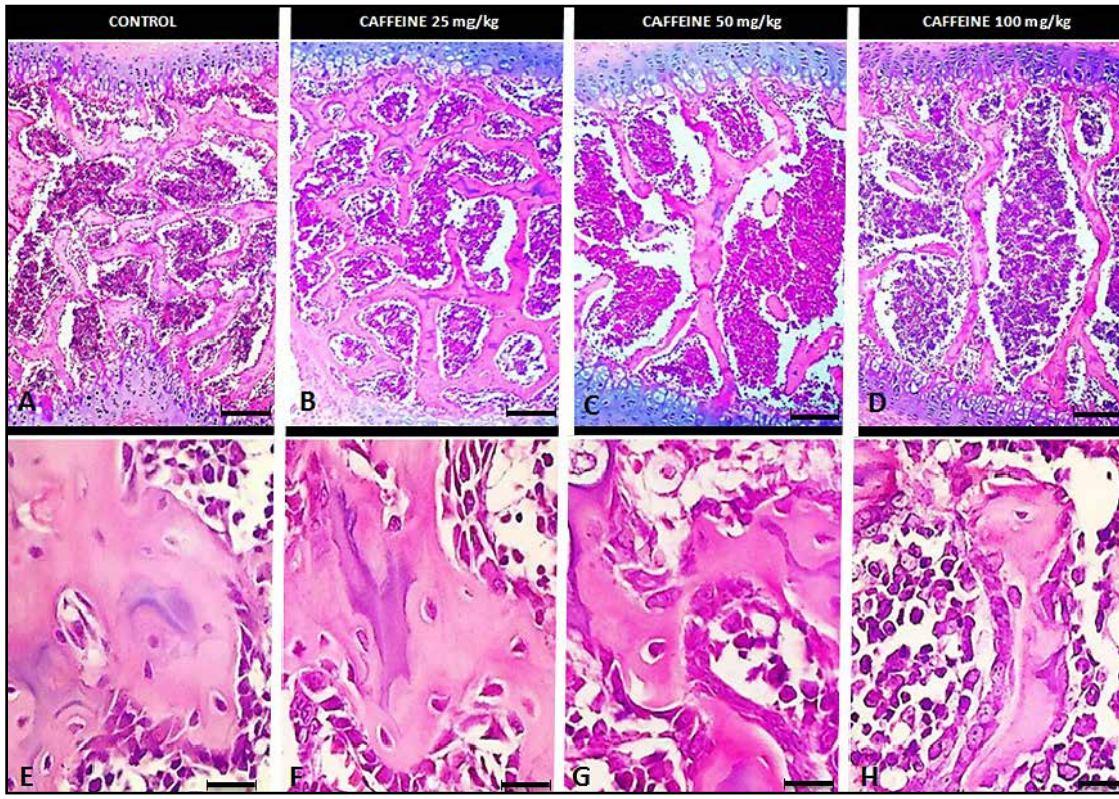

Figure 5. Primary spongy bone of the thoracic vertebrae 1-7 of rats at 21 days of age and Trabecular bone of the thoracic vertebrae 1-7 of rats at 21 days of age HE, bar = 94.59 µm. A, B, C and D) control group and groups treated with caffeine at doses of 25, 50 and 100 mg/kg, respectively. The percentage of trabecular bone in the groups treated with caffeine at doses of 50 and 100 mg/kg was lower when compared to the control group. E, F, G and H) Control group and groups treated with 25, 50, and 100 mg/kg caffeine, respectively. In all groups, the osteoblastic coating is uniform and formed by bulky osteoblasts arranged in one or more layers.

the fetal birth weight [15]. It is postulated that the vasoconstrictor effects of caffeine, via an increase in catecholamines, may influence placental development and reduce the fetal oxygen supply[26,27]. It is probable that caffeine also causes unknown changes in the placenta that may lead to low fetal birth weight. The gestational period in rats treated with 50 and 100 mg/kg of caffeine was one day less when compared to the control group (data not shown). This may be an alternative explanation for the lower birth weight of the offspring of these groups.

Another hypothesis that does not explain the low birth weight, but which might explain the low weight of caffeine-treated pups at weaning is the reduced nutritional quality of the milk. Caffeine has been shown to reduce the content of copper, zinc and iron in milk[28] and, consequently, the development of the feeding pup. Another possibility could be a change in the palatability of the milk. Studies have also shown that the palatability of animal feed can be affected by the addition of caffeine, leading to a decrease in food intake, and, consequently, the weight of the animals [29]. Elevated levels of cortisol in mothers treated with the highest dose of caffeine (100 mg/kg) may also have contributed to the low weight of the pups at weaning. Because high levels

Table 7. Percentage of trabecular bone in the epiphysis and primary spongiosa of the long bones and vertebrae of 21-day-old offspring of control rats and those treated with different doses of caffeine (mean±SD).

Bone site	Control	Caffeine 25mg/Kg	Caffeine 50mg/Kg	Caffeine 100mg/Kg
Femur-Ep.	23,510 ± 7,500 A	27,650 ± 6,740 A	25,130 ± 0,470 A	23,340 ± 0,003 A
Femur-Met.	35,420 ± 9,490 A	30,650 ± 6,030 A	30,790 ± 4,040 A	30,770 ± 5,820 A
Tíbia-Ep.	27,940 ± 9,340 A	24,080 ± 5,220 A	24,420 ± 6,650 A	21,820 ± 9,840 A
Tíbia-Met.	34,550 ± 3,770 A	38,810 ± 14,750 A	35,590 ± 5,940 A	35,800 ± 5,980 A
Humerus-Ep.	30,980 ± 14,520 A	23,990 ± 10,190 A	24,960 ± 5,470 A	25,990 ± 4,320 A
Humerus-Met.	34,140 ± 3,230 A	34,250 ± 5,390 A	33,240 ± 10,160 A	35,800 ± 8,380 A
VL1-3	24,140 ± 2,610 A	28,930 ± 18,060 A	22,890 ± 9,040 A	19,650 ± 8,730 A
VL4-6	26,530 ± 7,830 A	19,500 ± 7,030 A	20,120 ± 5,920 A	20,400 ± 7,930 A
VT1-7	31,620 ± 9,170 A	28,440 ± 8,760 AB	22,130 ± 5,880 B	18,310 ± 4,220 B
VT8-13	24,610 ± 5,680 A	18,210 ± 5,630 A	21,260 ± 4,680 A	22,440 ± 3,570 A

* Means with capital letters equal in the line do not differ by Student Newman Keuls test (SNK) ($p \geq 0.05$).
*Ep.: Epiphysis; *Met.: Metaphysis; VL: Lumbar vertebrae; VT : Thoracic vertebrae.

of this hormone affect the animals behavior[23] and increase both maternal and fetal stress levels [23,30], high cortisol levels can result in a decrease in the receptivity between mother and pup. Cortisol is passed to the pups both through the placenta and through milk, and can also affect the endochondral formation and the growth of pups, thus exerting negative overall effects on skeletogenesis[22,23]. However, serum cortisol concentrations in mothers after weaning were only higher in the group treated with 100 mg/kg of caffeine when compared to the other groups. Thus, it is postulated that cortisol levels may be responsible for the low birth weights observed only in the 100 mg/kg caffeine-treated group.

Beyond the direct effects of caffeine, high levels of plasma cortisol can pass to the fetus and intensify the effects of caffeine. The elevated cortisol levels can affect the fetus through at least two possible pathways. In the first pathway, the maternal cortisol crosses the placenta and directly exerts its effects on the fetus, acting on the hypothalamic-pituitary axis[31]. Fetal exposure to elevated cortisol, arising directly from the mother and indirectly affecting the hyper-activation of the fetal hypothalamic-pituitary axis, can then affect fetal growth. It increases the fetal caloric expenditure through an increase in neuromuscular activity and glycogenolysis[32]. In the second pathway, the maternal cortisol crosses the placenta and affects the regulation of related placental hormones such as corticotropin, leading to hyperperfusion of the placenta and resulting in reduced blood flow to the fetus [33,34].

The results of the present study demonstrate that caffeine, particularly at doses of 50 and 100 mg/kg, significantly affected endochondral growth, with a dose of 25 mg/kg resulting in milder changes. Because the 3-day-old rats exhibited caffeine-induced changes, it is postulated that prenatal endochondral bone formation may also have been altered. A delay in postnatal growth was confirmed in this study, but growth did not stop completely by the observation that there was no formation of the distal terminal bone plaque below the growth plate. There were also no moments of interruption and resumption of growth, as was evident from the absence of transverse bone trabeculae in the metaphysis. Interestingly, the growth reduction was most likely the result of a decrease in the growth rate. The inability to differentiate among the zones of growth, the degenerative changes or even the cell death observed in the cartilage indicates a reduction in growth rate. This reduction in growth had been previously observed by other researchers who reported that the offspring of rats treated with caffeine were smaller in size and also exhibited a reduction in the expression of some factors (IGF-1, histone, collagen I and II and aggrecan) involved in the mechanism of action of caffeine on endochondral ossification[15,16]. In addition, subcutaneous implantation of demineralized bone fragments in mice in which chondrogenesis was inhibited by a reduction in the expression of collagen II, histones and TGF beta also resulted in smaller sizes[14]. Regardless, the present study seems to be the first to detail and compare the morphological changes in the growth of cartilage tissue induced by caffeine. In addition, this study demonstrated that these changes occurred at low doses, such as 25 mg/kg, without the intervention of maternal cortisol. Moreover, compared with the caffeine levels tested in previous studies, the level lowest tested in this study is closer to those typically ingested by humans [11,14]. However it is difficult to estimate the actual human consumption, because the average consumption of caffeine varies widely per country, and the variety and quantity of individual human diet that can contain caffeine as coffee, tea, sodas, energy drinks, chocolates, cocoa based food, and drugs [1,2,3,4]. Searches can not account for all the sources of caffeine in the human diet, so our research also used higher doses. Also to be taken into account individual habits like smoking, drinking alcoholic beverage and drug use that may increase or decrease the time of clearance of caffeine[1,5]. The periods and amounts of caffeine used in this study are also related to the proportionality of the relationship: quantity supplied, lifespan of animals, including time of pregnancy and metabolism versus human consumption through the various sources of caffeine in diet, metabolism as well as life expectancy and gestation time longer.

By analysis of the trabecular bone percentage, the effects of caffeine were found to be more significant in the bones of 3-day-old rats than in 21-day-old rats. The percentage of trabecular bone in 3-day-old rats was lower in the tibia and vertebrae, even with administration of the lowest dose of caffeine (25 mg/kg). In 21-day-old rats, the percentage of trabecular bone was only minor in the 1-7 segments of the thoracic vertebrae at the 50 and 100 mg/kg doses of caffeine. This reduction in the percentage of trabecular bone below the cartilage was most likely due to a reduction in bone growth. There was no indication of osteoporosis in any of the animals; the trabeculae presented regular osteoblastic coverage and the presence of voluminous osteoblasts was observed, as in the control group. Caffeine is also considered a risk factor for osteoporosis and periodontal disease in adults[35]. In vitro, it has a negative effect on osteoblasts, in which it reduces cell viability and increases apoptosis [36,37,38,39]. Furthermore, the addition of caffeine to osteoblast cultures decreases protein synthesis and the expression of genes such as collagen, alkaline

phosphatase, osteocalcin, osteopontin, histone and Cbfa1/Runx-2 [36,39]. Other research indicate que caffeine has a beneficial effect on cultured primary adipose-derived stem cells and bone marrow stromal cells, enhancing differentiation to osteoblasts, this effect, which is mediated via RUNX2 activation at low doses is Significantly suppressed at high doses[40]. However, in this study, despite the reduced percentage of trabecular bone, which is generally a feature of osteopenia, there was no atrophy or osteoblastic hypoplasia, both of which are common morphological characteristics of osteoporosis.

Conclusions

We conclude that caffeine inhibits endochondral ossification in the offspring of rats treated with caffeine during pregnancy and lactation. The effects of caffeine are more damaging to cartilage growth in 3-day-old pups. In addition, the frequency and intensity of the changes are dose-dependent and low doses of caffeine (25 mg/kg) can cause changes in the cartilaginous tissue of the offspring after birth. Although the serum cortisol levels were not altered when the mothers were treated with low doses of caffeine, high doses of caffeine (100 mg/kg) increased the serum levels of maternal cortisol, and this is associated with more severe changes in the cartilaginous tissue related to endochondral ossification of the offspring.

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Declaration of interest

The authors declare that they have no conflict of interest.

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