Serum osteocalcin levels and bone mineral density in ovariectomized rats

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ABSTRACT:
Objective: This study aims to investigate ovariectomy effect on serum osteocalcin levels and bone mineral density in rats.
Methods: Forty five female Sprague Dawley rats were divided into 3 groups (n = 15 rats per group): sham-operated control, Ovariectomized, and Ovariectomized plus estrogen. After 3 months of experiment, rats were killed by decapitation after a 12 hour fast, obtained serum from rats' blood sample was kept at −80°C. For all groups of rats, serum osteocalcin levels were measured by ELISA. The rats' left femoral bone mineral density was measured by dual energy X-ray absorptiometry. Weights and lengths of the left femur were measured with electronic balance and Vernier caliper respectively.
Results: This study revealed an increase in serum osteocalcin levels in ovariectomized rats and in ovariectomized plus estrogen rats, alongside decrease in left distal femur bone mineral density in both groups was observed when compared to the control. The mean values of both serum osteocalcin and bone mineral density between the ovariectomized rats and the control were statistically significant. Estrogen administration in ovariectomized rats showed no significant changes in both serum osteocalcin levels and bone mineral density.
Conclusion: This study concludes that estrogen-deficiency by ovariectomy induces an increase in bone turnover with higher serum osteocalcin levels in ovariectomized rats and that the combination of serum osteocalcin levels and bone mineral density measurement may be a better predictor of the fracture risk.

KEYWORDS: estrogen, bone turnover, post menopause, osteoporosis, serum osteocalcin.

1   INTRODUCTION

Osteoporosis is another major public health problem; it has been well documented to be a progressive systemic skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1]. This disease has been reported to have a tremendous impact on the lives of many postmenopausal women. According to the World Health Organization, osteoporosis is second only to cardiovascular disease as a leading non-communicable health care problem. Worldwide, lifetime risk for osteoporotic fractures in women is 30-50% and in men, the risk is 15-30% [2]. Recent findings showed that high adipocyte count in bone marrow is directly related to bone loss as fat cells replace osteoblasts resulting in reduced bone mineral density (BMD) and increased propensity towards osteoporosis. This close relationship has a positive aspect whereby higher osteocalcin levels results in increased adiponectin production while the presence of adiponectin influences osteoblast proliferation and differentiation in a positive way [3]. Osteoblasts appear to regulate energy expenditure by acting on adipocytes and pancreatic islet cells via OC. In return, adipose tissue may also influence bone remodeling by regulating the activity of osteoblasts through adipokines, including leptin and adiponectin [4]. Further studies showed that an increase in OC concentration has been

associated with increased proliferation of β-cells, insulin gene expression, and division of adipocytes, leading to increased adiponectin production and hence better insulin sensitivity [5]. Research has also shown that in vivo, increase in serum OC has been found to have a beneficial effect on insulin secretion [6]. Osteocalcin is a bone matrix protein synthesized by mature osteoblasts, and constitutes approximately 15% of noncollagenous bone matrix proteins [7]. Moreover it has been well shown that 80 to 90% of the osteocalcin are adsorbed to bone hydroxyapatite, with a minor percentage leaking into the circulation [8]. Furthermore, Hsu et al. also showed that postmenopausal women with a higher percentage of body fat may have a higher risk for osteoporosis, osteopenia, and non-spinal fractures [9]. Although age-related bone loss has been reported to be caused mainly by increased bone resorption, impaired bone formation also contributes, and during menopause, bone resorption does increase more than bone formation [10-11]. Moreover, since the increased bone resorption and the impaired compensatory bone formation occur simultaneously at menopause; both are almost certainly induced by oestrogen deficiency. In addition, decreased oestrogen concentrations at menopause lead to lower intestinal absorption of calcium resulting in low serum calcium concentrations and increased osteoclastic resorption of bone. Both increase bone turnover and constitute risk factors for the development of osteoporosis [9]. Furthermore, Menopause and aging are associated with accelerated loss of cortical bone. Bone loss is the result of a negative remodeling balance due to impaired bone formation and/or increased bone resorption [12-13]. Osteocalcin has a high affinity for calcium and has a compact alpha helical conformation that is calcium dependent. The alpha-carboxyglutamic acid (Gla) residues of OC are capable of binding to bone matrix hydroxyapatite, thus leading to bone mineralization. Calcium and phosphorus deficient osteoporotic women may have a decreased rate of bone mineralization due to a reduction in hydroxyapatite crystal formation. In this condition, free OC may be present in the circulation, thus explaining the increased serum OC concentration in osteoporotic postmenopausal women [14-16]. Currently, biochemical markers of bone turnover are being used for predicting the bone loss rate and for assessing the risk of fractures in postmenopausal women. Estimation of bone turnover rates may be obtained through determination of the serum concentrations of certain proteins that are representative of the bone remodeling process. These proteins may be divided into bone formation markers and bone resorption markers. The most specific and sensitive bone formation markers include osteocalcin and bone alkaline phosphatase (AKP), which are indicative of osteoblastic activity, whereas bone resorption markers, such as tartrate resistant acid phosphatase (TRAP) reflect osteoclastic activity [17].

2 MATERIALS AND METHODS

2.1 ANIMALS AND DIETS

This study was realized on Forty five female Sprague Dawley rats of SPF. The rats were purchased from the Hercynian Pool-Rubicam Experimental Animal Co., Ltd., Shanghai. All rats were fed adaptively in one week, weight-matched and randomly divided into 3 groups: sham operation group (SHAM, n=15), ovariectomized group (OVX, n=15) and ovariectomized plus estrogen group (OVX+ E_2, n=15). Ovariectomized (OVX) rats and ovariectomized plus oestrogen group (OVX + E_2) were anesthetized with 2% sodium pentobarbital by intraperitoneal injection (0.2ml/100g), and after anesthesia, these rats underwent open surgery, bilateral ovaries were found and were completely removed; sham operation group was anesthetized with 2% sodium pentobarbital anesthetized by intraperitoneal injection (0.2ml/100g), and after anesthesia, underwent open surgery and the adipose tissues around the ovaries were removed. The size of adipose tissue around each ovary was similar to the ovary. After the surgery, normal drinking and eating was administered to the rats. Two weeks after operation, in the third week ovariectomized rats plus oestrogen group (OVX+ E_2) were daily gavaged with Estradiol-Valerate. Animals received suspension at a dose of 1ml/100g, the suspension concentration was 0.08 mg/ml. Ovariectomized group and sham operation group were also daily gavaged with saline at an amount of 1ml/100g. All three groups were fed ad libitum with normal chow and had free access to water under room temperature (20 ± 2) °C, relative humidity of (50 ± 10) % and simulating a 12-hour light-dark cycle for three months. Provided by Beijing China Fukang Biological Technology company Ltd, the chow contained 21.88% protein, 13.68% fat and 64.44% carbohydrates, with energy density of 3.29kcal/g. At the end of the experiment, rats were killed by decapitation after a 12 hour fast. Obtained serum from rats' blood was kept at −80°C for further analysis. Both animal facilities and protocols for this study were reviewed and approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

2.2 SERUM OSTEOCALCIN LEVELS

Serum osteocalcin concentration was measured by Enzyme-linked Immunosorbent Assay (Rat osteocalcin ELISA kit from Biological Technology Company, Ltd. Shanghai China) according to manufacturer's instructions.

2.3   BONE MINERAL DENSITY (BMD) OF THE LEFT FEMUR

Whole BMD measurements (g/cm^2) for the left femur were obtained by dual-energy X ray absorptiometry (DXA). The weights and lengths of the left femur were measured with electronic balance and Vernier caliper respectively.

2.4   STATISTICAL ANALYSIS

All data were entered to SPSS format using version 13.0 (SPSS Inc., Chicago, IL, USA). Variables were summarized using mean and standard deviation. One-way analysis of variance (ANOVA) was used to compare the mean deference in BMD and in serum osteocalcin levels. Post hoc analysis was performed using a LSD test or Dunnett's T3 test when appropriate. Significance was accepted at $P < 0.05$.

3   RESULTS

3.1   EFFECT OF OVARIECTOMY ON RATS' SERUM OSTEOCALCIN LEVELS

In this study, there were three sample groups randomized as follows: the control sham-operated group (SHAM), the ovariectomized group (OVX), and the ovariectomized plus oestrogen group (OVX +E$_2$). At the end of the experiment, serum osteocalcin was assessed and was estimated by ELISA (rat OC Enzyme-linked Immunosorbent Assay kit). The mean levels of serum osteocalcin showed a significant difference between the ovariectomized rats and the sham-operated control group (F= 6.563, P = 0.006), as shown in **[Fig-1].** Serum osteocalcin levels increased highly in OVX group compared with the SHAM group, and showed a statistical significance (P= 0.002). There were also increases observed in serum OC levels in the OVX + E$_2$ groups compared to the SHAM group with a significant difference (P = 0.011). But, when comparing the OVX group to the OVX + E$_2$ group, no statistical difference were found (P = 0.490).

Figure 1 Rats' serum osteocalcin levels measurement
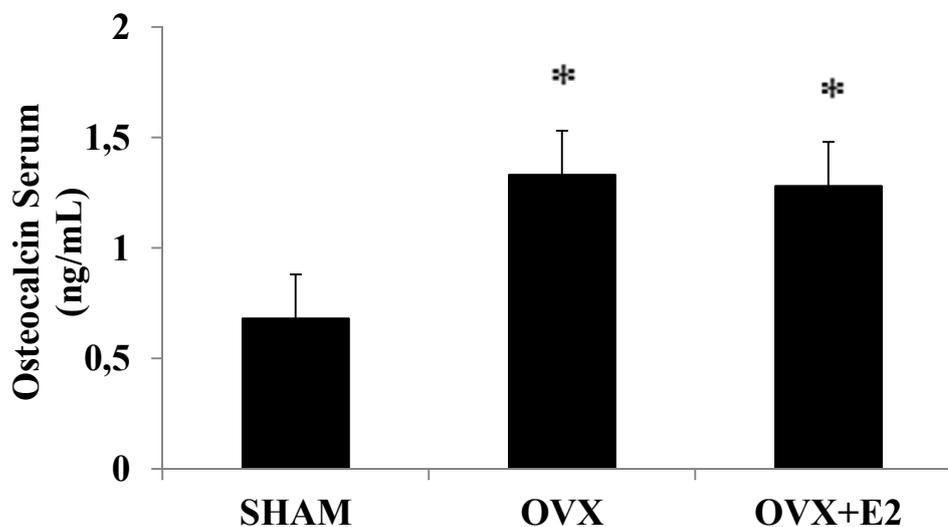
SHAM, sham operated group; OVX, ovariectomized group; OVX+E2, ovariectomized plus estrogen group; Serum Osteocalcin levels observed within the three groups. *OVX compared with SHAM group at P < 0.05; * OVX+E2 group compared to the SHAM group at P < 0.05. Values are means ± standard deviation (SD). A one-way ANOVA was used to analyze all data.

3.2   OVARIECTOMY EFFECT ON RATS' LEFT FEMUR WEIGHT, TOTAL BMD, AND DISTAL BMD

Both total BMD and distal BMD of the left femur were measured. There were no statistical differences observed in total BMD among groups (F = 1.022, P = 0.369). Also, no statistical difference (F = 0.799, P = 0.459) was observed in the left femoral weight among the different groups **[Table 1]**. But, the distal BMD showed a statistical significance between the ovariectomized rats and the sham-operated control group (F = 6.291, P = 0.004). In the ovariectomized rats (OVX and OVX + E$_2$ groups), the distal bone mineral density (BMD) of the left femur decreased compared to the Sham-operated control group

and showed a statistical significance in turn (P = 0.003 and P = 0.004) **[Fig-2]** but no statistical differences were found between OVX and OVX + E$_2$ groups (P = 0.901).

Table 1 Ovariectomy effect on rats' weight, total BMD and distal BMD of the left femur

Group	SHAM(n =14)	OVX(n =15)	OVX+ E2 (n =15)
Left femur weight (g)	0.64± 0.03	0.63± 0.04	0.63± 0.03
Total femoral BMD (g/cm2)	0.12± 0.01	0.11± 0.01	0.11± 0.01
Distal femoral BMD (g/cm^2)	0.14±0.01	0.13±0.01*	0.13± 0.01*

SHAM, sham-operated group; OVX, ovariectomized group; and OVX+E2, ovariectomized plus oestrogen group. BMD: bone mineral density; *OVX compared with SHAM group at P < 0.05; *SHAM group compared with OVX +E2 at P <0.05. Values are means ± standard deviation (SD). A one-way ANOVA was used to analyze all data.

Figure 2 The distal bone mineral density of the left femur
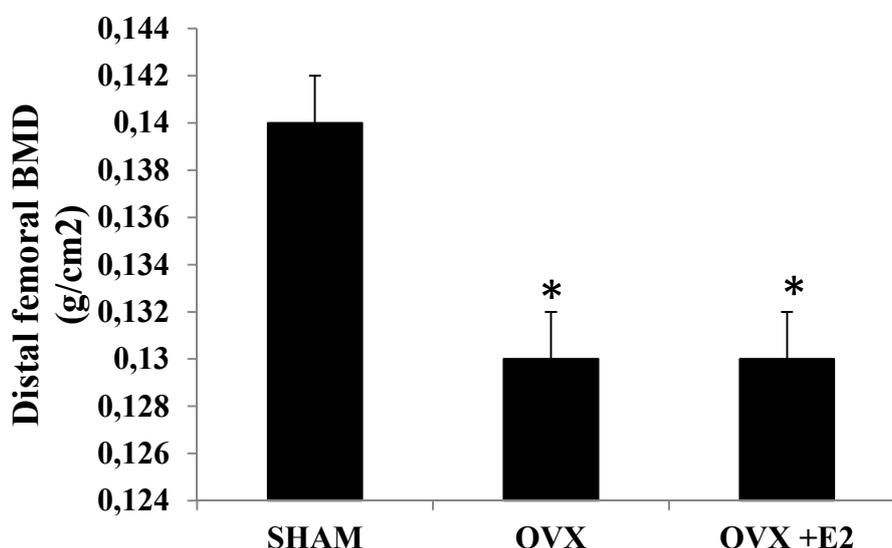
BMD, bone mineral density; SHAM, sham operated group; OVX, ovariectomized group; OVX+E$_2$, ovariectomized plus estrogen group. Distal BMD of the left femur from the three groups; *OVX compared with SHAM group at P < 0.05; * OVX+E$_2$ group compared to the SHAM group at P < 0.05. Values are means ± standard deviation (SD). A one-way ANOVA was used to analyze all data.

3.3   MEAN COMPARISON BETWEEN SERUM OC AND BMD OF THE LEFT FEMUR IN THE OVX AND IN THE SHAM RATS

In this study, the mean levels of serum osteocalcin and BMD showed a significant difference between the OVX group and the control group (F= 82.107, P = 0.000). While increased serum OC levels were observed in the OVX rats, a decrease in the left femoral total and distal BMD was observed alongside with statistical significance (P = 0.000) when compared to the control group **[Table 2].**

Table 2 Mean comparison between serum osteocalcin and bone mineral density

Group	n	SHAM	n	OVX
OC (ng/mL)	10	0.68 ± 0.44	7	1.33 ± 0.34
Total femoral BMD (g/cm2)	15	0.12 ± 0.01*	14	0.11 ± 0.01*
Distal femoral BMD (g/cm^2)	15	0.14 ± 0.01*	14	0.13 ± 0.01*

OC: Serum Osteocalcin, OVX: Ovariectomized group, and BMD: bone mineral density; the mean comparison in the OVX and in the control rats group. * Total femoral BMD compared with OC at P < 0.05; * Distal femoral BMD compared with OC at *P* < 0.05. Values are means ± standard deviation (SD). * indicate the OVX, and ##/# indicate the control group (SHAM). A one-way ANOVA was used to analyze all data.

4   DISCUSSION

To mimic the internal environment of postmenopausal women, ovariectomies were performed on female Sprague Dawley rats, an animal model with characteristics that are comparable with those of early postmenopausal trabecular bone loss [18]. It is now well established that increased bone turnover occurs during the early stages of oestrogen deficiency. Oestrogen deficiency in postmenopausal women induces the imbalance between bone resorption and bone formation, which reduces skeletal mass [19-20]. Hence, loss of oestrogen function is the single most important factor in the development of osteoporosis in postmenopausal women. Furthermore, oestrogen deficiency has been attributed for stimulation of increased bone resorption compared to bone formation [21-23]. In this present study, the left distal femoral BMD in OVX rats was found to be significantly decreased as compared to the control group **[Table 1/Fig 2]**, which is similar to the findings of Kalaiselvi et al. [24] and they reported that the bone mineral density was significantly decreased in postmenopausal osteoporotic than non-osteoporotic subjects. This result agrees with the findings of the study carried up by Johannes *et al* [25] and by Neetakumar *et al* [26]. In this study, the mean levels of serum osteocalcin were found also to be significantly increased twofold in OVX rats when compared with the control [Fig-1]. This increased levels of serum osteocalcin in the OVX rats group is attributed to the induced oestrogen deficiency. Therefore, this result agrees with others studies that reported in principle, a clear correlation between oestrogen deficiency and the especially elevated levels of serum osteocalcin, which is a marker of bone turnover [27-29]. It is well documented that osteocalcin is synthesized in the skeleton by osteoblasts, cells that are responsible for bone formation, [7] and osteocalcin is also a major and thoroughly studied and characterized non collagenous protein in mature human bone. It is a highly sensitive marker for bone formation. Osteocalcin has a high affinity for calcium and exhibits a compact calcium dependent Alpha helical conformation, in which the Gamma carboxyglutamic acid (Gla) residues bind and promote absorption to hydroxyapatite in bone matrix. Normal bone mineralization takes place in this condition. The deficiency of oestrogen in the OVX rats or during the menopausal period induces a lower intestinal absorption of calcium, resulting in decreased serum calcium levels and an increased osteoclastic resorption of the bone [14]. Both calcium and phosphorus deficiencies reduce hydroxyapatite crystals formation. When the bone mineralization decreases, free osteocalcin may be available for circulation in the blood. This may explain the increased concentrations of osteocalcin in the serum of osteoporotic postmenopausal women [30] or in the ovariectomized rats. Hence, deficiency in oestrogen and the reduction of hydroxyapatite crystals formation lead to increased bone turnover, thereby contributing as risk factors for the development of osteoporosis [9]. In addition, in almost all cases of osteoporosis, bone formation remains at least partially coupled to bone resorption; even the resorption rate can far exceed the formation. Therefore, during the states of elevated turnover, markers of bone formation should be increased. Furthermore, oestrogen deficiency also prolongs the resorption phase of remodeling cycle because of increased lifespan of osteoclasts and induces a high bone turnover, i.e. high bone turnover can disrupt the trabecular architecture and its deterioration is a contributory factor to the bone fragility, which increases the incidence of trabecular perforation and buckling, thus reducing the bone strength in osteoporosis, ultimately resulting in decreased levels of bone mineral density as showed the findings of this study **[Fig-2]**, BMD is the best quantifiable predictor of osteoporotic fractures. Serum osteocalcin being a dynamic marker, the efficacy of treatment can be assessed by repeating the estimation of osteocalcin and by comparing it with its original value. Thus, a single measurement of a biochemical marker of bone turnover may be unable to predict even short term individual fracture risk. However, the assessment of osteoporotic risk fractures can be done effectively by a combination of BMD, which provides a static feature of the skeleton and the biochemical marker, osteocalcin, which provides a dynamic measure of the bone remodeling unit, as was evidenced   from the study of Vanitha *et al*. [16] although the main effect of estrogen replacement is to suppress bone turnover, which prevents trabecular bone loss. However the decreased bone formation

observed in OVX rats treated with oestrogen **[Fig-1 & 2]** appears to be secondary to the reduced bone resorption since oestrogen administration was shown to stimulate bone formation in vivo [31-32]. Indeed, treatment with 17beta-estradiol prevented the decreased metaphyseal bone loss in OVX rats, the total bone mineral density measured was not completely corrected by oestrogen replacement **[Fig-1]**. The most likely explanation for the discordance between the effects of oestrogen on trabecular bone volume and BMD is that DXA measures combined cortical and trabecular bone, whereas the histomorphometric method measures only trabecular bone, which is mainly affected in OVX rats [33]. In this study, no significant difference was found between the OVX rats and OVX+ E2 rats in BMD and in serum osteocalcin levels [Fig-3 & 4]. While increased serum osteocalcin levels were observed in the OVX rats, in the same OVX rats, a decrease in the left femoral BMD was observed alongside **[Table 2]**. Therefore, these findings suggest a negative correlation between the serum osteocalcin levels and the BMD measurement supported by findings of Kalaiselvi Vs *et al* [24]. In addition, the high value of serum osteocalcin noticed in ovariectomized rats is also consistent with the study conducted by Vanitha *et al*. [16]; they reported that elevated levels of serum osteocalcin in postmenopausal women can be considered as prognostic marker of osteoporosis for better management of postmenopausal osteoporosis in women.

5   CONCLUSION

In this study, a negative correlation was observed between the serum osteocalcin levels and the bone mineral density measurement. The mean levels of both serum osteocalcin and bone mineral density between ovariectomized rats and sham-operated rats were statistically significant. This study also finds that ovariectomy decreases significantly the left femoral bone mineral density at the distal levels. We find that estrogen administration is not an effective treatment for preventing or controlling serum osteocalcin levels and bone mineral density. However, it is evident that a combination of serum osteocalcin levels and bone mineral density measurement may be a better predictor of bone fracture risk, which provides a static picture of the skeleton.

AUTHOR CONTRIBUTION STATEMENT

Camara Ce is the primary researcher and corresponding author under the supervision of **Prof. Nianhong Yang. Dong Yu** and **Yaowu Zhao** carried out operational procedures on the subjects, and Linyuan Zhou assisted in carrying out standardized laboratory procedures.

CONFLICT OF INTEREST

The study was conducted independently and there is no an ethical problem or conflict of interests.

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