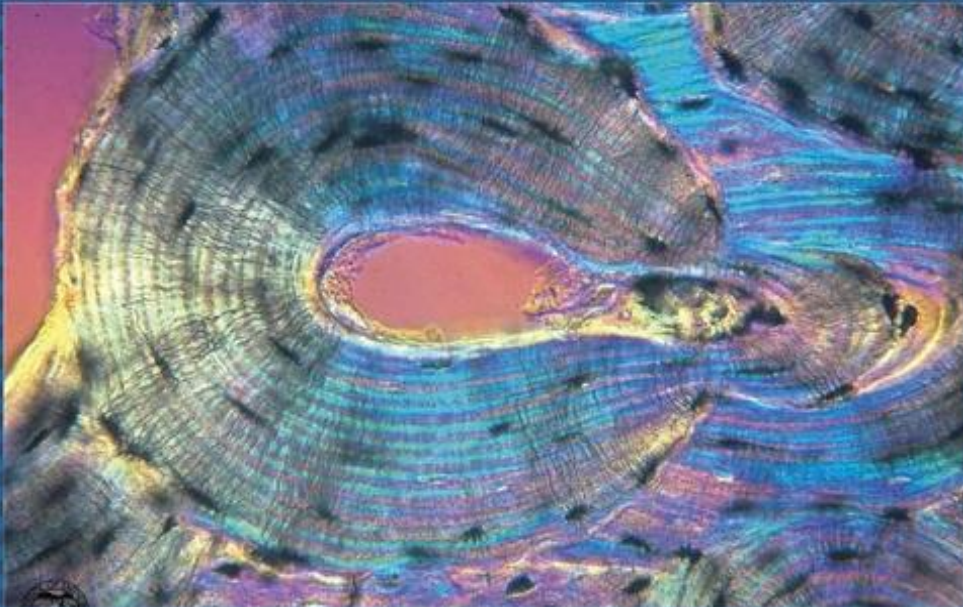




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## The Femoral Head Epiphysis of Ovariectomized Rats as A Site for Studies on Osteoporosis: Microstructural Changes Evaluations

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### ABSTRACT

**Background:** Osteoporosis is a systemic skeletal disease characterized by decreased bone mass, destruction of the microarchitectonics of bone structure and a high risk for fracture

**Objective:** To explore in detail the structural characteristics of cancellous bone from the femoral head epiphysis of normal and ovariectomized rats, and to characterise an alternative and complementary anatomic site to improve experimental researches on osteoporosis.

**Methods:** Twenty female Sprague-Dawley rats were randomly divided into 2 groups:

Group: 1 (sham-operated control group) and group: 2 (bilaterally ovariectomized group OVX for induction of osteoporosis) group 2 is further divided into 2-subgroups: (3-months OVX and 6-months OVX). Femur's heads were collected, examined by using different techniques such as scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX) and routine bone histology. Blood serum samples were collected for the determination of total calcium and phosphorus levels by using a spectrophotometer.

**Results:** Postmenopausal osteoporosis model was successfully established in OVX rat compared to sham-operated control rats. The femoral head epiphysis of OVX animals had decreased trabecular thickness, cortical thickness, with increased medullary width and porosity holes as determined by histological assessment and SEM. Moreover, EDX analysis of the femoral head epiphysis indicated a significant decrease of calcium concentration in epiphyseal regions of femoral heads in OVX rats ( $P < 0.05$ ) compared to sham-operated control rats. In contrast, serum levels of calcium in sham-operated group were significantly decreased compared to that of OVX 3 or 6 months groups ( $P < 0.05$ ).

**Conclusion:** The data from this study characterize osteoporosis induced in the rat 12 weeks after ovariectomy and present the epiphysial femur's head as a site for further investigations on osteoporosis and osteoarthritis.

## INTRODUCTION

Musculoskeletal conditions are the most common causes of severe short and long-term pain and physical disability, affecting hundreds of millions of people across the world, with costs approaching 3% of gross national product globally (Harvey *et al.*, 2010) and constituting the second greatest contributor to years lived with disability worldwide (Vos *et al.*, 2012). Osteoporosis affects one in three women and one in five men globally. This debilitating condition presents with a high incidence of low-trauma hip, spine, and other fractures, leading to immobility, associated comorbidity, and early death (Harvey *et al.*, 2010). More than 8.9 million fractures worldwide annually are caused by osteoporosis; these fractures are a significant cause of morbidity and mortality (Khired *et al.*, 2021). Causes of osteoporosis include increasing age, female sex, postmenopausal status, hypogonadism or premature ovarian failure, low body mass index, ethnic background, rheumatoid arthritis, low bone mineral density (BMD), vitamin D deficiency, low calcium intake, current smoking, alcohol abuse, immobilization, and long-term use of certain medications (Akkawi and Zmerly 2018). Experimental research can improve our understanding of pathogenesis and of the activity of pharmaceutical agents in the prevention or treatment of the disease (Talmage and Talmage 2007). Fortunately, the bones of rats are similar to those of humans, representing a dynamic tissue, which is constructed and reconstructed throughout life by bone modeling and remodeling (Karsenty 2017); (Kenkre and Bassett 2018), that is related to age progression in both cancellous and cortical bone (Dennison *et al.*, 2005).

Animal models of osteoporosis are suitable tools for studying new

prevention and treatment modalities. The first choice, and the one most commonly employed for such studies, is the Ovariectomized (OVX) rat model (Turner *et al.*, 2001). According to the Food and Drug Administration guidelines, the rat model of osteoporosis is an excellent preclinical model for postmenopausal osteoporosis (Organization 1994). The OVX rat model is a scientifically accepted model of postmenopausal (Namkung-Matthai *et al.*, 2001), the various pathological processes found in this model are similar to those found in humans. Various techniques are currently available for detection and diagnosis of osteoporosis in humans, but not all of them can be used for evaluation of osteoporosis in animals (Reddy Nagareddy and Lakshmana 2005). SEM is among the most frequently used instruments for examining bone. It offers the key advantage of very high spatial resolution coupled with a large depth of field (Shah *et al.*, 2019). In bone, the most frequent application of EDX is the measurement of extracellular matrix Ca and P content (and the Ca/P ratio) (Akesson *et al.*, 1994) (Obrant and Odselius 1985). The relative content of Ca and P, however, is critical for sustaining mineral homeostasis and bone metabolism and their co-dependence is evident for bone growth and development (Shapiro and Heaney 2003). It is, therefore, a suitable biomarker for the assessment of bone health (Coats *et al.*, 2003). In this study, we aim to assess osteoporosis in the femoral head epiphysis of a rat model using different techniques. To the best of our knowledge, this is the first study to apply these methods in the assessment of osteoporosis in epiphyses of femoral head of rats.

## MATERIALS AND METHODS

### 1. Experimental Design:

Twenty female Sprague Dawley rats (aged 12 weeks, weighing

200 ± 15g) were purchased from the laboratory animal research center, holding company for biological products & vaccines (Vacsera), Helwan, Egypt. All animals were acclimatized in the laboratory conditions with an illumination schedule (12L: 12D) for 7 days before they were used in the experiments. The room was maintained at a constant temperature of 24 ± 1°C, and 55 ± 1% humidity. Fresh tap water and standard rodent food pellets (proteins, lipids, fibers, NaCl, lysine, methionine, vitamins, salts, and wheat) were always available. All animal experiments were conducted in accordance with the requirements of the scientific research ethics committee for the faculty of science, Al-Azhar University, Assiut branch No:2/2021.

### **2.OVX-Induced Rat Model of Osteoporosis:**

After acclimation for one week, the rats were randomly assigned to three groups: 1- sham-operated control group, 2- experimental 3-months OVX and 3- experimental 6-months OVX (n = 10 per group). In brief, rats were anesthetized by intraperitoneal injection (IP) of 50 mg/kg ketamine, 5 mg/kg Xylazine (Ketamine Hydrochloride injection USP ROTEXMEDICA GmbH, TRITTAU, Germany), (Xyla-Ject, Adwia Pharmaceuticals, Cairo, Egypt). Bilateral ovariectomy was conducted in the OVX group under sterile conditions, as previously described (Chen *et al.*, 2015). The remaining sham-operated rats (10) had their ovaries surgically exteriorized then re-insertion without any surgical intervention.

### **3. Histological examination**

Rat's femur heads epiphyseal bone specimens from each experimental group were dissected, fixed in 10 % neutral buffered formalin washed and demineralized. Specimens were then processed for routine hematoxylin and eosin (H&E)

staining according to (Suvarna and Layton 2012).

### **4. Scanning electron microscopy (SEM):**

The femoral head samples were dissected out from the rats in the OVX and sham groups, after coating with gold, these tissue samples were examined with a scanning field emission SEM (ZEISS Sigma 500 VP), observations were done mainly on the epiphysial bone surfaces according to (Boyd 2019).

### **5. Energy-Dispersive X-Ray Microanalysis EDX:**

The femoral head samples were dissected out from the rats in the OVX and sham groups. The surface of all samples was coated with a conductive carbon layer using a JEOL JEE-4X Vacuum Evaporator. The distribution of calcium and phosphate in each sample was quantified using SEM-EDX, (ZEISS Sigma 500 VP) configured with an EDX detector Bruker Nano GmbH Berlin, Germany Esprit 2.0X Flash 630 operating at 15 kV accelerating voltage. The analysis was carried out according to (Obrant and Odselius 1985).

### **6.Determination of Serum Total Calcium and Phosphorus Levels:**

Serum calcium levels were determined by colorimetric method according to (Gindler *et al.*, 1972) using kits supplied by Biodiagnostic Co. (Cairo, Egypt). Serum phosphorus levels by the colorimetric method according to (Goldenberg and FERNFINDEZ 1966).

### **7. Statistical Analysis:**

Data were analyzed using SPSS (Statistical package for social science) software (version 26) statistical program at (0.05) level of probability, Levene's test for equality of variances was used to compare between means of bone mineral densities of femur's heads in three experimental groups and was negative, (p<0.05), showing the variances' homogeneity. The experimental design was established as a complete randomized block design with ten

replicates (Snedecor 1967). Results of three groups were subjected to one-way ANOVA with the least significant difference (LSD), and Tukey's test was presented using mean  $\pm$  standard error.

## RESULTS

### 1. Histological Examination Revealed Osteo-necrotic Changes with Increased Activity of the Osteoclasts in Femoral Head Epiphysis of OVX Osteoporotic Model:

Examination of (H&E) stained sections obtained from the epiphyseal region of rat's femur bone control group (Sham) showed an active covering cartilaginous cap formed from actively dividing chondrocytes which were either singly or in duplicates within lacunae. This cap over lined a normal anastomosing thick network of cancellous bone trabeculae having bone lamellae and osteocytes inside their lacunae. The smooth regular continuous endosteal surface of that trabeculae covered by osteoprogenitor and osteoblast cells was observed. Bone marrow spaces containing many hemopoietic cells were seen between trabeculae (Figs. 1 A & B). Examined sections of rat's femur head bone tissue of OVX-induced osteoporotic group showed osteo-dystrophic and osteoporotic reactions in the form of osteocytes apoptosis and necrosis and matrix resorption. Other bone lesions were represented hypo-cellular cavities with a relative increase in the osteoclastic reactivity. Bony and cartilaginous degenerative and necrotic changes with increased activity of the osteoclasts especially around the degenerated areas were seen. Moreover, multifocal osteoporotic lesions with bone rarefaction and the formation of cavities of different shapes and sizes were detected. The bone matrix of the affected areas appeared hypo-cellular with a deep bluish matrix denoting demineralization. The marrow cavities appeared wide irregular with early

dysplastic changes and hypo-cellularity (Figs. 1 C&D).

### 2. SEM Revealed Microstructural Changes in the Inner Femoral Epiphysial Bones of OVX Osteoporotic Model:

SEM was performed to investigate the microstructure and morphology of the trabecular region of the epiphyseal region of rat's femur bone after a 12-week for the sham and OVX groups. The Sham group exhibit dense femoral cortical bone, as well as a continuous and compact cancellous bone with thick trabeculae exhibiting highly definitive lamellae. The presence of numerous cement lines appears brighter in the boundaries between secondary osteons and interstitial bone, which are relatively hyper-mineralized trabeculae. Also, a number of resorption lacunae of various shapes were seen on the surface of the trabeculum, the collagen fibers of lacuna were resorbed (Figs. 2 A & B). However, the trabeculum was covered by well-arranged collagen fibers around the resorption lacuna multiple cavities were present in the cancellous bone in the OVX group with trabecular bone fracture and lower bone density (Figs. 2 C & D).

### 3. EDX Microanalysis Revealed A Significant Change in Calcium and Phosphorous Composition in OVX Femoral Head Compared to Sham-Operated Control Rats:

Calcium content in the epiphysis of the femoral head was estimated using EDX parameters in the three studied groups (Table 1). Results revealed a significant increase in calcium concentration in control group ( $P < 0.05$ ) ( $74.4 \pm 2.25\%$ ) compared to 3-months OVX group ( $60.96 \pm 1.01\%$ ) and 6 OVX months group ( $60.54 \pm 1.21\%$ ). It is worth mentioning that there was no significant difference ( $P > 0.05$ ) in calcium content between 3 and 6 OVX months groups. Also, phosphorus content in the epiphysis of the femoral head was estimated in the three studied groups. A significant

reduction in phosphorous content (P<0.05) of sham-operated control rats was observed compared to 3 OVX

months and 6 OVX months groups respectively.

**Table 1:** Statistical analysis of calculated concentration percent of calcium and phosphorus in epiphysis of femoral head using energy-dispersive x-ray microanalysis EDX parameters in the three studied groups.

Parameters	Control (Sham)	OVX (months)	
		3	6
Bone Ca EDX	74.4± 2.25 <sup>a</sup>	60.96 ±1.01 <sup>b</sup>	60.54 ± 1.21 <sup>b</sup>
Bone P EDX	28.47 ± 0.76 <sup>b</sup>	29.90±1.45 <sup>a</sup>	30.51 ± 1.29 <sup>a</sup>

According to Tukey's test, the different small letters (a, b) indicate that there is a significant difference between the three groups at probability level less than 0.05 (P <0.05).

**4. Biochemical Analysis of Serum Mineral Elements Revealed A Significant Change in Total Calcium and Phosphorous Levels in OVX Compared to Sham-Operated Control Rats:**

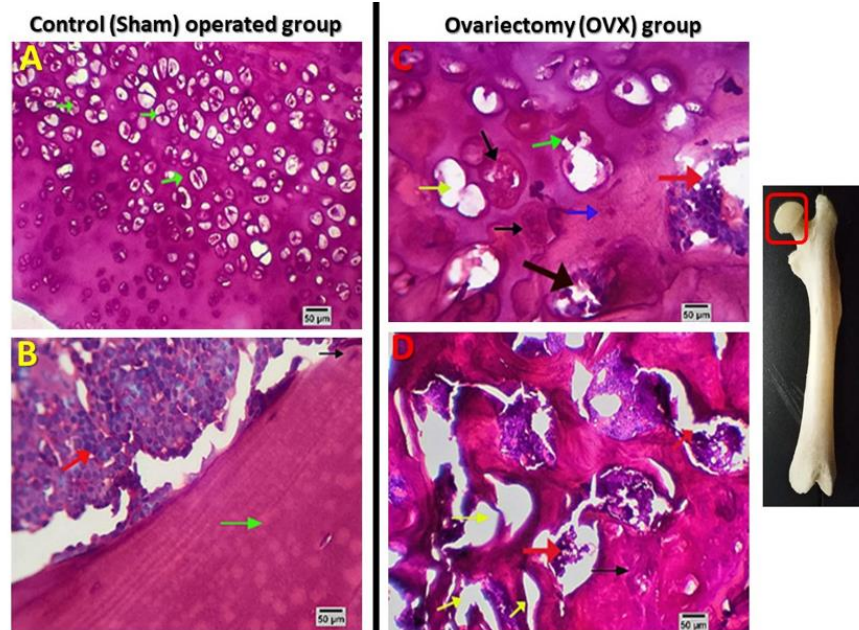
Serum levels of calcium in sham group (9.46± 0.96 mg/dL) were significantly low when compared to that of 3-months OVX group (10.61± 1.06 mg/dL) or 6 months OVX group (P>0.05) (10.11±1.26 mg/dL) . Notably, no significant difference (P>0.05) between sham control group,

the 3-months OVX group (10.61± 1.06 mg/dL) and the 6-months OVX group. Moreover, results of phosphorus serum levels analysis showed that the sham group had a slightly high phosphorus level (P<0.05) (5.66± 0.58 mg/dL) when compared to that of the 3-months OVX group (5.12± 0.26 mg/dL) or 6-month OVX groups (5.01±0.48 mg/dL). Also, no significant difference (P>0.05) was observed between 3 and 6-months OVX groups for phosphorus values (Table 2).

**Table 2:** Statistical analysis of serum levels of total Calcium and Phosphorus (mg/dL) in the three studied groups.

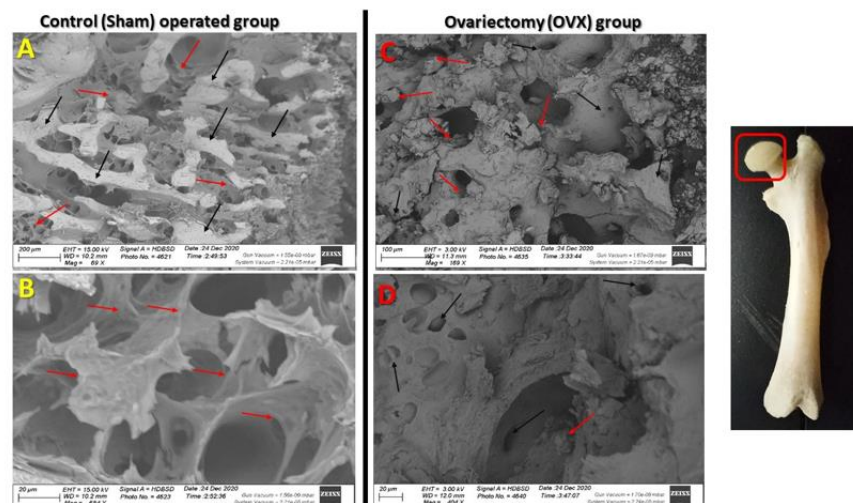
Parameters	Control (Sham)	OVX (months)	
		3	6
Serum Ca	9.46± 0.96 <sup>b</sup>	10.61± 1.06 <sup>a</sup>	10.11±1.26 <sup>ab</sup>
Serum P	5.66± 0.58 <sup>a</sup>	5.12± 0.26 <sup>b</sup>	5.01±0.48 <sup>b</sup>

According to Tukey's test, the different small letters (a, b) indicate that there is a significant difference between the three groups at probability level less than 0.05 (P <0.05).



**Fig 1.:** Photomicrograph of rat's femur epiphyseal bone of femoral head tissue of control (Sham) group A & B and OVX group C&D showing:

- (A) An active covering cartilaginous cap formed from actively dividing chondrocytes which appears either singly or in duplicates within lacunae (Green arrows).
- (B) Cartilaginous cap over lined a normal anastomosing thick network of cancellous bone trabeculae having bone lamellae and osteocytes inside their lacunae (Green arrows). Bone marrow spaces containing large number of hemopoietic cells are seen between trabeculae. (Red arrows).
- (C) Osteo-dystrophic and osteoporotic reactions in the form of osteocytes apoptosis and necrosis and matrix resorption (thin and thick black and blue arrows).
- (D) Bone lesions are represented by hypocellular cavities with relative increase in the osteoclastic reactivity (yellow and green arrows). The marrow cavities appear wide irregular with early dysplastic changes and hypo-cellularity (red arrows). Scale bars 50  $\mu$ m



**Fig. 2** Representative scanning electron micrographs of the longitudinal sections of the epiphysial (trabecular) region of femoral head of Control (Sham) group A&B and OVX group C&D showing:

(A) Cement lines with few remaining of cortical bones (black arrows) appear immediately proximal to the trabecular bones.

(B) Enlarged view showing trabecular remodeling site (red arrows) with normal architectures of trabecular bones without aggressive activity for osteoclastic sites.

(C) At low magnification trabecular bone can be clearly distinguished. An osteoclastic resorption field extends onto the trabecular bone surface. indicate that multiple cavities were present in the cancellous bone in the OVX group with trabecular bone fracture and lower bone density. In some of these (black arrows), evidence of a cutting cone of a remodeling system could be found.

(D) Higher magnification of the osteoclast resorption field in the undulating surface of the bone created by multiple osteoclastic resorption events can be easily distinguished. Many cells remain attached to the surface of the bone, most of which are associated with pits and canals in the bony surface (red arrows).

## DISCUSSION

The epiphyseal of the femur's head, which contains sub-chondral, cortical and trabecular bone, is exquisitely sensitive to estrogen deprivation and many studies have reported that ovariectomy results in rapid and profound osteoporosis in the femoral neck (Lelovas *et al.*, 2008). Also, definitive reports of bone loss in the epiphyseal of the femur's head are lacking which may be due to the difficulty of diagnosis in human and experimental osteoporotic models via traditional methods. Regarding the microstructure of cancellous bone, models involving large animals such as sheep or dogs seem to be superior to those involving small animals, since the murine trabecular thickness and separation is approximately three times smaller than in humans. Investigating fracture healing in small-animal osteoporosis models has proven to be a useful and reproducible model (Egermann *et al.*, 2005).

This prompted us to choose to study the ultrastructural characteristics the epiphyseal of the femur's head region for studying the trabecular bone loss using different techniques.

In the current study, osteoporosis was induced by bilateral OVX and bone mass attenuation was successfully demonstrated using SEM-EDX analysis. Compared to the sham group, a significant reduction in bone mineral density associated with an increase in trabecular bone separation was observed in OVX rats at 12-weeks post-operatively, mimicking postmenopausal osteoporosis in women after estrogen withdrawal. The current SEM observations for the sham group clarified that the presence of numerous cement lines appear brighter in the boundaries between secondary osteons and interstitial bone, and between individual trabecular packets are formed by cement lines, which are relatively hyper-mineralize. Previous studies showed that SEM-EDX

analyses of cement lines had either higher calcium content when compared to distant osteonal and interstitial bone (Skedros *et al.*, 2005). This may represent relative hyper mineralization or, alternatively, collagen deficiency with respect to the surrounding bone. Cement lines were first described as "kittlinien" (putty line or glue line) by (von Ebner 1875). Other investigators have published microradiographs showing that cement lines often have brighter gray levels (indicative of higher mineral content) than surrounding bone (Amprino and Engstrom 1952), (Jowsey 1960). Our data do not support the hypothesis that cement lines of secondary osteons are poorly mineralized (Burr *et al.*, 1988).

In cortical bone, enlargement of the marrow cavity is an indirect measure of bone loss. This enlargement in the diaphysis of long bones is due to increased endosteal bone resorption (Turner *et al.*, 2001) and periosteal bone apposition (Miller *et al.*, 1991). Endosteal resorption and the simultaneous periosteal bone formation result in a very slow rate of cortical bone loss (Kimmel and Wronski 1990). Analysis of the inner half area of the shaft in cortical bones is a very sensitive index because the bulk of bone loss occurs at this site. The earliest changes in the cortical bone width and the marrow cavity of the femoral and tibial shaft are noticed between 90 and 120 days after ovariectomy, (Danielsen *et al.*, 1993), (Ke *et al.*, 1993), (Yamamoto *et al.*, 1995) whereas cortical bone requires up to 180 days or longer after surgery to achieve steady-state (Jee and Yao 2001).

On SEM evaluation of the OVX osteoporosis group, we found that lamellae were preserved in some regions, while they extremely degenerated in other regions. In addition, less dense with more porous and larger spacing in the area of subchondral trabecular bones and the



area of the femur head in epiphyseal being very notable. Sporadic decomposition of lamellae and prevalence of homogenous material deposition between them were noteworthy. This finding agrees with (Gul *et al.*, 2013). In contrast, ovariectomy does not induce bone mass loss in the epiphyses of long bones, the distal tibia metaphysis, or caudal vertebrae (Li and Jee 1991) (Li *et al.*, 1996) (Li *et al.*, 1996) (Li *et al.*, 1996).

Histological examination of rat's femur heads bone tissue of OVX group showed bony and cartilaginous degenerative and necrotic changes with increased activity of the osteoclasts especially around the degenerated areas were seen. These results are in harmony with that described by (Zhang *et al.*, 2018) who indicated that the subchondral trabecular from epiphysial femur's head bones ultrastructure in the osteoarthritis model was characterized by the destruction of the network structure and collagen fibers. The subchondral bone ultrastructural damage caused by osteoporosis may change the mechanical properties of the upper cartilage and aggravate osteoarthritis cartilage. Moreover, multifocal osteoporotic lesions with bone rarefaction and the formation of cavities of different shapes and sizes were detected.

Our results can be explained in the light of published finding explains the (steady-state) effects on bone mass of ovariectomy, prostaglandin E2, estrogen treatment or altered mechanical usage can appear in 6 months or less in rats [(Li *et al.*, 1990) (Jee *et al.*, 1991), (Hori *et al.*, 1988), (Jee and Li 1990), (Ke *et al.*, 1992), (Wronski *et al.*, 1989). The total calcium and phosphorus analysis to some extent, in agreement with together these findings (Lieben *et al.*, 2012) indicate a mass transfer of calcium from bone to serum. Increased bone resorption, in response to the high circulating levels of PTH and 1,25(OH)2D3, contributes to the

trabecular and cortical bone loss and to the preservation of normal serum calcium levels. Indeed, suppression of osteoclastic bone resorption leads to better preservation of bone mass but concurrently reduces serum calcium levels. These diagnostic criteria of decrease in bone mineral density are in line with the results of OVX rat model of osteoporosis done by (Griffith *et al.*, 2010) and (Ce *et al.*, 2014) On the contrary, (Zhang *et al.*, 2018) indicated that Blood calcium content can help to analyze the etiology of osteoporosis but not as a basis for diagnosing osteoporosis.

Statistically, significance to bone loss is seen in the proximal tibial metaphysis after 14 days (Wronski *et al.*, 1988), (Wronski *et al.*, 1989), in the lumbar vertebrae bodies after 60 d and in femoral head compression test (Chon *et al.*, 2017), and for femoral neck after 30 d (Li *et al.*, 1997). From this context, we found that the epiphysial region has a strong and close relationship with the occurrence of osteoarthritis. Besides, the experimental assessment of osteoporosis in this boney site was negligible in the experimental studies, and this may be due to the interference of their histological structure with several other connective tissue components. Nevertheless, our study was limited to the analysis of cancellous as femoral head bone requires 3D imaging technology.

#### **Conclusion:**

Overall, our current data clearly showed that epiphyseal cancellous bone of induced OVX osteoporotic rat femur's head represents a valuable model for osteoporosis researches. Furthermore, our study added to the accumulating evidence that the microstructural changes in the subchondral and femoral head may, therefore, induce the development of osteoporotic osteoarthritis. Finally, the data from this study characterize osteoporosis induced in the rat epiphysial femur's head 12 weeks after ovariectomy and present a methodology

for further investigations on osteoporosis and osteoarthritis

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