

## **Histological and biochemical effects of caffeine on bone of growing rats**

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### **ABSTRACT**

The present study has been carried out to evaluate the histological and biochemical effects of caffeine on bone of growing rats.

A total of 25 rats (8 weeks old) were divided randomly into three groups: Group 1(n = 5), serve as the control group, group 2 (n = 10), caffeine-low dose; group 3 (n =10), caffeine high dose. The caffeine was fed by gastric tube daily and the high dose of caffeine=10 mg/100 g body weight/day, and the low dose of caffeine=2.5 mg/100g body weight/day. Body weight was measured weekly. After sacrifice, blood samples were collected in tubes, and serum samples were separated for the determination of Ca, ALP, Zn and Mn. Femoral bones of rats were used for histopathological assessment. The results showed that serum Ca, Zn and Mn levels for high dose group is significantly lower than the low dose than the control group. The Alkaline phosphatase in low dose group is not significant smaller than the control group, but the high dose group has significantly elevation value than the control group. Thickness of epiphyseal plate especially proliferating and hypertrophic zones compared to control group was observed in the group which received low dose of caffeine. Section of diaphysis of low-dose treated group showed thinning out of the outer compact bone with multiple osteoporotic cavities in the bone matrix. The epiphyseal plates of caffeine-high dose showed disturbance of bone growth as revealed by presence of numerous clefts among rows of cartilage cells of the epiphyseal plate, between the proliferating and hypertrophic zone and between calcified and ossified zones.

Thus, it can be concluded that the oral administration of caffeine lead to a significant reduction in serum Ca, Zn and Mn; and a significant elevation in serum ALP together with a dose dependent histological changes in the bone of treated rats according to the increase of caffeine dose.

**Keywords:** Caffeine, Ca, Zn, Mn and ALP levels, osteoporosis, histological changes and femoral growing rat bones.

### **INTRODUCTION**

Caffeine is one of the most commonly consumed drugs in the world (Nawrot *et al.*, 2003). Many popular beverages, such as coffee, tea, soda, and energy drinks contain caffeine, as

well as other products, such as chocolate and medications. Because of caffeine's significant physical, metabolic and psychoactive properties, there is considerable public health interest in its effects on humans (Larry, 2017). Caffeine-containing beverages are marketed directly to children (Bramstedt, 2007) and that caffeine use is growing among children (Frary *et al.*, 2005), it is important to understand the potential effects of caffeine use within this population. The people consuming high amounts of caffeine tend to carry a higher risk of developing bone problems, including osteoporosis, as well as problems in metal absorption, excretion and reabsorption processes in intestines and in kidney (Chen and Whitford, 1999; Massey, 2001; Borse *et al.*, 2002 and Pan *et al.*, 2003), and iron deficiency anemia (Hallberg and Rossander, 1982). Caffeine interact with adenosine receptors, phosphodiesterase, channels and intracellular  $Ca^{2+}$  release (Teramoto *et al.*, 2000; Montano *et al.*, 2003 and Daly, 2007). Caffeine has been shown to inhibit (Dall'Igna *et al.*, 2003; Alvira *et al.*, 2007 and Wang and Lu, 2007) or to stimulate the apoptosis of nucleated cells (Hirata *et al.*, 2006; Matsuoka *et al.*, 2006; Gabrielli *et al.*, 2007 and Lu *et al.*, 2007), an effect depending on the cell type and the presence of other challenges. Caffeine has several biological and pharmacological properties including; anti-inflammatory, anticarcinogenic, immune modulator and antioxidant activities (Abd El-Rahman and Sherif, 2015) and anti-apoptotic activity (Bavari *et al.*, 2016).

Caffeine is a well-known risk factor for osteoporosis and fractures in humans, as it causes bone mineral loss (You *et al.*, 2011). Studies of immature rats have demonstrated that chronic exposure to caffeine inhibits both bone mineral mass and long bone growth (Shin *et al.*, 2015; Choi *et al.*, 2016 and Choi *et al.*, 2017). Osteoporosis is a progressive and systemic metabolic bone disease characterized by a reduction in bone mineral density and microarchitecture of bone tissue, resulting in increased bone fragility, increased risk of fractures and other pathological diseases (Kanis, 2007). Serum levels of Alkaline phosphatase (ALP) provide a good impression of the extent of new bone formation and osteoblast activity (Van Straalen *et al.*, 1991). Zinc (Zn) is a structural constituent of some proteins, including enzymes belonging to cellular signaling pathways and transcription factors, that stimulates osteoblastic cell proliferation, differentiation and mineralization (Yang *et al.*, 2012). Manganese (Mn) is an essential trace element, which plays a role in lipid and carbohydrate metabolism. In the skeleton, manganese positively modulates RANKL/OPG (receptor activator of NF- $\kappa$ B ligand (RANKL) /osteoprotegerin (OPG) ratio in the process of bone formation, determining thickness of trabecular bone area and increasing trabecular number (Liu *et al.*, 2015). Like zinc; manganese accelerates growth via activation of somatomedin synthesis (Zofkova *et al.*, 2013). Caffeine increases urinary calcium excretion by reducing renal reabsorption and, possibly, reducing calcium absorption, leading to a negative calcium balance (Bergman *et al.*, 1990; Massey and Whiting, 1993 and Ilich *et al.*, 2002). Bone tissue consists of hydroxyapatite mineral, protein, water and lipids. Collagen is the principal matrix protein upon which hydroxyapatite is deposited. It also exists other non-collagenous proteins (27%), which are primarily synthesized by connective tissue cells (Jee, 1983 and Boskey, 1989). During growth, the alteration of the size

and shape of bones occurs by bone formation and resorption at different surfaces and at different rates. This modeling process leads to the final gross bone structure. Because mineralization lags behind matrix production by osteoblasts during bone formation, there remains a layer of unmineralized matrix that appears as osteoid seams (**Melsen and Mosekilde, 1988**).

The three major components of bone are osteogenic cells, organic matrix, and mineral. The osteogenic cells include osteoblasts, osteocytes, and osteoclasts, while the matrix consists predominantly of collagen and proteoglycans and constitutes approximately one third of the bone mass. The mineral that makes up approximately two thirds of bone is composed of calcium phosphate crystals deposited as hydroxyapatite. Caffeine may adversely affect osteoblasts, either directly or indirectly, by lowering trace mineral concentration's in plasma and bone, which in turn might lead to retarded bone development (**Wink et al., 1996**). In other words, if young, rapidly growing rats are exposed to caffeine, disruption of osteoblasts and retarded bone development occur, which could be related to the decreased plasma Cu level in the young animals.

This study has been conducted to investigate; the histological and biochemical effects of oral administration of low and high doses of caffeine on bone of growing rats.

## **MATERIALS AND METHODS**

### **Experimental animals:**

A total number of 25 albino rats (7-weeks old) with a mean body weight of  $100 \pm 10$  g. They were purchased from Egyptian Vaccine and Antibody Company (VACSERA, Giza, Egypt). The rats were maintained under standard conditions of light, ventilation, temperature, and humidity and allowed free access to standard pellet diet and tap water. After 1 week of adaptation; rats were fed with Purina Laboratory Rodent Diet (PMI; St. Louis, MO) (0.95% calcium) and distilled water ad-libitum. This approach was used because many younger adults and more than 50% of older adults living in the United States, including those who use calcium-containing supplements (**Ervin and Kennedy-Stephenson, 2002**). The experiment began when the rats were 8- weeks old. The experiments were accepted by the state authorities and it followed the Egyptian rules on animal protection, as well as specific local institutional laws for protection of animals under the supervision of authorized examiners.

### **Experimental design:**

The rats were randomly divided into three groups: Group (1); contains 5 rats and served as a control, group (2); contains 10 rats and each rat received (10 mg/100g body weight) caffeine via a gastric tube daily (caffeine-high dose group) and group (3); contains also 10 rats and each rat received (2.5 mg/100g body) caffeine via a gastric tube daily (caffeine-low dose group).

### **Caffeine treatment:**

Caffeine was purchased from Sigma Chemical Co., St. Louis, MO, USA. The product is provided as a concentrated exudate, dissolved in saline. 0.5% gm caffeine solution was prepared, the high dose of caffeine=10 mg/100g body weight/day, and the low dose of caffeine=2.5 mg/100g body weight/day. The dose of caffeine was delivered to each rat by a gastric tube daily throughout the duration of the experiment (8 weeks) (**Yeh and Aloia, 1986 and Glajchen et al., 1988**).

### **Sampling:**

Twenty-four hours after the last dose of caffeine treatment. Blood samples were obtained from retro-orbital plexus of vein by capillary tube then rats were killed by decapitation under light anesthesia with diethyl ether at the end of experimentation (8 weeks).

### **Biochemical study:**

Blood samples were collected, and sera were separated for measurements of Ca, ALP, Zn and Mn. Zn and Mn were measured by atomic absorption spectrophotometry (Perkin-Elmer 560, Norwalk, CT). Ca was measured by Colorimetric method based on formation of color complex between calcium and o-cresolphthalein in alkaline medium (**Young, 1997**). ALP was measured by "Optimized standard method" according to the recommendations of the German Clinical Association (Deutschen Gesellschaft für Klinische Chemie) (**Klin, 1972**). Ca and ALP are measured by Biosystem BTS 330 spectrophotometer analyzer.

### **Histological study:**

Femora of rats were dissected out by removal of muscles around, and fixed in 10% neutral buffered formalin for 5 days then decalcified by using 14% EDTA (**Dong et al., 2009**) and processed for making paraffin blocks. 5- $\mu$ m cross and longitudinal sections were cut and stained with hematoxylin and eosin (H&E) for routine histological study, Masson Trichrome stain for detection of collagen and Periodic acid Schiff (PAS) for detection of mucopolysaccharides (**Boncroft and Gamble, 2008**).

### **Statistical analysis:**

The results were expressed as mean  $\pm$  SD of different groups. The differences between the mean values were evaluated by one-way analysis of variance ANOVA followed by Tukey-Kramer multiple comparison test (**Armitage and Berry, 1987**) using Graph Pad Prism software. *P* values < 0.05 were statistically significant.

## **RESULTS AND DISCUSSION**

The effect of caffeine on bone is controversial, therefore, this study was performed to clarify the histological changes induced by caffeine administration on bone of young rats and to

estimate the serum levels of ALP, Ca, Zn and Mn by biochemical methods. At 8 week after caffeine feeding, Alkaline phosphatase (ALP), Calcium (Ca), Zinc (Zn) and Manganese (Mn) serum levels are measured. This is obvious in (**Table 1**) and (**Figure 1**).

In this study, the level of alkaline phosphatase in low dose group; recorded (**39.30±4.244<sup>ns</sup>**) UI/L and this value is slightly lower than its value in the control group (**39.75±1.258**) without a significant change, but the high dose group recorded (**50.40±5.168<sup>\*\*</sup>**) and this value is detected as an extremely significantly elevation compared with the control group. The low dose of caffeine induced a non-significant change of alkaline phosphatase level compared to control value; this finding is in agreement with (**Liu *et al.*, 2011**) who showed that the low concentration of caffeine did not affect the bone marrow cell viability and alkaline phosphatase activity during osteoblast differentiation from bone marrow stromal cells, but it effectively enhanced the osteoclastogenesis from bone marrow hematopoietic cells and the bone resorption activity by pit formation assay.

In the present study, the low dose of caffeine induced an extremely significant decrease in serum calcium level (**8.990±0.3957<sup>\*\*\*</sup>**) mg/dl; compared with the control values (**10.40±0.6325**). On the other hand, the high dose of caffeine induced also an extremely significant decrease in serum calcium level (**7.750±0.5126<sup>\*\*\*</sup>**) compared with the control values. Blood Ca levels in experimental groups revealed an extremely significant decrease in both low-dose and high-dose treated groups. These findings were in harmony with the results of (**Conlisk and Galuska, 2000**); who attributed the decrease in blood calcium level to the increase in urinary excretion of Ca by caffeine. Moreover, **Heaney (2002)**, added that caffeine has a clear depressant effect on intestinal absorption of calcium. Caffeine dose dependently decreases vitamin D receptor expression and alkaline phosphatase enzyme activity in human osteoblasts; constituting a possible mechanism by which caffeine may affect bone metabolism. The effect of caffeine on bone tissue is related to calcium metabolism. Where caffeine slightly impairs calcium absorption from intestines; however, it has no effect on calcium excretion with urine (**Heaney, 2002**). In addition, **Kamagata-Kiyoura *et al.* (1999)** demonstrated that caffeine has an inhibitory effect on the proliferation of osteoblast like cells *in vitro*. **Rapuri *et al.* (2007)**, concluded that 1,25- Dihydroxy vitamin D3 performs a fundamental 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 vitamin 1,25(OH)2D3 and controlled by vitamin 1,25(OH)2D3 activity of human osteoblast cells by reducing alkaline phosphatase activity.

Serum Zn level for the low dose of caffeine (**78.90±4.533<sup>\*\*\*</sup>**) µg/dl recorded an extremely significant reduction in serum zinc level, compared with the control values (**96.80±7.294**), and the high dose of caffeine also, induced also an extremely significant reduction in serum zinc level, compared with the control value (**70.30±3.802<sup>\*\*\*</sup>**). Zinc plays an important role in the maintenance of cell membrane structure and function (**Bettger and O'Dell,**

**1993**). Zinc deficiency causes a reduction in osteoblastic activity, collagen and chondroitin sulfate synthesis, and alkaline phosphatase activity (**Calhoun et al., 1974**). In the presence of caffeine, the elements; such as zinc, copper, magnesium and calcium have relatively low intestinal absorption efficiency and are excreted primarily in feces (**Avioli, 1980; Li and Vallee, 1980 and Shils, 1980**).

Serum Mn level for low dose of caffeine ( $3.450 \pm 0.4327^*$ )  $\mu\text{g/dl}$  was significantly decreased compared with the normal values ( $4.120 \pm 0.4324$ ), and the high dose of caffeine ( $3.170 \pm 0.4739^{**}$ ) also, induced a significant reduction in Mn values, compared to normal values. Manganese absorption is influenced by many factors such as chemical form, presence of chelating or complexing agents and interactions between different micronutrients. It has been suggested that manganese and iron share a common mechanism of absorption and transport in the digestive tract (**Keen and Zidenberg-Cherr, 1996**). This finding is in consistent with **Leach and Harris (1997)**, who found that Mn and Fe share mechanisms of transport and cell uptake.

Histopathological studies of the bone sections of control and treated rats with low and high doses of caffeine; are shown in (**Figures 2-11**), these were carried out to examine the effect of oral administration of low and high dose of caffeine on bone of growing rats. Section of lower end of the femur of control rats; showing the normal histological structure of the epiphyseal plate and metaphyseal spongy bone (**Figure 2**). The longitudinal growth was evidently observed in four success zones, resting, proliferating, hypertrophic and diaphysis showing periosteum (P), endosteum (E) and bone matrix containing osteocytes (**Figures 2&3**). In this study, marked histological changes are observed in caffeine treated rats. Thickness of epiphyseal plate especially proliferating and hypertrophic zones compared to control group was observed in a group which received a low dose of caffeine (**Figure 4**). The diaphysis of a low dose treated group showed thinning out of the outer compact bone with multiple osteoporotic cavities in the bone matrix (**Figure 5**). Moreover, diaphysis of the same group showed an increase thickness of the outer fibrous layer of periosteum and proliferation of the inner osteogenic cells of the periosteum (**Figure 6**). Numerous osteoporotic cavities filled with a granulation tissue are also seen in the matrix (**Figure 7**). The present study revealed that, the administration of a low dose of caffeine induce an increase of long bone growth as evident by thickness increase of the epiphyseal plate due to thickness increase of the proliferating and hypertrophic zones. However, the proliferating zone showed abnormal multiple areas of fusing rows of chondrocytes which leads to appearance of numerous areas of lost cells. Similar results were also recorded by **Huang et al. (2002)**, who attributed these changes to the stimulation of growth hormone release from pituitary gland by the effect of caffeine that enhances and increase the activity and growth of chondrocytes of the epiphyseal plate. The epiphyseal plate section of a caffeine-high dose revealed a disturbance of bone growth as emphasized by presence of numerous clefts among rows of cartilage cells of the epiphyseal plate, between the proliferating and hypertrophic zone and between calcified and ossified zones (**Figure 8**). Cancellous bone trabeculae appeared thin, short with a few collagens in their matrix and separated by numerous wide bone marrow cavities.

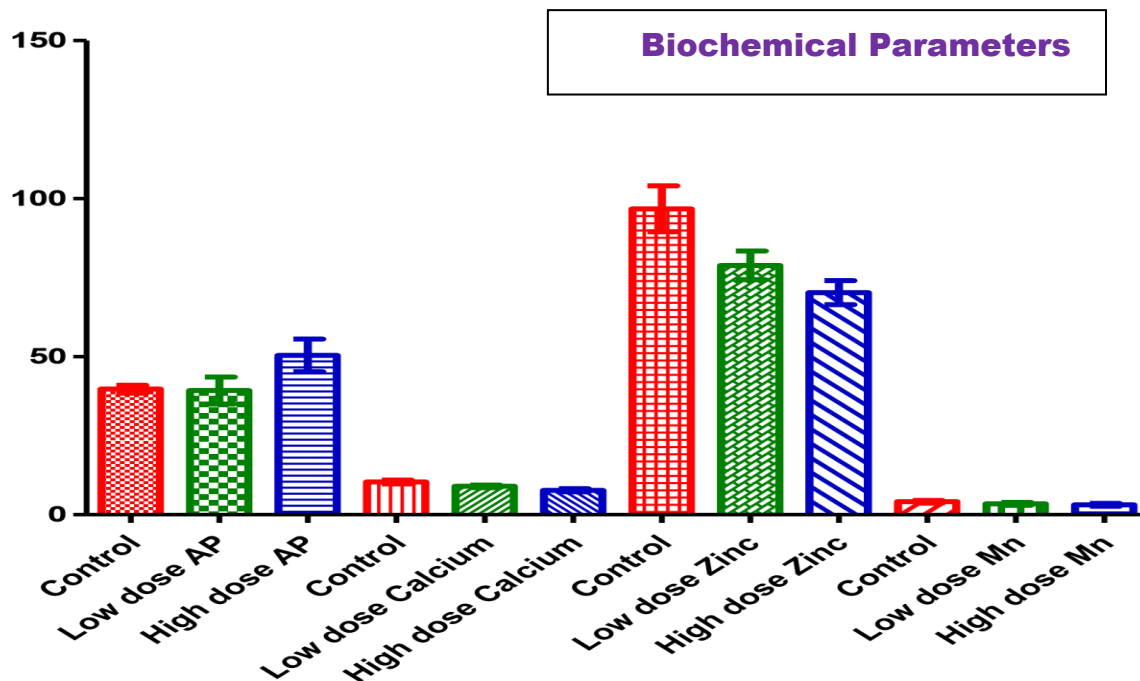
The diaphysis revealed multiple osteoporotic cavities of variable size and shape filled with a granulation tissue, some of these cavities open on the surface (**Figure 9**). The periosteum showed increased thickness mainly in its fibrous layer which invaginate deeply inside bone matrix. Irregular basophilic areas appeared inside acidophilic bone matrix denoting presence of defects along cement lines (**Figure 10**). Also, the bone matrix showed lightly stained areas denoting defect in mineralization and lack of collagen in these areas (**Figure 11**). With high dose of caffeine intake, the epiphyseal plate showed reduction in length mainly; the proliferating and hypertrophic zones. This, could be explained by the negative feedback effect of caffeine on growth hormone level (**Spindle et al., 1984**). The transverse section of diaphysis of caffeine-treated groups; showed an apparent reduction in the cortical width of the bone matrix and thinning of the cortical bone with appearance of multiple resorption cavities and multiple defects in cement lines which were dose-dependent. The outer surface of the bone shaft displays irregularities overlaid by thickened periosteum and some of the resorption cavities open on the surface, with hyperplasia of the outer fibrous and inner osteogenic layer of the periosteum. **Tsuang et al. (2006)** concluded that, caffeine may induce apoptosis and decrease the viability of osteoblasts. **Focking et al. (2005)** showed that, caffeine amplifies the expression of the glucocorticoid receptor in osteoblastic cells. It has been reported that glucocorticoids induce the apoptosis of osteoblasts and, therefore, the glucocorticoid receptor is one of the most important factors in the induction of osteoporosis in humans. **Conradie et al. (2007)** reported that, the decrease number of osteocytes in bone matrix of caffeine treated group could be explained by the inhibitory effect of caffeine on osteoblast differentiation which result in defect in transformation of osteoblasts into osteocytes and subsequently result in defect in bone mineralization. The presence of non-homogenous matrix, numerous osteoporotic cavities, osteoclasts and thin bone trabeculae could be explained by deficiency of Ca, Zn and Mn which induces a defect in bone mineralization, as discussed by **Wink et al. (1996)** who found that, Mn and Zn deficiency impair the ability of osteoblasts to manufacture bone matrix. However, **Durate et al. (2009)** explained that, the high doses of caffeine did not alter bone mineral density after two months, but it disturbs the early stage of bone healing. Cavities filled with granulations and fractures; which are remarked in some sections at definite sites; could be explained by the direct effect of caffeine on bone as reported by **Ohta et al. (1999)** who added that early caffeine administration resulted in alteration in the mechanical properties of bone under applied load suggesting that caffeine may be a possible risk factor for fracture and this risk was dose and time-dependent (**Kiel et al., 1990**).

It is concluded that oral administration of caffeine leads to a significant reduction of serum Ca, Zn and Mn and significant elevation of serum ALP together with destruction changes in the bone histological structure and this was dose dependant. Nowadays, it is impossible to avoid caffeine intake, and its effect on bone tissue is not fully understood. Further studies are in progress to elucidate cellular and biochemical mechanisms of effects of caffeine on bone metabolism.

**Table (1):** The effect of low and high doses of caffeine on the levels of ALP, Ca, Zn and Mn respectively; on bone of growing rats.

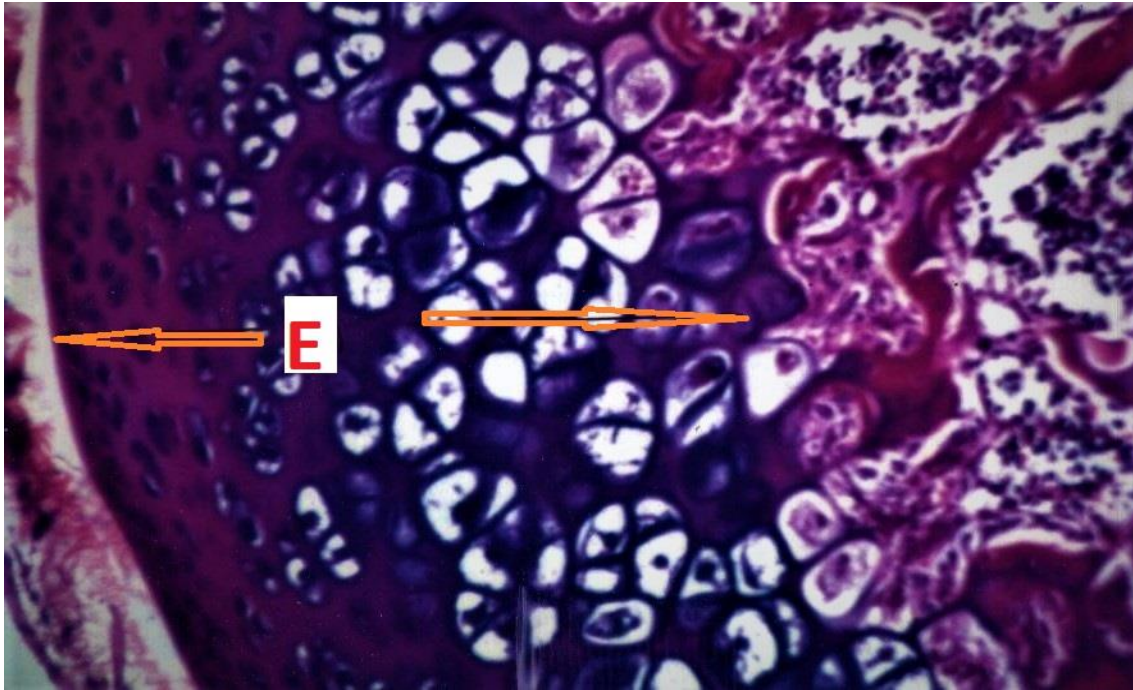
Biochemical Parameters	Control	low dose of caffeine	high dose of caffeine
Serum ALP (UI/L)	39.75±1.258	39.30±4.244 <sup>ns</sup>	50.40±5.168 <sup>**</sup>
Serum Ca (mg/dl)	10.40±0.6325	8.990±0.3957 <sup>***</sup>	7.750±0.5126 <sup>***</sup>
Serum Zn (µg/dl)	96.80±7.294	78.90±4.533 <sup>***</sup>	70.30±3.802 <sup>***</sup>
Serum Mn (µg/dl)	4.120± 0.4324	3.450±0.4327 <sup>*</sup>	3.170±0.4739 <sup>**</sup>

All data are expressed as mean ± SD. (\*\*\*) Extremely Significant  $P \leq 0.001$ ; (\*\*) Very Significant  $P \leq 0.01$ ; (\*) Significant  $P \leq 0.05$  and (<sup>ns</sup>) Not Significant  $P > 0.05$ .

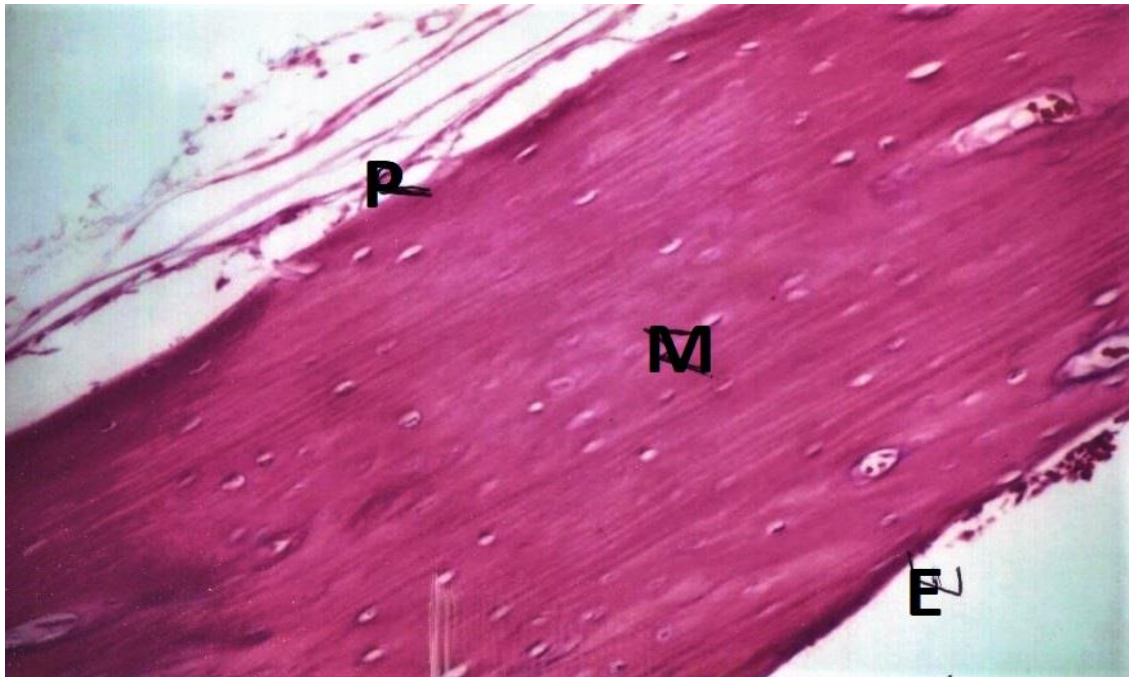


**Fig. (1):** A histogram illustrates the effect of low and high doses of caffeine on the levels of ALP, Ca, Zn and Mn respectively; on bone of growing rats.

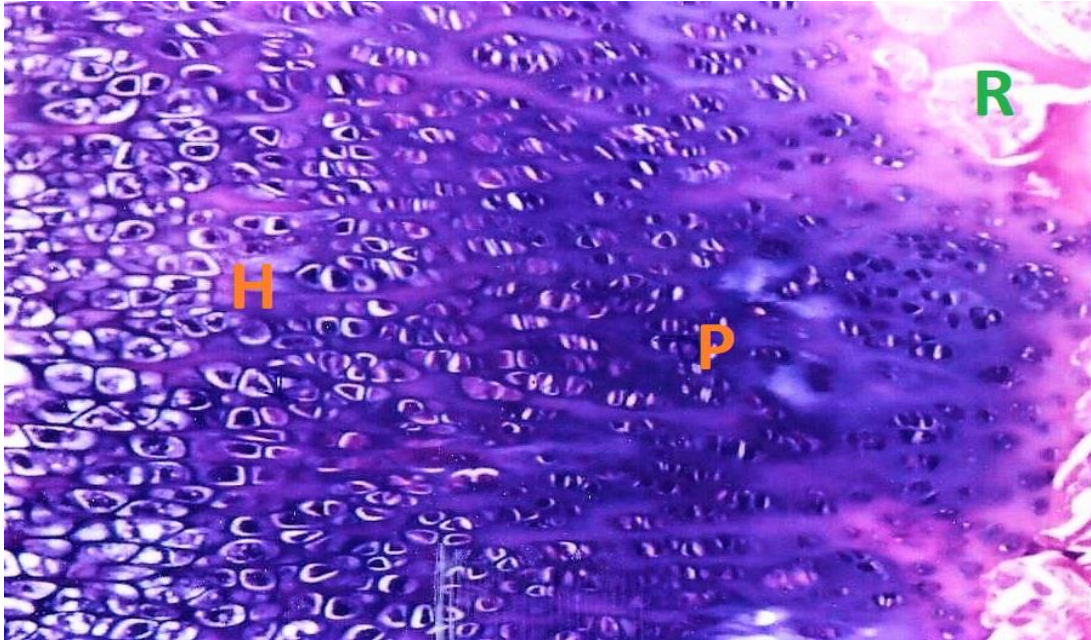




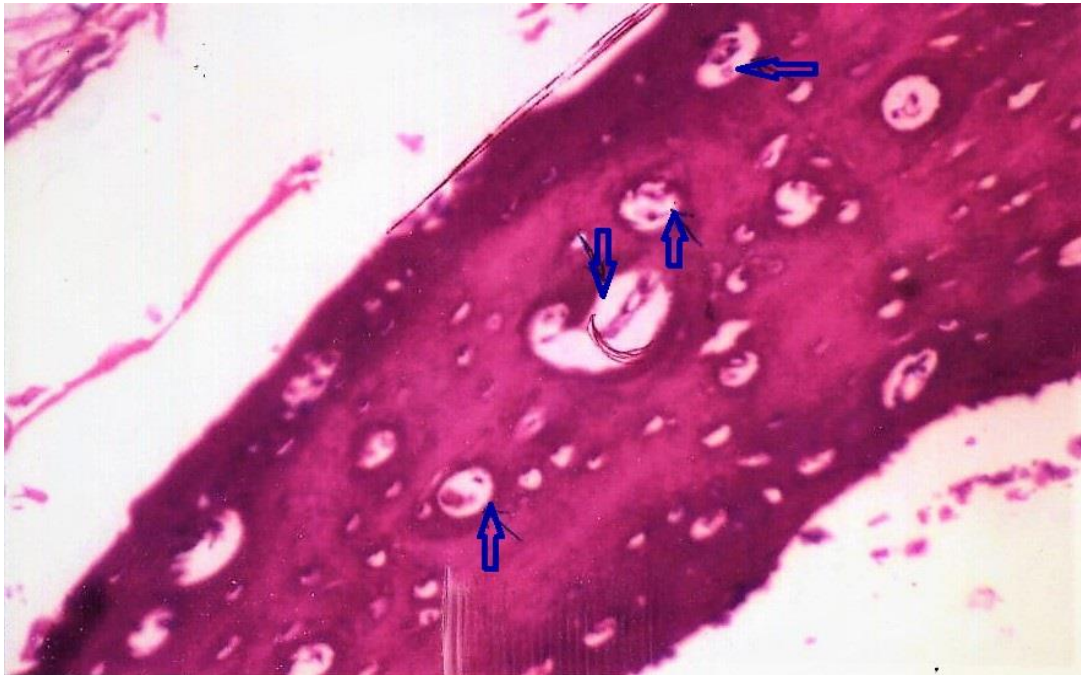
**Fig. (2):** A photomicrograph of epiphyseal plate (E) of a control rat showing normal structure of epiphyseal plate (H&E X400).



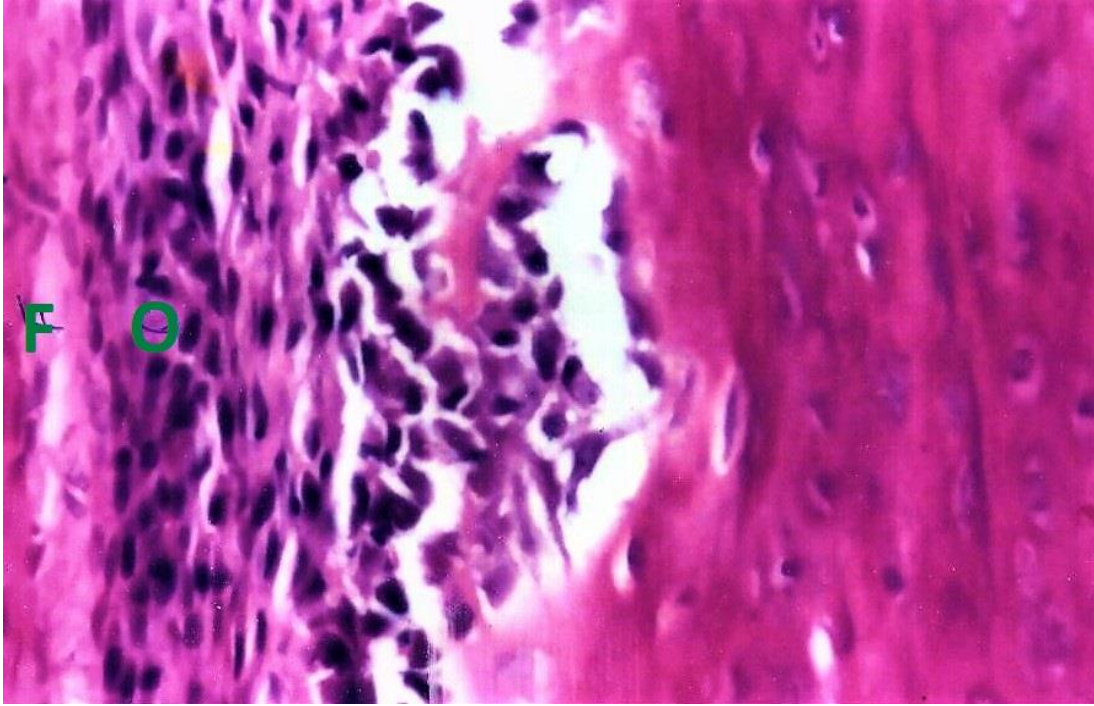
**Fig. (3):** A photomicrograph of diaphysis of a control rat showing periosteum (P), endosteum (E) and bone matrix containing osteocytes (M) (H&E X200).



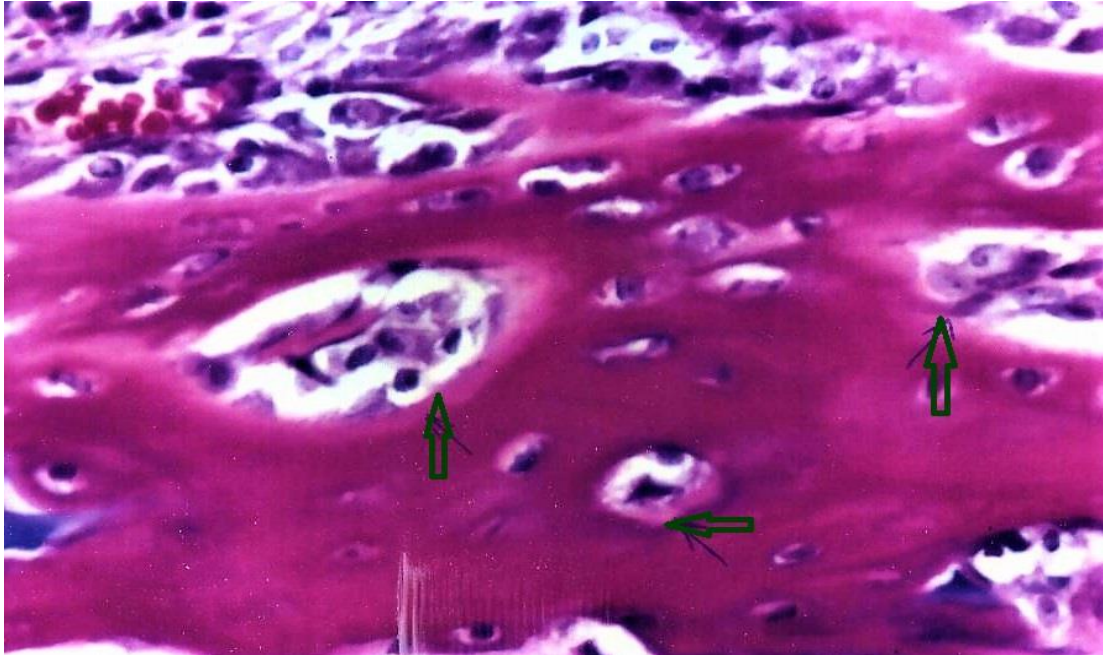
**Fig. (4):** A photomicrograph of bone section of low dose –treated group showing increase thickness of epiphyseal plate especially; reserve (R), proliferating (P) and hypertrophic zones (H) (H&E X100).



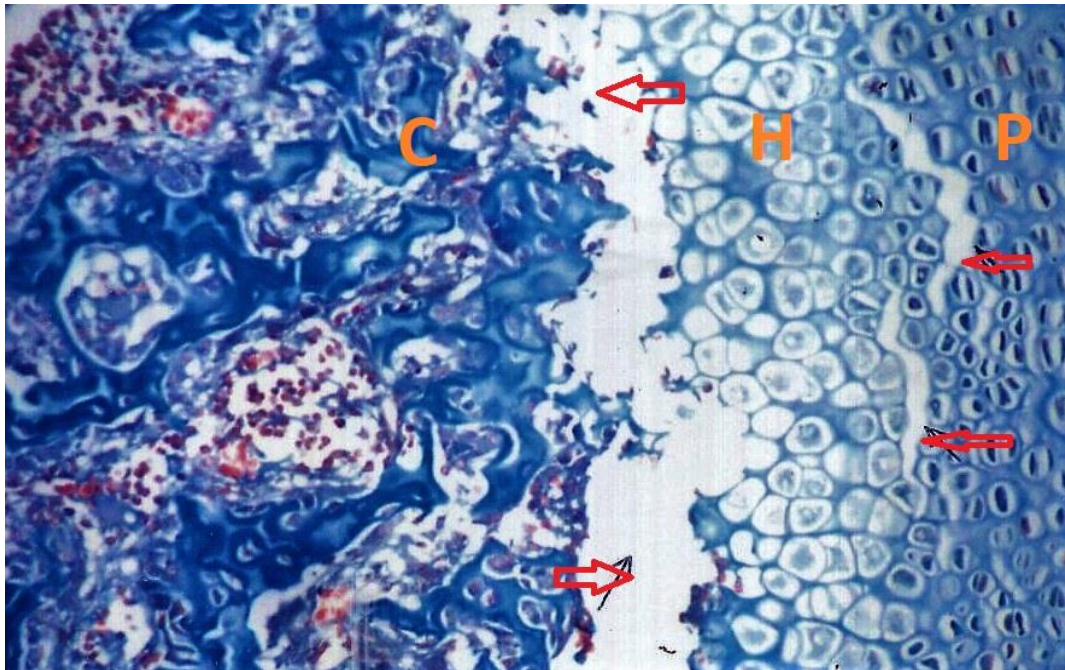
**Fig. (5):** A photomicrograph of a diaphysis section of caffeine low dose treated rats showing thinning out of the compact bone with multiple osteoporotic cavities in the bone matrix (↑) (H&E X200).



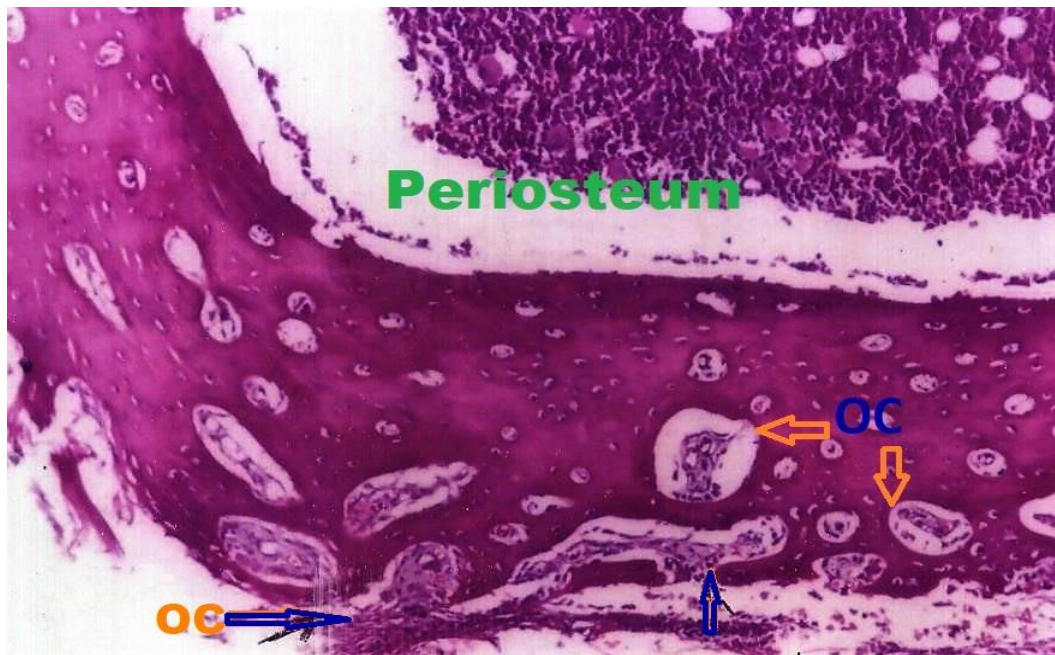
**Fig. (6):** A photomicrograph of a diaphysis section of caffeine low dose treated rats showing increase thickness of outer fibrous (F) and inner osteogenic (O) layers of periosteum (H&E×400).



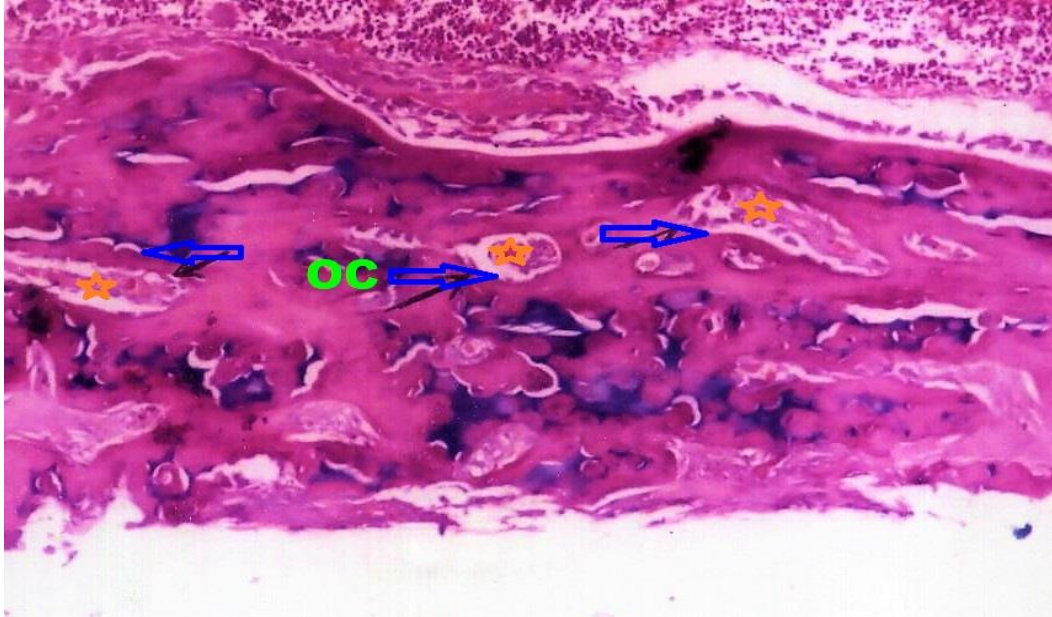
**Fig. (7):** A photomicrograph of a bone section of caffeine low dose treated rats showing multiple osteoporotic cavities filled with a granulation tissue (↑) (H&E X400).



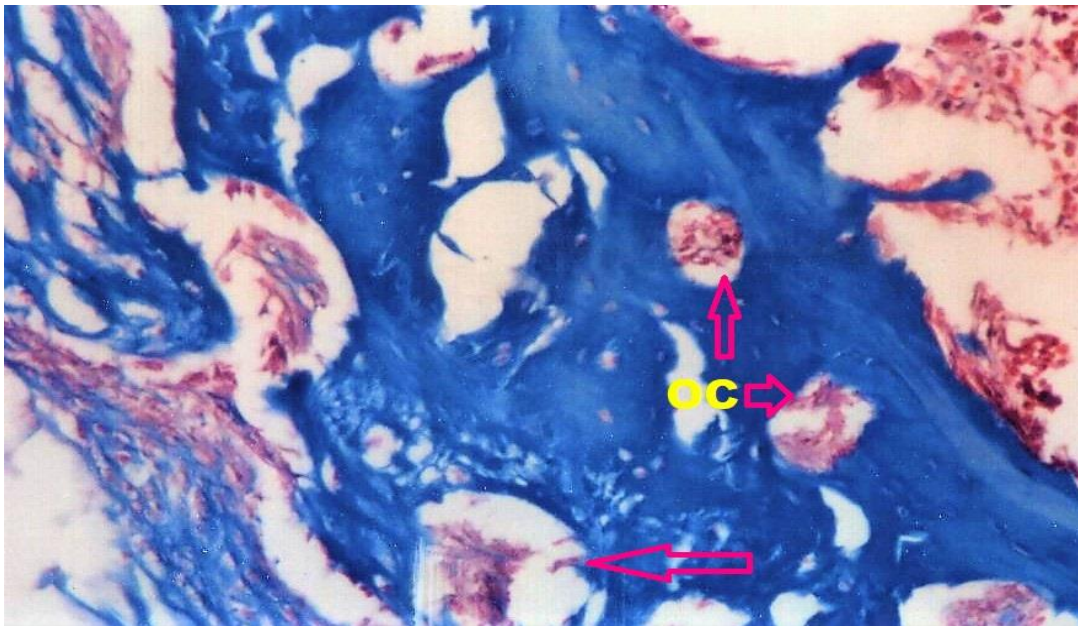
**Fig. (8):** A photomicrograph of a bone section of caffeine high dose treated rats showing disturbance of bone growth as revealed by presence of numerous clefts (↑) among cells of epiphyseal plate between proliferating (P) and hypertrophic zones (H) and between hypertrophic (H) and calcifying zones (C) (Mt X200).



**Fig. (9):** A photomicrograph of a bone section of caffeine high dose treated rats showing multiple osteoporotic cavities (OC;↑) and thinning out of periosteum and some of these cavities open on the bone surface (↑) (H&E×100).



**Fig. (10):** A photomicrograph of a bone section of caffeine high dose treated rats showing non-homogenous matrix with multiple osteoporotic cavities (↑) filled with a granulation tissue ★ (H&E X100).



**Fig. (11):** A photomicrograph of a bone section of caffeine high dose treated rats showing non-homogenous matrix denoting defective mineralization and presence of numerous osteoporotic cavities (↑) (Mt X200).

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