Iranian Journal of Veterinary Surgery

ORIGINAL ARTICLE

Evaluation of Protective Effects of Pomegranate Seed Oil on Experimental Osteoporosis in Rats

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ARTICLE INFO

Article History:

Received: 23 October 2023
Revised: 14 November 2023
Accepted: 16 December 2023

Keywords:

Estradiol
Histopathology
Osteoporosis
Pomegranate

ABSTRACT

Osteoporosis is characterized by a reduction of bone mass and destruction of bone structures, followed by high bone fragility and susceptibility. This study aims to evaluate the protective effects of pomegranate seed oil (PSO) on experimental osteoporosis in rats. Twenty-four female Wistar rats were divided into four groups: sham-operated (Sham = 6), Sham with PSO treatment (Sham+PSO = 6), ovariectomized (OVX = 6), and ovariectomized with PSO treatment (OVX+PSO = 6). OVX+PSO and Sham+PSO groups received 0.1 ml of pomegranate juice daily, and OVX and Sham groups received the same amount of paraffin oil. After eight weeks, the femur and tibia bones were removed, and the structure and metabolism of samples were assessed by histological examination. The average thickness of femoral neck trabeculae in group OVX was significantly lower than in groups Sham+PSO and Sham ($p < 0.05$). Regarding the number of trabeculae in the neck of the femur, a significant difference was observed between groups OVX and Sham+PSO ($p < 0.05$). Furthermore, trabecular separation in group OVX was significantly more than in the other three groups ($p < 0.05$). The trabecular separation in group OVX+PSO compared to groups Sham+PSO and Sham was significantly higher ($p < 0.05$). A histopathologic examination of the upper metaphysis of the tibia indicated that the number of bone trabeculae in Sham+PSO was only statistically significant in the OVX group ($p = 0.018$). It was also found that the average thickness of bone trabeculae in the OVX+PSO and Sham groups was significantly lower than in the Sham+SO group. The results of the present study suggest that pomegranate seed oil, having estrogenic compounds, can prevent osteoporosis in rats caused by ovariectomy.

Introduction

Osteoporosis is a disease characterized by an increase in the porosity of the skeleton due to a decrease in bone mass.[1,2] This leads to increased bone fragility and susceptibility to fracture.[3] This disease may be limited to a specific bone, such as osteoporosis caused by the disuse of an organ, or may affect the entire skeleton, such as a manifestation of metabolic bone disease.[4,5] Diffuse osteoporosis can be primary due to old age.[6] Primary osteoporosis is caused by estrogen deficiency and constitutes 95% of all cases.[7] Secondary osteoporosis may be caused by overusing glucocorticoids and heparin, renal failure, hyperthyroidism, primary hyperparathyroidism, and hyperadrenalism.[8,9] The most common reasons for osteoporosis are old age.[10] The maximum bone mass is obtained in youth, but with the beginning of the third or fourth decade in both sexes, bone absorption increases from its deposition.[11,12] Bone loss with age (on average 7% per year) is a natural biological process.[13,14] This state often occurs in areas containing large amounts of spongy bone (trabecular) and is more evident in the femoral neck and vertebrae.[15]

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https://doi.org/10.30500/ivsa.2023.422083.1371

For this reason, these areas are more prone to fractures in people with osteoporosis.[16] Regardless of the underlying cause, the progressive loss of bone mass is clinically important due to the increased fracture risk.[17,18] The main symptom of osteoporosis is bone loss in parts of the skeleton that contain high trabecular bone.[19,20] Bone trabeculae become thinner and more scattered than usual, increasing the susceptibility to fracture.[21] In osteoporosis, bone loss is severe, especially in the vertebrae, leading to fractures and collapse.[22,23] Similarly, the bone is destroyed in other bones that bear weight, such as the femoral neck.[24] The main microscopic changes are the trabeculae's thinning and the Haversian ducts' widening.[25]

Estrogen modifies the production of bone-resolving cytokines such as interleukin 1 and 6, bone-stimulating factors such as insulin-like growth factors 1 and 2, colony-stimulating factor, osteoprotegerin, and the number of vitamin D receptors in bone.[26,27] Phytoestrogen is a descriptive term for various non-steroidal substances that either has estrogenic activity or are metabolized to substances with estrogenic activity.[28] The oil in pomegranate (*Punica granatum*) seeds has also been studied in recent years regarding industrial use and supply of essential fatty acids.[29] Most components of pomegranate seed oil (PSO) are saturated and unsaturated fatty acids.[30] PSO is a unique natural product containing various fatty acids, about 80% of which are 18-carbon fatty acids with three bonds.[31] The fatty acid composition of PSO includes panicic acid, palmitic acid, stearic acid, oleic acid, and linoleic acid.[32] In addition to the mentioned fatty acids, it is rich in steroidal such as gamma-tocopherol, 17-alpha-estradiol, sigmasterol, sitosterol, beta-sterol, and testosterone.[33,34] Recently, it has been determined that the main steroidal estrogen in PSO is 17-alpha-estradiol, a biosimilar to estrogen that is less dangerous than other forms of estrogen.[35,36] The wide range of safe phytoestrogens makes PSO exceptional [37].

The recent trend towards using phytoestrogenic compounds in medicine to prevent and treat osteoporosis caused by estrogen deficiency increases the possibility that PSO and its extracts can be effective. Therefore, the aim of this study is to evaluate the effect of PSO on bone lose in ovariectomized rat model of postmenopausal osteoporosis.

Materials and Methods

Housing and Grouping

This research was carried out at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. In this study, 24 female three-month-old Wistar rats weighing 175-200 grams were obtained from the laboratory animal breeding center. The rats were kept in the laboratory for one week in order to adapt to the new environment, reduce the stress of transportation, and ensure their health. Then the rats were randomly divided into four groups in standard cages including sham-operated (Sham = 6), Sham with PSO treatment (Sham+PSO = 6), ovariectomized (OVX = 6), and ovariectomized with PSO treatment (OVX+PSO = 6). Drug-treated groups (OVX+PSO and Sham+PSO) were treated orally with 0.1 ml PSO using a stomach tube (gavage) once a day, for eight weeks after surgery. Rats in the non-treated groups (OVX and Sham) received paraffin orally simultaneously in a volume similar to PSO.

Pomegranate Seed Oil (PSO)

The PSO used in this study was produced, and generously donated by Orum Narin Co. (Urmia, Iran) and estrogen content was determined by immune-radiometric assay (IRMA) using commercial kits (Biosource, Dorest, Belgium) and Dream Gamma- 5 gamma counter (Shin Jin Medics Inc., Korea).

Surgery

Under general anesthesia with isoflurane double dorsolateral approach was used to harvest the ovaries. With scalpel number 15, two 1.5 cm long incisions were made on the skin parallel to the spine on each side. We separated the muscles, entered the abdominal area, and gently pulled out the fatty tissue with forceps up to the ovaries. The ovaries and the surrounding fat were slowly removed from the incision site, then the end of the uterine horn was ligatured with 5-0 Vicryl thread, and the ovaries were removed entirely. Tramadol (Darou Pakhsh pharmaceutical, Tehran, Iran) was injected at a dose of 10 mg/kg, IM, every 12 hours for 2 days and enrofloxacin 5% (Razak, Tehran, Iran) at a dose of 10 mg/kg, SC, every 12 hours for 2 days was administrated.

Pathology Samples

At the end of the study (eight weeks after PSO administration), the animals were euthanized, and femur and tibia bones were removed. The samples were fixed in 10% formalin and decalcified in 5% nitric acid (5 cc of 65% nitric acid was added to 95 cc of distilled water) for 5 days (changed daily), washed, dehydrated in ethyl alcohol and cleared by xylol. Then the bones were cut and embeded in paraffin and serial sections 5-µm thick were made and stained using hematoxylin and eosin. Under the light microscope, the number of trabeculae with 10 magnification and thickness and distance between trabeculae (separation) with 100 magnification were measured by a pathologist who was blinded to the group assignment of the samples.

Statistical Analysis

The comparison of the groups in terms of the average

number, thickness, and distance between trabeculae in the microscopic view of the femur and tibia was evaluated by ANOVA statistical test, and pairwise comparison of groups was made by Tukey post hoc test (SPSS Software version 26). The discrepancy was considered statistically significant at the *p-value* < 0.05 for all the analyzed data.

Results

Estrogen Contents of PSO

The results of IRMA showed that PSO contains estrogen (1.3 µg/ml) and the intra-assay coefficients of variability (CVs) in all IRMA runs were less than 3.4%. Based on the estrogen levels measured in PSO as well as the normal levels of estrogen in female rats, we selected the amount of PSO based on their importance in current study model.

Histopathologic Indicators of Femoral Neck

The results obtained from the histological analysis of the femoral neck are shown in Table 1, Figures 1 and 3. Histopathologic analysis of the femur of the Sham+PSO group showed that the number of trabeculae was more than in groups OVX+PSO, OVX, and Sham. A statistically significant difference was found in group OVX ($p = 0.037$). The average number of bone trabeculae in group OVX+PSO was more than in group OVX on average, but this difference did not reach statistical significance ($p > 0.05$) (Figure 1A). It is also shown that the average thickness of bone trabeculae in group OVX is lower than in the other three groups. This difference is statistically significant in Sham ($p = 0.013$) and Sham+PSO groups ($p = 0.004$) (Figure 1B). Additionally, in the OVX group, the average of trabecular separation was lower significantly than in other groups; OVX+PSO ($p = 0.007$), Sham+PSO ($p = 0.000$) and Sham ($p = 0.000$). Furthermore, compared to other groups, the OVX+PSO group has a lower trabecular separation; Sham+PSO ($p = 0.000$) and Sham ($p = 0.005$) (Figure 1C).

Histopathologic Indicators of the Upper Metaphysis of the Tibia

The results obtained from the histological analysis of the tibia bone are shown in Table 2, Figures 2 and 4. A histopathologic examination of the upper metaphysis of the tibia indicated that the number of bone trabeculae in the Sham+PSO group was significantly higher than in other groups and that this difference was only statistically significant in OVX group ($p = 0.018$) (Figure 2A). The average thickness of bone trabeculae in the OVX group was significantly lower than in the other three groups; OVX+PSO ($p = 0.002$), Sham+PSO ($p = 0.001$), and Sham ($p = 0.000$). It was also found that the average thickness of bone trabeculae in the OVX+PSO and Sham groups was significantly lower than in the Sham+PSO group (Figure 2B). The average trabecular separation in the OVX group was significantly lower than other groups; OVX+PSO ($p = 0.007$), Sham+PSO ($p = 0.000$) and Sham ($p = 0.000$) (Figure 2C).

Discussion

This study demonstrated that administration of PSO for eight weeks markedly improved OVX-induced osteoporosis in rats. In histomorphometric analysis,

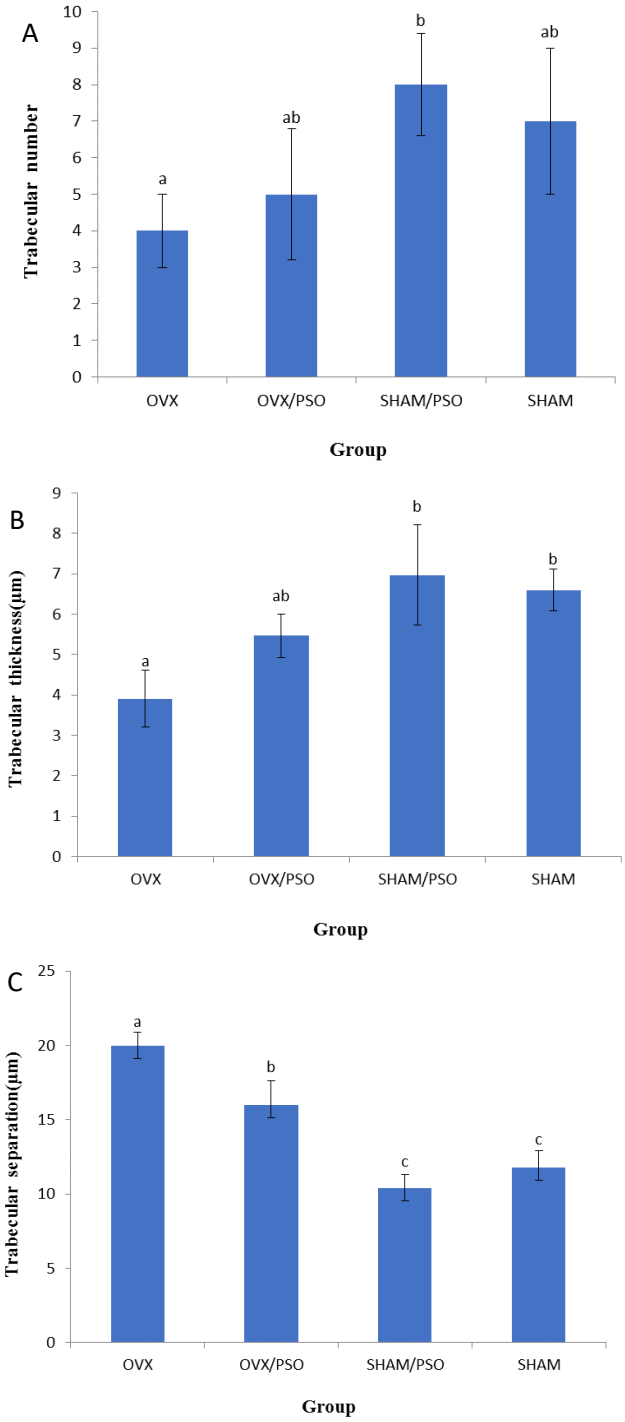

Figure 1. Effects of OVX, and PSO on the number of trabeculae (A), the thickness of trabeculae (B), and the distance between trabeculae (C) in femoral neck. Bars are means ± SEM. Values with different letters have statistically significant differences ($p < 0.05$). n=6/group.

Table 1. Bone trabecular number, thickness and separation (mean ± SD) of the femur of OVX, OVX+PSO, Sham+PSO, and Sham groups.

Group	Number of Trabeculae	Thickness of Trabeculae	Distance between Trabeculae
OVX	4 ± 1[a]	3.91 ± 0.7[a]	19.99±0.87[a]
OVX+PSO	5 ± 1.82[ab]	5.47 ± 0.4[ab]	15.99±1.61[b]
Sham+PSO	8 ± 1.41[b]	6.97 ± 1.24[b]	10.39±0.94[c]
Sham	7 ± 2[ab]	6.60 ± 0.52[b]	11.77±1.17[c]

Each column with different letters have statistically significant differences ($p < 0.05$). n = 6/group.

Table 2. Bone trabecular number, thickness and separation (mean ± SD) of the proximal metaphysis of the tibia of OVX, OVX+PSO, Sham+PSO, and Sham groups.

Group	Number of Trabeculae	Thickness of Trabeculae	Distance between Trabeculae
OVX	3.66 ± 1.15[a]	4.25 ± 0.25[a]	35.88 ± 4.5[a]
OVX+PSO	7 ± 1.63[ab]	5.87 ± 0.47[b]	21.25±2.36[b]
Sham+PSO	8 ± 2[b]	7.16 ± 0.28[c]	16.16±1.46[b]
Sham	7 ± 0.81[ab]	6.17 ± 0.47[b]	21.28±1.18[b]

Each column with different letters have statistically significant differences ($p < 0.05$). n = 6/group.

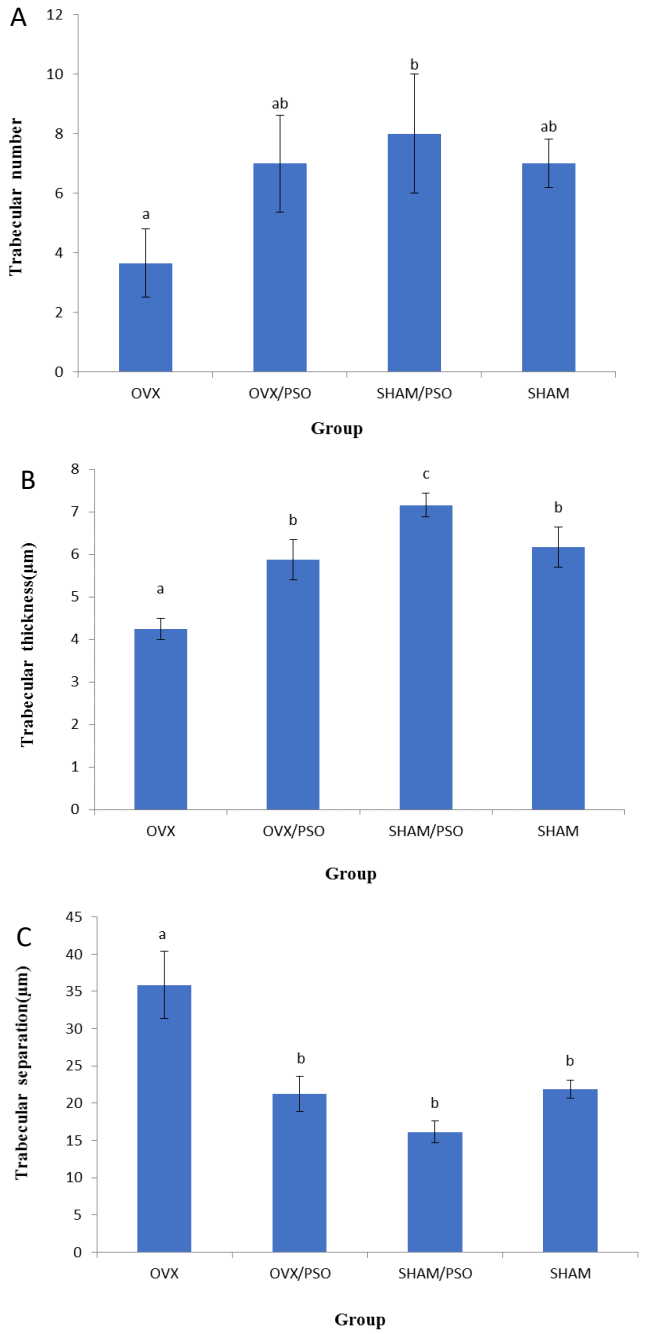

Figure 2. Effects of OVX, and PSO on the number of trabeculae (A), the thickness of trabeculae (B), and the distance between trabeculae (C) in tibia bone. Bars are means ± SEM. Values with different letters have statistically significant differences ($p < 0.05$). n = 6/group.

ovariectomized rats with no treatment showed significant osteopenia and osteoporosis.

The World Food and Drug Administration has recommended that ovariectomized rats are a good model for studying osteoporosis and discovering a new drug's preventive and therapeutic effects.[38] Ovariectomy in rats is a method that is often used to simulate osteoporosis because it is fast and causes complete osteopenia in bones with trabecular and cortical parts such as the femur.[39,40] Lack of estrogen following ovariectomy is responsible for disturbing the balance in bone regeneration in such a way that resorption occurs more than formation.[41,42]

Osteoporosis manifests as bone pain and secondary fractures caused by decreased bone mass or density.[43] In some bone areas, such as the femoral neck, the trabecular bone greatly contributes to bone strength. Osteoporosis reduces trabeculae's thickness and their number.[44]

Femoral neck fracture caused by osteoporosis is one of the main causes of bone complications and mortality in the elderly. Therefore, the upper part of the femoral neck in ovariectomized rats may be clinically a more suitable region than other parts of the appendicular skeleton (such as the upper part of the tibia) to investigate new treatment methods for osteoporosis.[45]

Previous studies showed that bone mass loss could be seen in the femur approximately 30 days after removing the rat's ovaries.[45,46] In the current study, the average thickness of femoral neck trabeculae in the OVX group was significantly lower than in Sham+PSO and Sham groups after eight weeks. These results can be attributed to the effectiveness of ovarian removal in reducing the thickness of trabeculae and causing osteoporosis. Yamamoto *et al.* in 1995 reported that the reduction of estrogen causes a decrease in bone density in spongy bone; also, the femoral neck of rats in a short time after ovariectomy.[47] Another study reported that bone density decreases in both spongy and cortical parts of the femoral neck within one year after ovariectomy.[48] This information is important for the design and preparation of clinical studies to determine the effectiveness of therapeutic agents to prevent or reverse the process of decreasing bone density caused by reducing estrogen.

In comparing the groups, in terms of the number of trabeculae in the neck of the femur, a significant

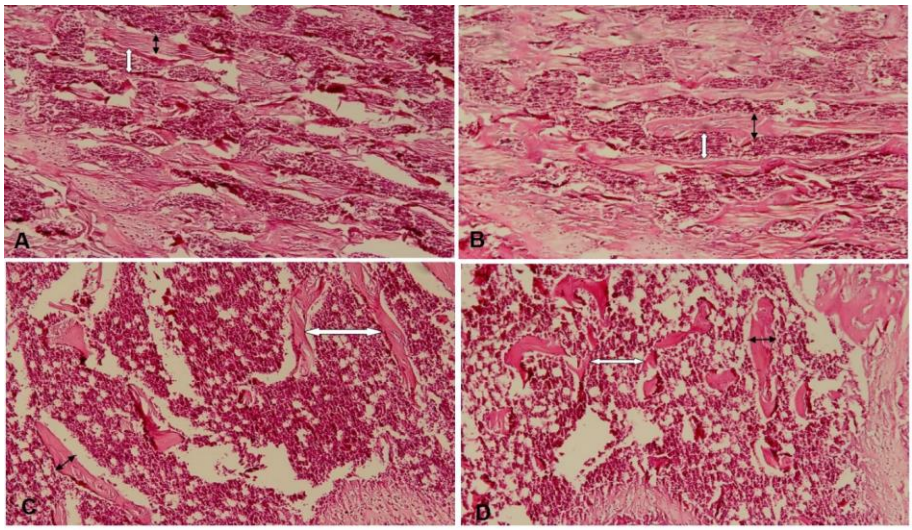

Figure 3. Histopathology of the femoral neck of the Sham (A), Sham+PSO (B), OVX (C), and OVX+PSO (D). The thickness of trabeculae (black arrow) and the distance between trabeculae (white arrow). H&E staining, magnification ×100.

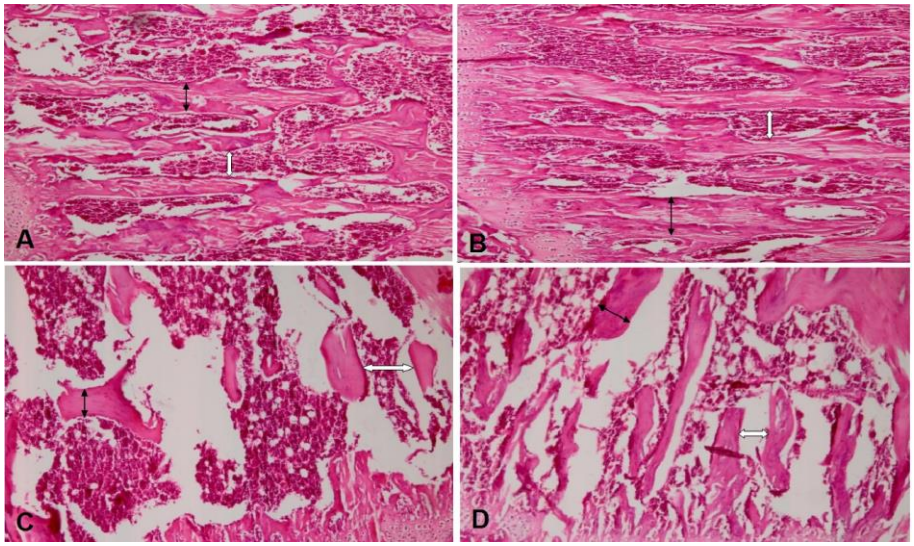

Figure 4. Histopathology of the proximal metaphysis of the tibia bone of the Sham (A), Sham+PSO (B), OVX (C), and OVX+PSO (D). The thickness of trabeculae (black arrow) and the distance between trabeculae (white arrow). H&E staining, magnification ×100.

difference was observed only between groups OVX and Sham+PSO. Furthermore, the average distance between femoral neck trabeculae in group OVX was significantly more than the other three groups. The trabecular separation in group OVX+PSO compared to groups Sham+PSO and Sham was significantly higher. These results can indicate the effectiveness of ovarian harvesting in causing osteoporosis and the role of PSO.

Furthermore, the increase in the number, thickness and trabecular separation in the comparison between group Sham+PSO and other groups can signify the effectiveness of PSO in preventing osteoporosis. The results obtained from comparing the thickness and the distance between the trabeculae in the present study are similar to the study by Chiba *et al*. in 2003.[49] Their study used the 4-week administration of alpha-glucosyl hesperidin, a phytoestrogen. The exact mechanism of the inhibitory effect of these phytoestrogens on bone resorption is unclear.

Another study showed that the administration of *Pueraria radix* extract, which contains a large amount of isoflavonoids such as daidzein and genistein, in ovariectomized rats with three doses of 5%, 10%, and 20% of the diet for a period of 4 weeks completely prevents the reduction of the thickness of trabeculae and the increase trabecular separation.[50] Monsefi *et al.* showed that administering pomegranate extract in pregnant rats increased the bone calcium content and femur length of the fetus on the 19th day of pregnancy. Finally, the results of this study showed that pomegranate could facilitate and strengthen the bone formation.[51]

In ovariectomized rats, the first changes of density reduction usually occur in the upper part of the tibia, usually 14 days after the surgery. In the present study, the average number of trabeculae in the upper metaphysis of the tibia in group OVX was significantly lower than in group Sham+PSO. A significant increase in the number of trabeculae in group Sham+PSO can be a sign of the

effectiveness of PSO in preventing osteoporosis. This result was similar to the results of the number of trabeculae in the neck of the femur in this study and the study by Okamato *et al.,* 2004 which indicates the effectiveness of ovarian removal in reducing the number of trabeculae and causing osteoporosis.[52] However, whole pomegranate extract (pomegranate juice with seed oil) was used in Okamato's study.

In the study by Peng *et al.* in 2008, following the 12-week administration of phytoestrogens derived from the *Epimedium* plant (mainly including daidzein and genistein) to ovariectomized rats, there was no significant difference in the number of trabeculae between the treated and ovariectomized groups like current study. They explained that the increase in bone volume can be due to the thickening of existing trabeculae rather than the production and creation of new trabeculae.[53] However, in the studies of Yao *et al.* in 2005, it was found that the increase in bone volume following the treatment with fibroblast growth factor in ovariectomized rats was due to the increase in the number of trabeculae.[54] Peng explains this difference because the effects of fibroblast growth factor and phytoestrogens derived from epimedium on trabeculae are probably different.[53]

In the current study, the average trabecular separation of the upper metaphysis of the tibia in group OVX was significantly higher than the other three groups, which was utterly similar to the result of the trabeculae distance in the neck of the femur. Okamato and Peng's study support current results.[52,53] These results indicate the effectiveness of ovarian harvesting in the osteoporosis and the role of PSO in preventing osteoporosis.

The results of the present study showed that PSO reduced the histological effects of osteoporosis in the bones of ovariectomized rats, and it can be concluded that it would be helpful as estrogen replacement therapy in osteoporosis.

Acknowledgments

We thank the research deputy of the Ferdowsi University of Mashhad for supporting us.

Conflict of Interest

The authors declare that they have no conflict of interest.

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