

Bone Quality Associated with Daily Intake of Coffee: A Biochemical, Radiographic and Histometric Study

Suzie Aparecida LACERDA
Renata Inahara MATUOKA
Rander Moreira MACEDO
Sergio Olavo PETENUSCI
Alessandra Aparecida CAMPOS
Luiz Guilherme BRENTGANI

*Department of Morphology, Stomatology and Physiology, Ribeirão Preto Dental School,
University of São Paulo, Ribeirão Preto, SP, Brazil*

Caffeine induces loss of calcium and influences the normal development of bone. This study investigated the effects of coffee on bone metabolism in rats by biochemical measurement of calcium, bone densitometry and histometry. Male rats, born of female treated daily with coffee and with coffee intake since born, were anesthetized, subjected to extraction of the upper right incisor, and sacrificed 7, 21 and 42 days after surgery. Blood and urine samples were taken, and their maxilla radiographed and processed to obtain 5- μ m-thick semi-serial sections stained with hematoxylin and eosin. The volume and bone quality were estimated using an image-analysis software. The results showed significantly greater amount of calcium in the plasma (9.40 ± 1.73 versus 9.80 ± 2.05 mg%) and urine (1.00 ± 0.50 versus 1.25 ± 0.70 mg/24 h) and significantly less amount in bone (90.0 ± 1.94 versus 86.0 ± 2.12 mg/mg bone), reduced bone mineral density (1.05 ± 0.11 versus 0.65 ± 0.15 mmAL), and lower amount of bone (76.19 ± 1.6 versus 53.41 ± 2.1 %) (ANOVA; $p \leq 0.01$) in animals treated with coffee sacrificed after 42 days. It may be concluded that coffee/caffeine intake caused serious adverse effects on calcium metabolism in rats, including increased levels of calcium in the urine and plasma, decreased bone mineral density and lower volume of bone, thus delaying the bone repair process.

Key Words: densitometry, coffee, calcium, bone repair.

INTRODUCTION

Bone is a dynamic tissue that is continuously undergoing resorption and formation, mediated by osteoclasts and osteoblasts, respectively, in a process known as bone remodeling. The bone act as a physiological vital tissue, representing a readily available source of calcium for the maintenance of normal calcium plasma concentration levels (1). For this reason, calcium is probably the most studied nutrient in bone health and is important in prevention and treatment of osteoporosis (2). Adequate consumption of calcium and vitamin D from food and/or supplements is necessary to ensure the maximum peak of bone mineral density in younger individuals to reduce the rate of bone loss in old age (3).

Several endogenous and exogenous factors control the formation, absorption and remodeling of bone tissue (4), such as consumption of caffeine. The nutritional aspect is important in the development and maintenance of bone mass (5). Components of the daily diet are necessary for normal bone metabolism (6,7). Considering that coffee is a widely consumed beverage and several dental treatments involve alveolar bone, it is important to clarify the structural changes on bone repair in individuals that consume coffee daily.

Although some studies have suggested that the effects of coffee/caffeine on bone metabolism are still controversial (8), others have reported that caffeine, a major constituent of coffee, is associated with a significant increase of periodontal diseases, decreased

bone mineral density, increased risk of fractures and negative influence on calcium retention (9,10). Recent data show direct effect of caffeine on protein expression of the vitamin D receptor and osteoblast activity, indicating a probable molecular mechanism for the role of caffeine in osteoporosis (11).

This study evaluated the effects of daily consumption of coffee on bone metabolism in rats by biochemical measurement of calcium in the blood, urine and alveolar bone, analysis of bone mineral density (bone densitometry) and histomorphometry.

MATERIAL AND METHODS

All animals in this study were treated in accordance with the ethical principles for animal research defined by the local Committee of Ethics for Animals Experimentation (04.1.957.53.0), and received a vermifuge for animal use (Systemex; Shering of Brazil, São Paulo, SP, Brazil) during 3 days.

Treatment of Animals

Forty-two Wistar rats (*Rattus norvegicus*) weighing 250-300 g were used. The animals were fed a balanced diet 6003 Nuvilab VC-1 (Nuvital, Colombo, PR, Brazil) composed of corn grain, soybean meal, meal wheat, calcium carbonate, dicalcium phosphate, sodium chloride, vitamin mineral premix, amino acids, and water. They were housed in plastic boxes (40 x 32 x 17 cm) under controlled lighting (12 h light/12 h darkness) and temperature (21-25°C), and were allocated to either a treatment group (coffee) or a control group (water).

Adaptation Model to the Intake of Coffee

Seven female rats were adapted to the ingestion of 50 mg/mL (1.2 mL of infusion coffee/day) of roasted and ground coffee (Utam SA, Ribeirão Preto, SP, Brazil) for 30 days, by reducing the offer of water, as follows: 1st week: 50 mL water/rat and infusion coffee *ad libitum*, in separate bottles; 2nd week: 35 mL water/rat and infusion coffee *ad libitum*, in separate bottles; 3rd week: 25 mL water/rat and infusion coffee *ad libitum*, in separate bottles; 4th week: 10 mL water/rat and infusion coffee *ad libitum*, in separate bottles.

The vaginal smear of females was used to determine the mating cycle. The last doses (10 mL water and infusion of coffee *ad libitum*) were maintained

during mating and pregnancy. Females that did not get pregnant were removed from the study. Coffee intake was maintained for the pups since birth up to adult age, at which time were operated. The amount of coffee ingested by the animals was estimated based on the human daily consumption of 4 cups (240 mL) *per* day for a person weighing 60 kg.

Surgical Procedure, Sacrifice and Material Collection

After 60 days of birth and coffee intake, the animals were anesthetized with an intraperitoneal injection of 2,2,2 tribromoethanol (Tribromoethanol; Sigma-Aldrich, St. Louis, MO, USA; 25 mg *per* 100 g body weight). After antiseptis of the area, disconnection of the surrounding gingiva and luxation, the upper right incisors were extracted. The soft tissues were sutured with Mononylon 4.0 (Ethicon, São José dos Campos, SP, Brazil) and the animals received 0.2 mL of veterinary pentabiotic intramuscularly in a single dose (Wyeth, São Bernardo do Campo, SP, Brazil).

One day before sacrifice, urine was collected in metabolic cages during 24 h for calcium measurement. The animals were sacrificed 7, 21 and 42 days (n=7/period) after extraction by anesthetic overdose. At sacrifice, a blood sample was collected by cardiac puncture and immediately processed for calcium measurement.

The right side of the maxilla was separated from the left side by a sagittal incision along the intermaxillary suture. A cut with straight scissors tangential to distal face of molars provided a piece that was immersed in 10% formalin for fixation. After radiographic projections for bone densitometry, the maxillas were decalcified for 4 days in 20% sodium citrate and 30% formic acid, washed for 24 h in running water, dehydrated, cleared, embedded in paraffin, and oriented to provide 15 5- μ m-thick longitudinal cuts, distant 50 μ m from each other, and stained with hematoxylin and eosin.

The left maxilla was dissected, and their teeth extracted and immediately incinerated at 700°C for 14 h. The ashes obtained were dissolved in appropriate volume of 0.1 N hydrochloric acid, and an aliquot taken for determination of calcium.

Levels of Calcium in the Plasma, Urine and Maxilla

Calcium concentration in plasma, urine and maxilla ashes was measured by a specific colorimetric method for using a commercial kit Labtest (Diagnostic

Systems Labtest Ltda, Belo Horizonte, MG, Brazil).

Densitometric Analysis of the Maxilla

The analysis of bone mineral density was performed by the method of radiographic optical densitometry (12). An aluminum scale (alloy 6063, ABNT) with 8 steps ranging from 2 mm to 2 mm until the eighth step, served as reference for densitometric radiographic analysis. The standard x-ray machine was set to 50 kVp and 10 mAs, 1 meter focus-film distance and 24 -pulse exposure time (Weber x-ray unit; The Weber Dental Mfg. Co., Canton, OH, USA). Radiographic films (Kodak Min-R 2000 film in Kodak Min-R Cassettes; 18x24 cm) were used and developed manual (Kodak X-OMAT Developer and Replenisher).

The images were scanned on a professional scanner (Epson model - Expression 636) controlled by a computer program (Epson scanner II 32, version 2.10E 1994), and then saved in TIFF format. Using Adobe Photoshop CS3 software (Adobe System Corporation Inc., San Jose, CA, USA), each image was analyzed by the histogram of the intensity of tone scales in the "light channel". In each radiograph (standard-size areas in the center of the alveoli), a value of the average of brightness intensity of each step of densitometric reference was obtained. The values obtained by the densitometric scanner HP Scanjet 4C were converted to values of thickness of aluminum, according to the methodology described by Louzada et al. (12).

Histometric Analysis of the Alveoli

A Leica DM LB2 optical microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) equipped with a digital video camera Leica DFC 280

Table 1. Mean values and standard deviation of the calcium dosages in controls and animals subjected to daily intake of coffee.

Parameters	Control	Treated
Ca concentration in the plasma (mg%)	9.40 ± 1.73	9.80 ± 2.05*
Ca concentration in the urine (mg/24 h)	1.00 ± 0.50	1.25 ± 0.70*
Ca concentration in the maxilla bone (mg/mg bone)	90.0 ± 1.94	86.0 ± 2.12*

*Statistically significant difference (ANOVA; $p < 0.05$)

(Leica Microsystems AG Imaging, Cambridge, England) were used for image capture. The images were processed using Qwin Leica software (Leica Imaging Systems Ltd, Cambridge, England) to obtain the bone volume fraction (%). Five fields were analyzed in each third of the alveolus (cervical, middle and apical), totalizing 15 fields per alveolus, in order to estimated the bone percentage volume through a method of differential count of 1,500 points per alveolus ($n=6$) (13).

Statistical Analysis

Data were expressed as means ± standard deviation. Differences between groups were analyzed by ANOVA ($\alpha=0.05$), using GMC statistical software (available from <http://www.forp.usp.br/restauradora/gmc/gmc.html>).

RESULTS

The animals treated with coffee did not loose weight or appetite, but were more excited on manipulation, an they bled more than controls during the surgical procedure.

The biochemical results showed that the levels of calcium in the urine (mg/24 h) and plasma (mg%) were statistically significant ($p < 0.05$) in animals treated with coffee when compared to controls (Table 1). The calcium in the maxilla bone (mg Ca/mg bone) showed lower values ($p < 0.05$) in animals that ingested coffee throughout the experimental period (Table 1).

The densitometric values converted to values of

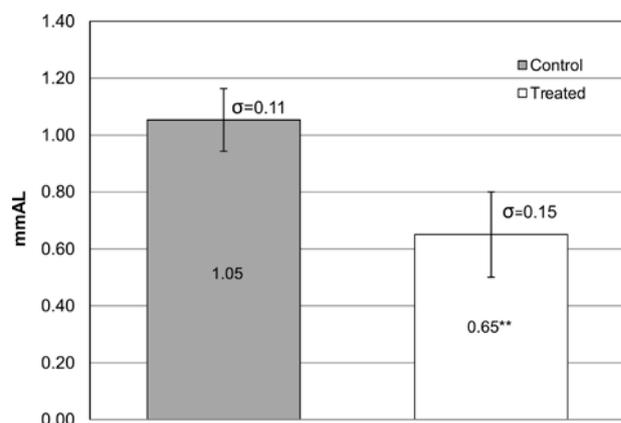


Figure 1. Mean values (number in column) and standard deviation (σ) of bone mineral density (in mmAL) in the alveoli of controls and animals subjected to daily coffee intake. ** (ANOVA; $p < 0.01$)

thickness of aluminum (mmAL) showed reduced values ($p < 0.01$) in animals treated with coffee (Fig. 1).

The radiographic evaluations of alveolus showed more radiotransparency in animals treated with coffee when compared with controls (Fig. 2).

The various stages of bone repair were recognized histologically in controls and rats treated with coffee from 7 to 42 days after dental extraction (Fig. 3). At the end of the 1st week (7 days), immature bone trabeculae were seen surrounded by osteoblasts, mixed with blood clot remnants and abundant connective tissue rich in newly formed capillaries. With progressive bone formation at the 6th week (42 days), the bone defect was filled with compact trabecular bone surrounding small areas of mature connective tissue (Fig. 3A). A possible delay in the osteogenic process was apparent in the alveoli of rats treated with coffee, which had lower volume bone density

from the 7th to the 42nd day (Fig. 3B) after surgery.

The histometric analysis provided quantitative data showing bone formation along with gradual decrease in the percentage of connective tissue up to 42 days after dental extraction. However, the amount of bone in the alveoli of animals treated with coffee (Table

Table 2. Mean values \pm standard deviation of newly formed bone in controls and animals subjected to daily intake of coffee during at 7, 21 and 42 postoperative days (n=7).

Period	Control	Treated
7 days	16.16 \pm 1.68	6.88 \pm 1.52**
21 days	54.13 \pm 2.76	37.30 \pm 1.98**
42 days	76.19 \pm 1.66	53.41 \pm 2.19**

** Statistically significant difference (ANOVA; $p < 0.01$).

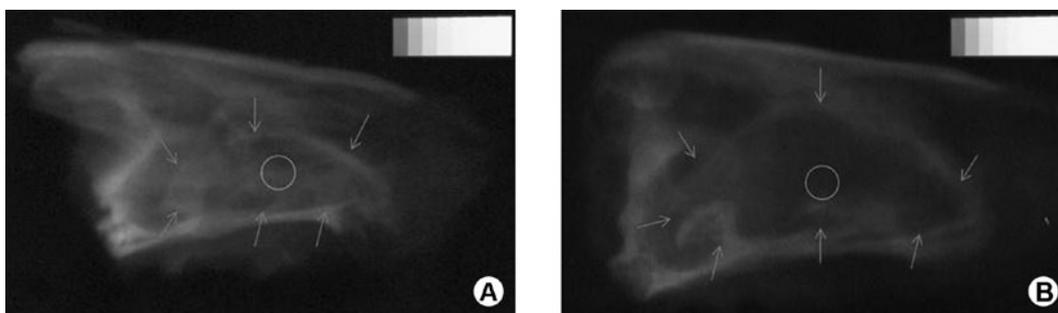


Figure 2. Radiographs of the alveolus of a control animal (A) and an animal subjected to daily intake of coffee (B) 42 days after dental extraction. Arrows show de alveolus limit and the circle represents the standard area used to measure radiographic bone density.

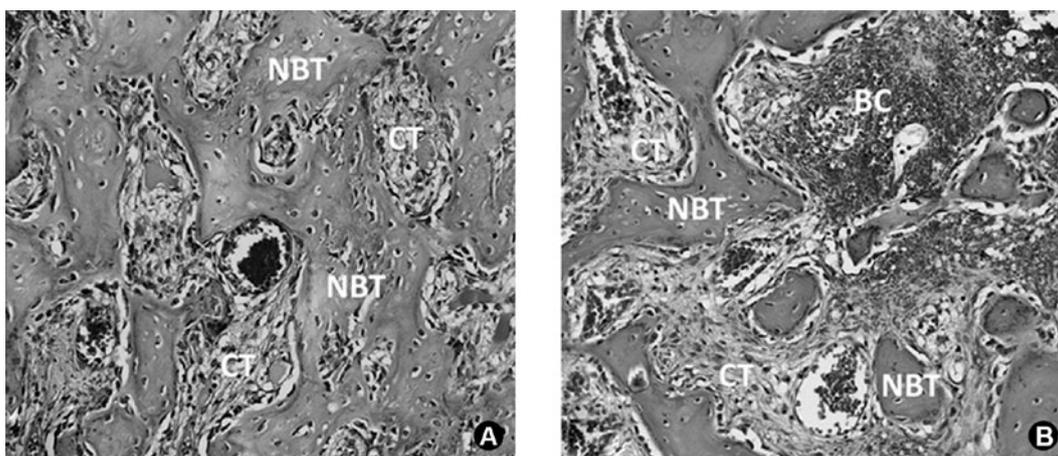


Figure 3. Histologic aspects of bone repair in alveolus of a control animal (A) and an animal subjected to daily intake of coffee (B) 21 days after surgery. NBT = Newly formed bone tissue; CT - Connective tissue; BC - Blood clot. HE, 250 \times magnification.

2) decreased by 10-20% ($p < 0.01$) within the same period.

DISCUSSION

The results of this study provide evidence that the daily intake of coffee affected the repair process of bone defect produced by dental extraction in rats.

The alveolar bone repair offers an appropriate model for the study of bone formation in rats and may be considered a sensitive indicator of bone damage in different experimental conditions (14). The present methodology used an atraumatic technique that allowed the extraction of the tooth without postoperative complications, and in the biochemical, histometric and densitometric pattern methods, to provide a numeric characterization of bone repair process (13-15).

Coffee is one of the most psychoactive beverages consumed throughout the world, but its effect on bone metabolism is still controversial (8). Studies have suggested that caffeine, a major constituent of coffee, is associated with a significantly increased risk of fractures, osteoporosis and periodontal disease (9-11).

Although the effects of caffeine on bone metabolism are not yet conclusive, in general, the potential impact of this drug on bone is due to its ability to increase the excretion of calcium (10). Although some studies using animals have not been able to demonstrate a definitive effect of caffeine on the development of the bone, others have shown negative effects of caffeine on normal growth and bone development and decrease in calcium content of bone (10,15).

Caffeine acts antagonizing the adenosine receptors (A_1 , A_{2A} , A_{2B} and A_3) by changing the function of the enzyme adenyl cyclase and activating the kinase A protein (PKA) by increased concentrations of intracellular cAMP mediator (16).

Caffeine has a variety of cellular responses and pharmacological actions on bone metabolism, resulting in an increase in the excretion of calcium in urine (10,17). In this study, increased amounts of calcium were found in urine and plasma, and decreased amount was found in the alveolar bone in animals subjected to a chronic coffee treatment. These results suggest that the deposition of calcium in the bone was affected in exposure to caffeine, by changing the extracellular matrix required for mineral deposition.

Histological analysis showed that the administration of coffee produced a delayed formation of granulation tissue when compared to controls. The

bone defect of the animals treated with coffee had large amounts of blood clot even at 21 days (Fig. 3). As a result, differentiation in bone tissue was lower than in controls. Caffeine can modulate various aspects of the inflammatory response and adaptive and innate immune response (18). The results of this study suggest that a change has occurred in the role of macrophages by reduction of their activity and/or apoptosis, as well as a decrease in the production of interferon-gamma (IFN- γ) by T lymphocytes (19), with consequent decrease in stimulating effects of this mediator on the phagocytic and secretory functions. Once the reduced viability of macrophages in the repair site, many of the cytokines stimulating fibroblasts tend to have lower levels. As several of these growth factors act on chemotaxis, proliferation, production of collagen and formation of granulation tissue, perhaps a deficiency in the formation of granulation tissue and maintenance of blood clot might have occurred by inhibiting the reparative functions of fibroblasts.

Interference on differentiation, proliferation, production of bone matrix, and mineralization by osteoblasts and osteocytes (9) could also act as a synergistic factor, increasing the intensity of changes in the dynamic process of alveolar bone repair (19).

Although it is not clear in the literature the negative effects of caffeine on bone repair (8), in the present work using the intensity of shades on light scales of radiographs, it can be show a decrease in bone mineral density in dental alveoli of animals treated with coffee.

The histometry showed that alveolar bone repair was significantly impaired in animals that ingested coffee for a long time, demonstrating a direct effect of caffeine on the activities of osteoblasts and formation of bone matrix. These data suggest that the effect of coffee on the osteoblasts caused a delay in the formation of a competent extracellular matrix and its subsequent mineralization especially reflected by the decrease of calcium in bone. There is a clearly functional relationship between the parameters observed in this study and coffee. However, it was not possible to define which parameter was first modified by coffee and which are affected secondarily.

The findings of this work show the important relationship between coffee/caffeine intake and the repair of bone defects in rats. The outcomes do not fully explain the mechanisms involved in the molecular action of caffeine on bone cells, but they suggest its possible interaction with host cells, acting in one of the regulatory

processes of cell growth and differentiation.

It may be concluded that coffee/caffeine intake caused serious adverse effects on calcium metabolism in rats, including increased levels of calcium in the urine and plasma, decreased bone mineral density and lower volume of bone delaying the bone repair process.

RESUMO

A cafeína induz perda de cálcio e influencia no desenvolvimento ósseo normal. Este estudo investiga os efeitos do café sobre o metabolismo ósseo em ratos através de avaliações bioquímicas do cálcio, densitometria e histometria óssea. Ratos machos, nascidos de fêmeas tratadas diariamente com café, e com ingestão de café desde o nascimento, foram anestesiados, submetidos à extração do incisivo superior direito e sacrificados 7, 21 e 42 dias após a cirurgia. Amostras de sangue e urina foram colhidas, suas maxilas radiografadas e processadas para se obter cortes semi seriados (5 µm) e corados pela hematoxilina-eosina. Através de um programa de análise de imagens, o volume e a qualidade do osso foram avaliados. Os resultados demonstraram maior quantidade de cálcio no sangue ($9,40 \pm 1,73$ versus $9,80 \pm 2,05$ mg%) e urina ($1,00 \pm 0,50$ versus $1,25 \pm 0,70$ mg/24 h) e menor no osso ($90,0 \pm 1,94$ versus $86,0 \pm 2,12$ mg/mg osso), densidade mineral óssea menor ($1,05 \pm 0,11$ versus $0,65 \pm 0,15$ mmAL), e menor quantidade de osso ($76,19 \pm 1,6$ versus $53,41 \pm 2,1$ %) estatisticamente significativa (ANOVA $p \leq 0,01$) nos animais tratados com café sacrificados após 42 dias. Conclui-se que a ingestão de café/cafeína causou sérios efeitos adversos no metabolismo de cálcio em ratos, incluindo aumento dos níveis de cálcio na urina e no plasma, diminuição da densidade mineral óssea e menor volume de osso atrasando o processo de reparo ósseo.

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Accepted June 22, 2010