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The effect of caffeine on some indicators of bone metabolism in rats

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ABSTRACT

The propose of this article is to evaluate the effect of caffeine on some indicators of bone metabolism in rats by biochemical measurement of minerals, bone densitometry and histometry. Forty eight Wistar albino male rats, age 6-8 weeks and weighing 100±0.11 g were randomly divided into four groups (12 rats each). Each group of animals received balanced diet; the second, third and fourth groups received pure caffeine dissolved in distilled water with different oral doses (0.35, 0.43 and 50 mg/day) for 12 constitutive weeks. Blood samples were withdrawn at 3, 6, 9 and 12 weeks. Serum and urinary calcium, phosphorus, magnesium and caffeine were estimated. Bone density and bone length were measured. Bone minerals were also estimated. The data revealed that the bone density was significantly decreased ($p \leq 0.05$) in the fourth set (1.05±0.10 g/cm^3) for right femur rats. The length of right femur increased with more doses of caffeine and it was highly significant in the fourth group (3.40±0.12 cm). The proportion of each calcium, phosphors and magnesium in bone ash was significantly lower ($p \leq 0.05$). Serum levels of calcium, phosphors and magnesium were decreased with increasing the dose over time. The levels of urinary calcium and magnesium were increased significantly ($p \leq 0.05$) in group 4, but phosphors was raised ($p \leq 0.05$) in all groups. In conclusion, intakes of caffeine in amounts >300 mg/dl significantly affected the quantitative composition of the bone and this finding lead to be at a greater risk for bone loss. These results suggested that appropriate lifestyle changes to conserve bone mineral density (BMD) by reducing the consumption of caffeine and need further studies to elucidate the mechanism that caffeine effects on bone metabolism.

1. Introduction

Bone is a dynamic tissue undergoing a process known as bone remodeling that is a process of continuous re-sorption and formation, mediated by osteoclasts and osteoblasts cells. Several endogenous and exogenous factors control the formation, absorption and remodeling of bone tissue [1], such as nutrition, lifestyle, exercises, consumption of caffeine and genetics; these factors may be contributed to the pathogenesis of osteoporosis. Caffeine is consumed regularly by most of the Saud population. The intake of caffeine-containing beverages has unwanted health consequences but it can also produce several conceivable health benefits. Caffeine, is associated with a significant increase of periodontal diseases, induce loss of bone mineral density (BMD) with 5 mg net loss of calcium per cup of coffee [2], increased risk of fractures and negative influence on calcium retention [3,4]. Direct effect of caffeine on protein expression of the vitamin D receptor and osteoblast activity may be indicating a probable molecular mechanism for the role of caffeine in osteoporosis [5].

Caffeine is a risk factor on calcium balance and bones, loss of calcium rarely compensate with low intake of calcium and the net result increased caffeine consumption with increased calcium excretion in urine [6]. In woman, increased calcium excretion through an hour of caffeine consumption may be more sensitive that caffeine was effect on bone metabolism, so caffeine may represent a risk factor on bone in women [7,8]. A study conducted for women college age 19-28 years which taking 106±103 mg/day caffeine (equivalent to one cup of coffee/day) and 334±831 mg/day of calcium. It found that caffeine did not effect on bone density in the spine, but led to a high calcium extract in urine. This may represent a risk factor with increasing age. The rise in caffeine consumption in women which do not take the recommended amounts of Ca with increased of motor activity had a positive effect on bone mineral content [9].

A negative impact of caffeine on bone metabolism and calcium balance depends on the amount of calcium intake, age, length of stay of caffeine in the body. If the amount of Ca intake was not less than 800 mg/dl and the amount of caffeine was no more than 300 mg/dl, caffeine had no effect on bone density [10]. Although there is no relationship between caffeine consumption and loss of bone density in those taking enough calcium and there is a relationship between caffeine and the risk of hip fracture. Therefore it is recommended that no more than caffeine consumption as 300 mg/dl caffeine [11]. In a study conducted on the health status of Saudi women with age between 45 to 60 years living in Jeddah, it was found that osteopenia and fragile bones were prevalent among the women, as well as the lack of vitamin D was clear [12].

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Table 1. Bone density (g/cm^3) and length (cm) of femur in animals subjected to daily intake of caffeine *.

Parameters	Control	Low dose	Medium dose	High dose	Probability
Bone density	1.19±0.09 **	1.16±0.15 **	1.11±0.15	1.05±0.10	$p \leq 0.05$
Femur length	3.60±0.09 **	3.45±0.01 **	3.44±0.05 **	3.40±0.12 **	$p \leq 0.05$

* Values were express as means±S.E.; comparison between control group within caffeine groups was done by analysis of covariance; multiple comparisons were done by using post-hoc test.
** Significantly different between groups by one way Anova; significantly different, $p > 0.05$.

In recent decades, food habits has many changed in the Middle East, this change has been associated with many social changes. The prevalent of fast food based on refined foods and animal products is non-health as it is low in fiber, vegetables and fruits, essential nutrients such as vitamins and some minerals. Depending on these changes, unbalanced nutrition resulting increased the prevalent of diseases and health problems related to food and nutrition such as diabetes, high blood pressure, cardiovascular disease, obesity, and the fragility of bone in Ashia and Arab countries. This is expected to increase with the population increase in the world and the high average age. Therefore, osteoporosis become epidemic that threatens the health of the Kingdom of Saudi Arabia and is expected to increase the incidence of fractures due to bone fragility from 1.66 million to the world in 1999 to 6.26 million by the year 2050 [13-15]. This study was evaluated the efficacy of caffeine on some indicators of bone quality in rats by biochemical measurement of Ca, P and Mg in blood and urine; bone densitometry and histometry.

2. Experimental

2.1. Chemicals

Caffeine in the form of pure $C_8H_{10}N_4O_2$ powder was purchased from Scharlau Chemis.com (SA). The oral dose was given in rats equivalent to as much an adult weighing 60 kg [16] They are three different levels as follows: minimum daily intake of caffeine in Kingdom of Saudi Arabia (KSA) (258 mg/dl); Average daily intake of caffeine in KSA (211 mg/dl) and the upper limit for daily intake of caffeine in KSA (305 mg/dl).

2.2. Animals

This study was done during 2011-2012. Forty eight Wistar albino rats, age 6-8 weeks and weighing 100±0.11 g were used. The rats were obtained from Laboratory and Experimental Animal Care Center, College of Medicine, King Saud University. Animals have been kept in special cages, and maintained on a constant 12 h light/12 h dark cycle with air conditioning and 22 °C and humidity (60%). Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the King Saud University, College of Pharmacy. After one week acclimation, the rats were randomly divided into four groups (12 rats each). As the forth groups (G) received balanced diet [17] (Table 1) with free access to tap water ad libitum for one week before the experiment for acclimatization and during the period of the experiment. The second, third and fourth groups received pure caffeine dissolved in distilled water with different oral doses (0.35, 0.43 and 50.00 mg/day) for 12 constitutive weeks from the beginning of the experiment.

After an overnight fasting, rats were anesthetized, sacrificed and blood was withdrawn by heart puncture in tubes protected from light, then centrifuged at 3000 rpm for 10 minutes at 4 °C. Serum was immediately isolated, aliquoted, and stored at -80 °C until analyzed. Urine samples were collected in the last 24 hours from the end of each period, then filtered using Whatman paper No. 42, and kept at -30 °C until analyzed. At the end of the experiment, right and left femur bones were disarmed, cleaned well and washed with distilled water, then with sodium hydroxide (10%), and rinsed again with distilled water and dried by filter paper until completely dried, then frozen at -80 °C until analyzed.

2.3. Biochemical analysis

Serum and urinary Ca, P and Mg were estimated by enzymatic method as cited in the United Diagnostics Industry. Serum and urinary caffeine was estimated according to the method of Lui *et al*. [18], using liquid chromatography apparatus (HPLC).

2.4. Bone density measurement

Bone density was measured physically by the way of reference [19], right and left femur bones were disarmed, cleaned with a solution 10% sodium hydroxide, then washed with distilled water. Cut the femur in the middle of the head grandeur and output the bone marrow by needle and washed again. Placed each bone in a tube filled with distilled water under vacuum for 90 min to remove the air inside the reserved grandeur, then directed all the grandeurs and dried well on filter paper, then weighed and returned to the tube containing distilled water. The weight were taken in water and density was calculated (g/cm^3) as equation; the density of the body = submerged weight of the body in the air ÷ (body weight in the air - body weight in water).

2.5. Femur length measurement

Bone Length was measured along the right femur bone at the trial and during the time periods by the X-ray and at the end of the experiment according to the method [19].

2.6. Minerals determination in femur ash

Dry ashing of bone was made by incineration in Muffle Furnace. Calcium, phosphorus and magnesium were measured in the ash after sample preparation using the atomic absorption spectroscopy, Perkin Elmer A. Analyst 400 Model AAS [20].

2.7. Statistical analysis

SPSS version 7.0 computer program was used for statistical analysis. All numeric data were expressed as means ±S.E. Data were analyzed using ANOVA test. Unpaired *t*-tests were used to compare the data before and after the managements. The probability $p \leq 0.05$ was considered as significant

3. Results and discussion

Bone density in tissue is one of the basic parameters which determine its quality and susceptibility to fractures. Low bone mass and increased fracture risk has been associated with caffeine consumption in some, but not most, observational studies. The effects of caffeine on bone metabolism are still controversial. The present result show that caffeine had affected on bone density in rats. The mean value of bone density of right femur in rats with upper dose of caffeine was lower (Group 4, 1.0±0.10 g/cm^3) compared to control group (Group 1, 1.19±0.09 g/cm^3) (Table 1).

Table 2. The percentage of calcium, phosphors and magnesium in femur bone ash in rats *.

Parameters	Control	Low dose	Medium dose	High dose	Significance
Ca%	25.36±0.35	23.26±0.10	23.26±0.15	23.29±0.18	$p < 0.05$
P%	0.40±0.10	0.37±0.06	0.38±0.05	0.39±0.15	$p < 0.05$
Mg%	11.13±0.15	10.67±0.05	10.17±0.05	10.44±0.16	$p < 0.05$

* Values were express as means±S.E.; comparison between control group within caffeine groups was done by analysis of covariance; multiple comparisons were done by using post-hoc test; significantly different between groups by one way Anova; Significantly different: $p > 0.05$.

Caffeine-treated rats led to an increase in femur length in Group 4 for the last time period of the experiment (Table 1).

Some researchers [21,22] observed a relation between caffeine intake and bone loss in women. Conlisk and Galuska [22] conducted the effect of caffeine intake in doses of 100 mg/dl on bone tissue of young women. BMD was decreased of femoral neck and lumbar vertebral column 0.0069 g/cm^2 and 0.0119 g/cm^2, respectively. There was an association between caffeine consumption and elevated risk of fractures after menopause in women aged 50-59 who intake caffeine with a dose of 400 mg/dl [23]. Higher risk of fractures was observed in women aged 40-49 who consume more than nine cups of coffee/d with a low Ca intake. This indicated that caffeine was a risk factor for osteoporotic fracture development [24].

Liu *et al*., [24] stated that caffeine may reduce BMD in growing rats through the enhancement in osteoclastogenesis and it may possess the ability to enhance a cyclooxygenase-2 (COX-2)/prostaglandin (PG)E_2 production-regulated RANKL-mediated osteoclastogenesis. Caffeine was decreased bones by molecular mechanism of vitamin D3. 1,25-Dihydroxyvitamin D3 (1,25(OH)2D3) performs a basic role in the regulation of bone metabolism. A receptor for this vitamin (VDR, Vitamin D Receptor) occurs in osteoblast cells. This means that a high caffeine dose may influence VDR expression stimulated by 1,25(OH)2D3 and controlled by 1,25(OH)2D3 activity of human osteoblast cells by reducing alkaline phosphatase activity [5].

The present data concerning bone length, caffeine-treated rats led to an increase in femur length in Group 4 for the last time period of the experiment (Table 1). The caffeine consumption led to increase in growth of length for bone in rats, although it led to a decrease in bone density [25].

According to the present data, the percentage of Ca in femur bone had a significant decrease (23.26±0.10, 23.53±0.15, 23.29±0.18 *vs* 25.36±0.35; $p \leq 0.05$) between groups (Table 2). These results indicated that the reduction increases with increasing caffeine dose, which means that caffeine's role for reducing Ca content in bone. In the present study with regard to Mg and P, there was a significantly declined ($p < 0.05$) in the percentage values of Mg and P (Mg; 0.37±0.06, 0.38±0.05, 0.39±0.15 *vs* 0.40±0.10 and P; 10.67±0.05, 10.17±0.05, 10.44±0.16 *vs* 11.13±0.15, respectively) between groups (Table 2). Therefore, caffeine led to a low content of mineralization of organic matrix for femur bone rats. The impact of caffeine on bone minerals was related to Ca metabolism. Caffeine consumption was associated with slightly impairs Ca absorption from intestines; however it has no effect on Ca excretion in urine. Still its intake causes a decrease of bone mass and an increase of bone fracture risk. Caffeine has no destructive influence on the bone minerals of people with sufficient calcium intake [26]. The perimenopausal women who intake high caffeine (> 450 mg/dl) had increased loss of BMD, however this is only in cases of low Ca intake (< 800 mg/dl).

Bone density of Saudi's people was lower than their counterparts Americans and Northerners European [27]. Coffee is the highest sources of caffeine consumption in the KSA and it is known that the most common type of coffee consumption in Saudi Arabia is the Arabic coffee and instant coffee [15], the instant coffee was the highest types of caffeine, followed by Arabic coffee 3.89% and 2.00%, respectively. This leads us to consider consumption coffee in the quantities tested in the present study; (equivalent to 258, and 211 and 305 mg/day) was a risk factor for bone mineral content and BMD in healthy Saudis in process of growth with the length of consumption time [28].

The obtained results showed the negative impact of caffeine on the blood of rats ($p \leq 0.01$) (Figure 1). It was found that the Caffeine-time (3) increased the same amount almost in Group 2 and 3 with an advantage few of Group 3 (2.62 and 2.65, respectively), and that the Caffeine-time (6) increased more significantly in Group 3 (2.50) while no increase in Group 2 and 4. The caffeine-time (9) had increased more significantly in Group 2 (1.49) while no increase in Group 3 and 4 (Figure 1). In the present study regards to urine, the level of caffeine showed an effect of interaction between caffeine-treated rats and increasing time ($p \leq 0.01$) (Figure 2). It was found that the Caffeine-time (3) increased more significantly in the Group 2 (199.00), as well as in time (6 and 9; 303.22 and 450.98; respectively).

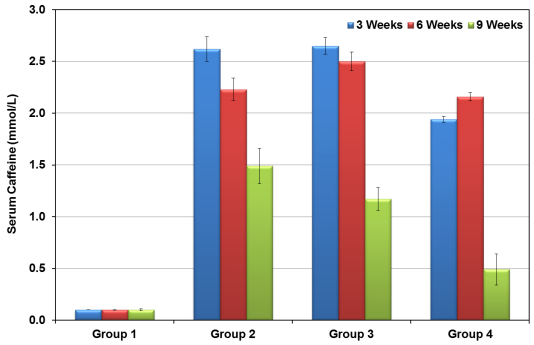

Figure 1. Serum caffeine level at 3, 6 and 9 weeks of control and animal groups (n = 12) subjected to daily coffee intake. Data show a significant increase of caffeine after consumption of caffeine at each time point. Values were express as means±S.E.; Comparisons between groups were performed using ANOVA ($p < 0.01$, significantly different from the control group).

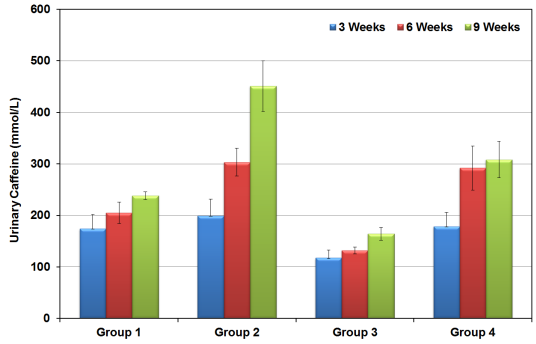

Figure 2. Urinary caffeine at 3, 6 and 9 weeks in the controls and animal groups (n = 12) subjected to daily coffee intake. Data show a significant increase of caffeine after consumption of caffeine at each time point. Values were express as means±S.E.; Comparisons between groups were performed using ANOVA ($p < 0.01$, significantly different from the control group).

In general the levels of metals were very close at the end of the experiment in serum, the level of Ca, P and Mg were decreased with increasing the dose over time (Table 3). This was contrary to a study of Chen and Whitford, [29], who states that caffeine had no effect on the balance of Ca and P, as well as it had no effect on the removal of minerals from bone.

Table 3. Serum calcium phosphorus and magnesium (mg/dl) levels in animals subjected to daily intake of caffeine with time *.

Parameters	Time	Groups			
		1	2	3	4
Ca (mg/dl)					
	0	9.8± 1.11	9.8±1.11	9.8±1.01	9.8±0.97
	3	9.0± 1.43	6.8±1.43	6.3±0.73	8.7±2.03
	6	8.2±1.71	8.2±1.71	7.6±1.43	9.1±1.01
	9	10.1±2.22	8.3±2.22	7.9±1.91	9.8±1.32
Mg (mg/dl)					
	0	0.94±0.29	0.96±0.29	0.96±0.12	0.92±0.33
	3	0.82±0.16	0.71±0.59	0.67±0.36	0.84±0.06
	6	0.85±0.11	0.83±0.18	0.85±0.17	0.85±0.59
	9	0.94±0.09	0.85±0.13	0.82±0.03	0.95±0.06
P (mg/dl)					
	0	3.83±0.29	4.24±0.29	3.47±0.12	2.83±0.33
	3	2.74±0.16	2.19±0.59	2.21±0.36	2.65 ±0.06
	6	2.54±0.06	2.55±0.11	2.83±0.46	2.08±0.58
	9	2.57±0.09	2.51±0. 13	2.54±0.36	2.49±0.06

* Values were express as means±S.E.; comparison between control group within caffeine groups was done by analysis of covariance; multiple comparisons were done by using post-hoc test; significantly different between groups by one way Anova; Significantly different: $p < 0.05$.

Table 4. Urinary calcium, phosphorus and magnesium (mmol/L) in animals subjected to daily intake of caffeine with time *.

Parameters	Times	Control	Low dose	Medium dose	High dose	Significance
Ca (mmol/L)						
	3	0.62±0.56 [a]	1.12±0.22 [a]	1.32±0.72 [a]	0.28±0.13 [a]	$p \leq 0.05$
	6	0.17±0.06 [a]	0.37±0.46 [a]	0.30±0.37 [a]	0.25±0.18 [a]	$p \leq 0.05$
	9	0.16±0.05 [a]	0.34±0.05 [a]	01.4±0.04 [a]	0.36±0.45 [a]	$p \leq 0.05$
	Final	0.17±0.06 [a]	0.31±0.15 [a]	0.11±0.03 [a]	0.40±0.32 [d]	$p \leq 0.05$
P (mmol/L)						
	3	1.25±1.14 [a]	0.76±0.07 [a]	0.37±0.19 [c]	0.43±0.08 [d]	$p \leq 0.05$
	6	0.41±0.28 [a]	0.86±0.62 [b]	0.27±0.05 [a]	0.29±0.19 [a]	$p \leq 0.05$
	9	0.43±0.32 [a]	0.18±0.08 [a]	0.31±0.18 [a]	3.01±0.63 [a]	$p \leq 0.05$
	Final	0.45±0.35 [a]	0.21±0.06 [a]	0.25±0.14 [a]	1.54±0.30 [d]	$p \leq 0.05$
Mg (mmol/L)						
	3	2 71±0.18 [a]	3.54±2.13 [a]	5.65±1.21 [c]	4.19±2.22 [d]	$p \leq 0.05$
	6	4.34±1.00 [a]	4.04±1.67 [a]	5.01±1.29 [a]	4.94±1.49 [a]	$p \leq 0.05$
	9	4.25±1.09 [a]	6.01±0.72 [a]	5.02±1.52 [a]	6.70±1.54 [d]	$p \leq 0.05$
	Final	4.39±1.12 [a]	6.64±0.88 [b]	5.52±1.08 [c]	6.64±1.29 [d]	$p \leq 0.05$

* Values were express as means±S.E.; comparison between control group within caffeine groups was done by analysis of covariance; multiple comparisons were done by using post-hoc test.
** Significantly different between groups by one way Anova; [a,b,c,d] significantly different, $p < 0.05$.

Also, the findings of a study [30] reported that there was no any difference in the amount of bone mineral in serum, although the Arabic coffee had a negative effect on BMD.

The obtained data concerning the level of minerals in urine, the mean value of Mg was significant declined for each Group 3 and 4 whereas, the mean value of P was significantly raised in Group 3 (p <0.05) compared to the control group (Table 4). There was no significant difference in the values of Ca between three caffeine-treated groups compared to control group. When comparing the level of Ca and P in urine during the second period with the control group, it noticed that no significant differences in the three groups, but the level of Mg was increased significantly in Group 2 during the same period ($p \leq 0.05$). Whereas, in the third period, the level of Ca and Mg had no significant differences among the study groups, but the level of (P) was increased significantly ($p \leq 0.05$) in Group 4. The urinary metals at the end of the experiment was clear compared to the control group, the level of Ca and Mg were increased significantly ($p \leq 0.05$) in Group 4, but p was raised ($p \leq 0.05$) in all groups.

Some authors observed a relationship between caffeine intake and change of minerals content in bone [8,31,32]. Consumption of caffeine increase Ca and Mg excretion in urine and this increase dependent on dose manner [30]. Sakamoto *et al.*, [33] measurement the levels of urinary Ca and Mg in three time periods of two groups (low and high-caffeine dose), caffeine did not effect on Ca excretion when comparing groups with the three time periods, whereas output of P did not affect by caffeine during the first and second periods but increased significantly ($p \leq 0.05$) during the third period. Other authors were contrary, they stated that caffeine led to a defect in Ca balance and the process of subtraction, but its effect on the increase of urinary Ca and P excretion did not lead to any effect on the absorption of Ca or P [34].

4. Conclusion

Intakes of caffeine in amounts >300 mg/dl significantly affected the quantitative composition of the bone and lead to a greater risk for bone loss. These results suggested that appropriate lifestyle changes to conserve BMD by reducing the consumption of caffeine and need further studies to elucidate the molecular mechanism that caffeine effects on bone metabolism.

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