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PREVENTION OF BONE LOSS BY PANAX GINSENG IN A RAT MODEL OF INFLAMMATION-INDUCED BONE LOSS

U. AVSAR[1]✉, E. KARAKUS[2], Z. HALICI[3], Y. BAYIR[4], H. BILEN[5], A. AYDIN[6], U. Z. AVSAR[7], A. AYAN[8], S. AYDIN[9] AND A. KARADENIZ[10]

[1] Department of Family Medicine, Ataturk University Faculty of Medicine, Erzurum, Turkey
[2] Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ataturk University, 25240, Erzurum, Turkey.
[3] Department of Pharmacology, Ataturk University Faculty of Medicine, Erzurum, Turkey
[4] Department of Biochemistry, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey.
[5] Department of Internal Medicine, Ataturk University Faculty of Medicine, Erzurum, Turkey
[6] Department of Orthopedics and Traumatology, Ataturk University Faculty of Medicine, Erzurum, Turkey
[7] Department of Medical Education, Ataturk University Faculty of Medicine, Erzurum, Turkey
[8] Department of Nuclear Medicine, Ataturk University Faculty of Medicine, Erzurum, Turkey
[9] Department of Family Medicine, Kafkas University Faculty of Medicine, Kars, 36000, Turkey
[10] Department Physiology, Faculty of Veterinary Medicine, Ataturk University, 25240, Erzurum, Turkey

Abstract

This study evaluated the protective effect of Panax Ginseng (PG) on bone metabolism in an experimental ovariectomy (OVX) model of osteoporosis in which inflammation was induced by subcutaneous magnesium silicate. The groups were: sham control (Group1, SH), sham+inflammation (Group2, SHinf), OVX (Group3), OVX+inflammation (Group4, OVXinf), OVX+inflammation+PG 100 mg/kg (Group5, OVXinf+PG1), OVX+inflammation+PG 200 mg/kg (Group6, OVXinf+PG2), OVX+PG 100 mg/kg (Group7, OVX+PG1), OVX+PG 200 mg/kg (Group8, OVX+PG1). After the OVX surgery, all the groups were allowed to recover for two months. On the 59th day after the OVX, inflammation was induced in Groups 2, 4, 5, and 6 by subcutaneous injections of magnesium silicate in the back of the animals. Groups 5 and 7 were administered oral PG 100 mg/kg, and Groups 6 and 8 were administered oral PG 200 mg/kg from the 60th to the 80th day. PG 200 mg/kg was able to restore BMD, up to values measured in both the OVX and the SH animals. The levels of OC and OP decreased in OVXinf+PG1 and OVXinf+PG2 groups. The serum levels of TNF-α, IL-1β, and IL-6 were increased significantly in the OVXinf rats compared with the SH group. The present data showed that PG protected against in the OVX model and in inflammation-induced bone loss rat model.

Key words: Panax Ginseng, Bone mineral density, Ovariectomised rat, Inflammation, Magnesium silicate.

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✉ **Corresponding author**
Tel: + 90 442 2312766
Fax: + 90 442 236 09 68
E-mail: umit.avsar@atauni.edu.tr

INTRODUCTION

The incidence of osteoporosis continues to increase in postmenopausal women due to progressively aging populations. Currently, it is estimated that over 200 million people worldwide have osteoporosis (17). In the United States and the European Union, about 30% of all postmenopausal women have osteoporosis (2). Fragility fracture is a major risk factor in osteoporosis, affecting about 40% of women and 13% of men in their lifetime (54). Osteoporosis is a multifactorial skeletal disease, characterized by a reduction in bone mass and structural deterioration of bone tissue that causes mortality and morbidity in the aged and results in high costs of medical care in the European Union (41). Many experimental studies are focusing on osteoporosis due to its complex pathogenesis and the associated dramatic decline in quality of life, high incidence of the disorder (especially in postmenopausal women), financial costs, and high mortality (51). Thanks to such studies, scientists are beginning to understand the pathogenesis of this condition and to develop new types of treatment (48). Many factors other than sex hormones play a role in the formation of osteoporosis (4). However, the main cause is aging-induced estrogen loss. This leads to an uncontrolled chronic inflammatory cascade, which is modulated by various cytokines and growth factors in bone tissues (62).

Compston (2001) demonstrated a relationship between estrogen and inflammatory cytokines, showing that estrogens reduce bone resorption directly by inhibiting osteoclasts and indirectly by suppressing osteoblastic production of various proresorptive paracrine factors, such as IL-1β, IL-6, and TNF-α (16). Moreover, estrogens may also inhibit the inflammatory reaction by decreasing the expression of specific markers and attenuating the degree of inflammation and tissue damage (2, 16, 18, 19, 65, 66). Estrogen replacement therapy (HRT) has been used as an effective strategy for prevention and treatment of osteoporosis in postmenopausal women (26, 31). However especially in postmenopausal women, HRT is associated with numerous adverse outcomes such as an increased risk of breast cancer and cardiovascular disease (57). Therefore, the potential of alternative and complementary medicine has been a popular subject of osteoporosis research in recent years, especially in relation to menopausal women who are known to use alternative medicine (40).

The ovariectomized (OVX) rat is the closest animal model of inflammation-induced osteoporosis in humans. Inflammation is induced by administering subcutaneous magnesium silicate, which mimics the inflammatory and oxidative status that occurs with aging (44).

There are many Asian and Chinese phytotherapeutic agents on the market. One of the most popular is Panax ginseng (PG), which is indigenous to Korea, China (Panax ginseng C.A. Meyer) (43). Three species with medicinal properties are currently recognized: *Panax ginseng* (Korean ginseng), *P. quinquefolius* (American ginseng), and *P. japonicus* (Japanese ginseng). This herb has been used in the Orient for 5,000 years as a tonic (14). Ginseng is known to have antioxidant (73), anti-aging, immunoenhancement, anti-tumor, anti-stress, and organ-protective effects (6, 25, 53). According to traditional Chinese medicine's "philosophy of opposites" (20), the root has been used as a treatment for asthenia, atherosclerosis, blood and bleeding disorders, colitis, and relief of symptoms associated with aging, cancer, and senility (3). Ginseng is also widely believed to be an aphrodisiac (72). Lee et al. (1995) demonstrated than it has protective effects against lipid peroxidation in the liver and brain (46). Other studies have reported its anti-carcinogenic, anti-diabetic, and anti-inflammatory effects (36, 38, 79).

Our studies and other studies have shown that PG has various anti-apoptotic effects in the liver (42, 56, 77), brain, kidney, and heart (33), as well as in bone marrow stromal cells (64), and that it has protective effects against cancer (80), diabetes (38), and inflammation(8).

This study was performed to evaluate the possible protective effect of PG on bone metabolism in an experimental OVX model of osteoporosis in which inflammation was induced by subcutaneous magnesium silicate to mimic the inflammatory and oxidative status that occurs with aging. Markers of inflammation, IL-1β, IL-6, and TNF-α, and markers of osteoporosis, ostepontin (OP) and osteocalcin (OC), were measured.

MATERIALS AND METHODS

Chemicals

The Korean Society of Ginseng (Seoul, Korea) kindly gifted the *P. ginseng*. All other reagents and chemicals were of analytical grade and purchased from commercial suppliers. Talc powder (magnesium silicate) was purchased from Sigma. This study was performed in the Laboratory of Pharmoclogy at Ataturk University, School of Medicine, Department of Pharmacology.

Animals

For the purpose of this study, 64 adult male albino Wistar rats ($n = 8 \times 8$) with an approximate weight of 220–250 g, were used. The rats were kept in individual cages (360 × 200 × 190 mm) for 15 days prior to the experiment in a room at a constant temperature 23 ± 2 °C, relative humidity 55%, with 14/10 hours light and dark cycles. Each cage contained two or three animals. The animal care and experimental protocols were approved by the Experimental Animal Ethics Committee, Atatürk University, Erzurum, Turkey.

Experimental groups

They were randomized according to their body weight either to sham-operated (sham: controls; $n = 16$) or ovariectomized groups (OVX; $n = 48$). Ovariectomy was performed under anesthesia with a thiopentalsodium injection 20 mg/kg, intraperitoneally. After the ovariectomy, the rats were given 25 mg/kg metamizol sodium as an analgesic

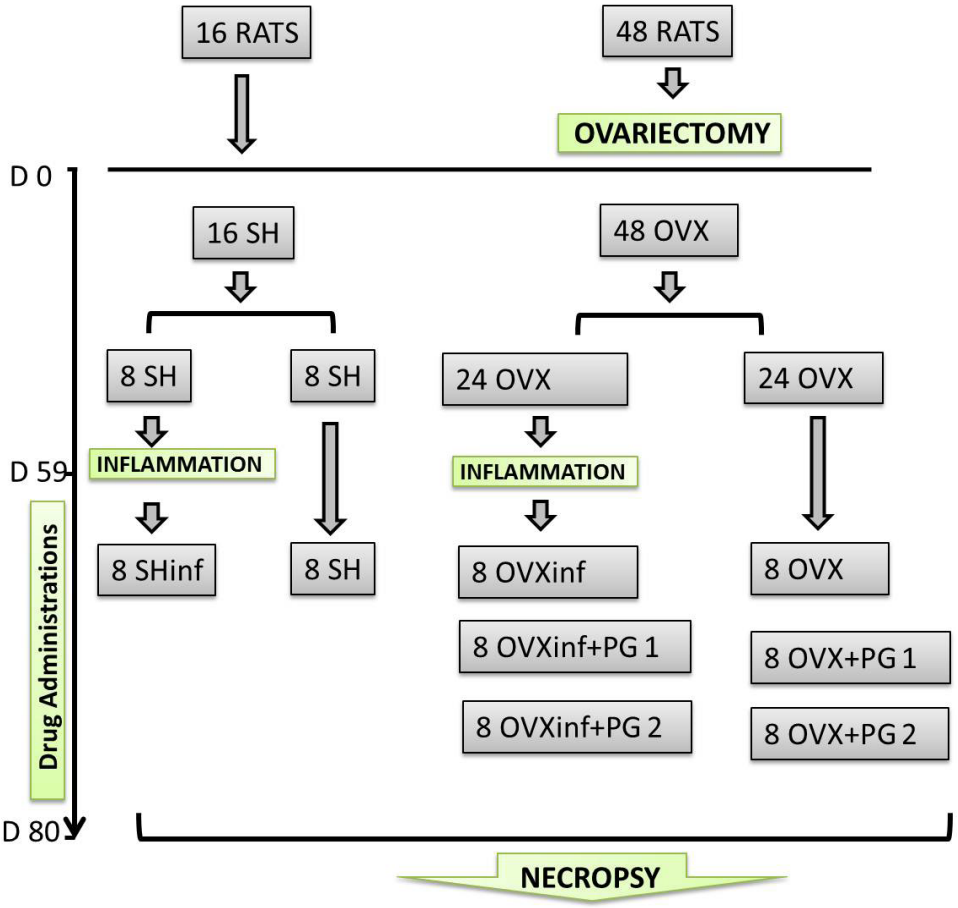

Figure 1. Experimental design: SH; Sham, OVX; Overiectomised rat, Inf; inflammation-induced rat, PG; Panax ginseng administration (PG 1; 100 mg/kg, PG 2; 200 mg/kg).

for two days, and 1.75 mg/kg amoxicillin. The rats were divided into eight groups, each containing eight rats: sham-operated control (Group 1, SH), sham + inflammation (Group 2, SHinf), ovariectomy (Group 3, OVX), ovariectomy + inflammation (Group 4, OVXinf), ovariectomy + inflamation + PG 100 mg/kg (Group 5, OVXinf + PG1), ovariectomy + inflammation + PG 200 mg/kg (Group 6, OVXinf + PG2), ovariectomy + PG 100 mg/kg (Group 7, OVX + PG1), ovariectomy + PG 200 mg/kg (Group 8, OVX + PG1). The groups were maintained separately in different cages. After the ovariectomy surgery, all the groups (3, 4, 5, 6, 7, and 8) were allowed to recover for two months. On the 59th day after the OVX, inflammation was induced in Groups 2, 4, 5, and 6 by four separate subcutaneous injections of talc (magnesium silicate: 3.2 g in total per animal) in sterile saline in the back of the animals. Groups 5 and 7 were administered oral PG 100 mg/kg, and Groups 6 and 8 were administered oral PG 200 mg/kg from the 60th to the 80th day. Experimental desing and all animal groups showed clearly in Fig. 1 (63). On day 80 after the beginning of study, the rats were sacrificed by an overdose of thiopental sodium (50 mg/kg). Blood samples were collected from the abdominal aorta and centrifuged (3,500g for 5 min at 41°C). The plasma was then frozen at -20 °C until OC and OP concentrations were measured to evaluate osteoblastic activity. To determine inflammation and oxidative stress, levels of IL-1β, IL-6, and TNF-α were measured in the collected plasma. Tissue was cleaned from the left and the right femurs. The left femurs were separated for bone mineral density (BMD) measurements, and the right femurs were separated for histopathological study and stored at -80 °C in 4% paraformaldehyde.

Dual-energy X-RAY absorptiometry (DEXA) estimations

The femur bones of the rats were evaluated in vitro after being surgically removed. The bone mineral content (BMC) and the BMD were analyzed by the DEXA method using Discovery Wi (Hologic Inc., Bedford, MA, U.S.A.) equipped with appropriate software for bone assessment in small animals. The same researcher performed each measurement, and all analyses were done using the same region of interest (ROI) window size.

Measurements markers of oxidative stress/ bone

Sera from the all the animal groups were separated and stored at −80 °C until they were thawed for the assay. IL-1β, IL-6, TNF-α, OP, and OC from each sample were measured with ELISA kits; eBioscience-bms630 (San Diego, CA, U.S.A.), Invitrogen- KRC0061 (Grand Island, USA), eBioscience-bms622 (San Diego, CA, U.S.A.), USCNK-E90899RA (Houston, U.S.A.), and USCNK-E90471RA (Houston, U.S.A.), respectively. The concentrations of OP and OC were measured with kits specifically designed for rat cytokines, and all measurements were performed according to the manufacturer's instructions. Cytokine assays for each animal and its correlated control were run in the same lot.

Statistical methods

Data are expressed as means ± standard deviation (SD). To test for any difference among the groups, a one-way analysis of variance test (ANOVA) and Duncan's tests were performed. $P<0.05$ was accepted as significant statistically.

RESULTS

Bone mineral density

Eighty days after the ovariectomy, there was a significant decrease in femoral BMD (BMD g/cm2) compared with the sham group, 0.229 ± 0.01 and 0.253 ± 0.01, respectively ($P<0.05$; Fig. 2). Inflammation via talc aggravated this bone loss in the OVXinf groups (0.213 ± 0.01), whereas the sham groups showed no effect (SHinf: 0.237 ± 0.03). PG 200 mg/kg administration was able to restore BMD, up to values measured in both the OVX and the SH animals. Comparing the results for each therapy, the higher dose (200 mg/kg) of PG showed a greater increase than the lower dose of 100 mg/kg (0.241 ± 0.01 and 0.228 ± 0.02). Both doses of PG, 100 mg/kg and 200 mg/kg, ameliorated the BMD in the OVX only group: 0.240 ± 0.01 and 0.246 ± 0.01, respectively.

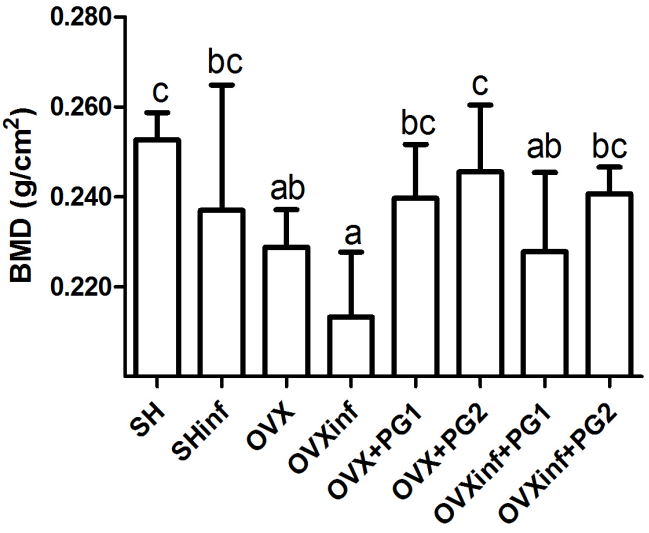

Figure 2. Femoral (BMD) bone mineral densities measured in sham-operated (SH) and ovariectomized rats (OVX) with or without inflammation (OVXinf and SHinf) and in rats supplemented with PG at 100 (OVX + PG 1 and OVXinf + PG1), 200 (OVX + PG2 and OVXinf + PG2) mg/kg body weight per day. Results are means ± SD. Means in the same column with the same superscript are not significantly different compared with Duncan's test ($P = 0.05$). $P<0.05$.

Bone turnover

As shown in Figure 3, osteoporosis induction significantly increased bone turnover markers (OC and OP) in the OVXinf groups when compared with the sham group ($P<0.05$). The OC and OP levels were 39.77 ± 8.23 and 16.49 ± 2.32, respectively, in the sham group, but these levels increased to 60.38 ± 8.58 and 30.79 ± 3.64, respectively, in the OVXinf groups exposed to the talc. Following PG 100 or 200 mg/kg administrations, the levels of OC and OP decreased to 40.66 ± 5.88 and 23.89 ± 1.24 ($P<0.05$) and to 40.22 ± 7.90 and 22.49 ± 1.59 ($P<0.05$) in the OVXinf + PG1 and the OVXinf + PG2 groups, respectively. In addition, the increased level of the bone turnover markers in the OVX alone groups was ameliorated with PG administration.

Markers of oxidative stress

To determine talc-induced inflammation changes in the cytokines in the serum, we examined levels of TNF-α, IL-

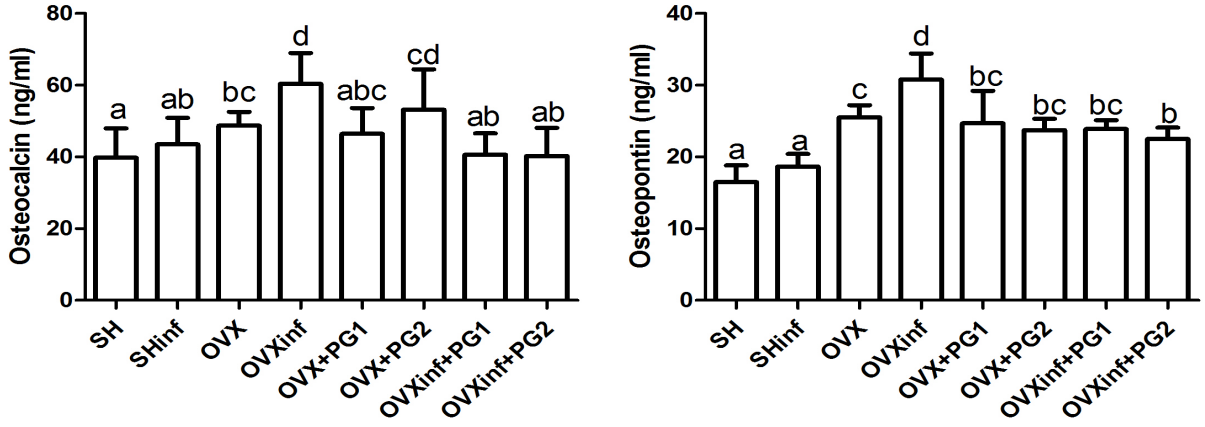

Figure 3. Serum osteocalcin (OC) and osteopontin (OP) concentrations (A and B, respectively) measured in sham operated (SH) and ovariectomized rats (OVX) with or without inflammation (OVXinf and SHinf) and in OVX rats with or without inflammation supplemented with PG at 100 (OVX + PG1), 200 (OVX + PG2), 100 (OVXinf + PG1), 200 (OVXinf + PG2), mg/kg body weight per day. Results are means ± SD. Means in the same column with the same superscript are not significantly different compared with Duncan's test ($P = 0.05$).

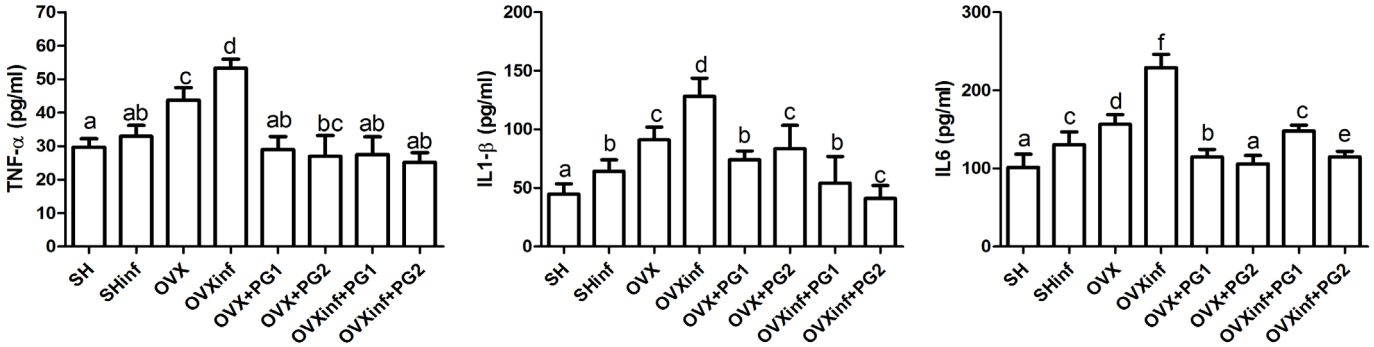

Figure 4. Effects of 12-week treatment with *Panax ginseng* (PG) on the serum level of IL-1β, IL-6, and TNF-α in the rats. Results are means ± SD. Means in the same column with the same superscript are not significantly different compared with Duncan's test ($P = 0.05$).

1β, and IL-6 in the serum using ELISA (Fig. 4). The serum levels of TNF-α, IL-1β, and IL-6 were increased significantly in the OVXinf rats (53.32 ± 2.64, 128.29 ± 15.46, and 229.23 ± 16.92, respectively) compared with the SH group (29.74 ± 2.49, 44.74 ± 8.67, and 101.34 ± 16.84, respectively). The observed elevations of TNF-α, IL-1β, and IL-6 in the serum of the OVXinf rat groups decreased significantly in response to both PG 100 mg/kg (27.51 ± 5.28, 54.24 ± 22.64, and 147.99± 7.47, respectively) and 200 mg/kg (25.17 ± 2.93, 41.08 ± 11.02 and 114.82 ± 6.95, respectively) compared with the OVXinf group. Moreover, both PG treatments decreased these serum cytokines in the OVX + PG1 and the OVX + PG2 rat group compared with the OVX only group (Fig. 4).

DISCUSSION

The data obtained in the present study suggest that PG, which is a popular phytotherapeutic agent, may play a positive role in bone health during aging. The results clearly show that PG prevented bone loss induced by ovariectomy/inflammation in a traditional experimental model of osteoporosis and an extensively used model of postmenopausal bone loss.

Overiectomy surgery of the rat is the most widely used experimental model for postmenopausal osteopenia. It is a suitable model for investigation of human menopausal osteoporosis due to many similarities in their pathophysiological mechanisms of bone impairment (21, 39, 58, 76). In the postmenopausal period, estrogen deficiency can impair the regulation of bone turnover by increasing bone resorption (60) and suppressing bone formation (5). Anabolic agents, especially estrogen derivates, are specifically recommended to treat bone loss (9). When studying problems relating to hormonal deficiency-induced bone loss, it should be considered that senile osteoporosis is not only associated with hormonal loss, but also with impairment in the inflammatory status due to aging (7). The absence of estrogen associated with aging has been shown to induce an unregulated chronic inflammatory process by increasing the local production of various cytokines and growth factors located within the bone microenvironment (62). Compston (2001) demonstrated a relationship between estrogen and these cytokines (16).

In our study, we investigated ovariectomy-induced bone loss aggravated by chronic inflammation with talc. After overiectomy, BMD, which is a useful marker for bone loss and risk of fracture, markedly decreased due to an increase in bone turnover in the OVX rats compared with the sham rats. In contrast, PG given in oral form in the OVX-induced osteoporotic rats produced a marked increase in BMD compared with the OVX group. The lowered BMD found in the OVXinf group supports the conclusion that magnesium silicate-induced inflammation in the rat reduced the BMD in a manner similar to that found in human chronic inflammation-induced osteoporosis. The BMD was decreased in the magnesium silicate-induced inflammation OVX rat group compared with the OVX only group, and PG resulted in a marked increase in the BMD in all the groups. Both OC and OP are sensitive markers of bone health and BMD. They can also be valuable in osteoporosis studies to improve the assessment of the efficacy of the

treatment when BMD measurement by itself does not provide a clear answer. The combined use of BMD values and bone markers can aid the study of experimental or clinical osteoporosis. OC, which is a very important biomarker of bone formation involved in regulating mineralization in the bones, has been used as a preliminary biomarker of the effectiveness of a given drug (12). OC is produced by osteoblasts (49) and increases in OVX-induced osteoporosis in rats and in postmenopausal women (30). In one study, reduced serum OC levels were suggested to be associated with a decrease in osteoblastic function (10). In our study, the serum level of OC peaked 80 days after surgery in the OVX rats and was higher than that in the sham control group. The highest level of OC level was found in the OVXinf group, and PG treatment at doses of 100 and 200 mg ameliorated the level of OC. PG treatment was also more effective in the OVXinf group. This finding suggests that PG has positive effects on osteoblast activation following magnesium silicate-induced inflammation in osteoporosis. OP is highly expressed in bone and has been implicated as an important factor in bone remodeling (13). Bone mineralization studies have suggested that it plays a role in normal bone in anchoring osteoclasts to the mineral matrix and in inhibiting the formation of hydroxyapatite (32, 33, 67). Yositake et al. showed that OP knockout mice were resistant to bone loss after overiectomy (78). Our findings relating to OP are similar to those of previous studies (62). As expected, ovariectomy greatly increased the production of OP in the serum of rats compared with the SH group. These results are in agreement with those of Kwak et al. and Wada et al. (45, 74). The level of serum OP increased in both the OVX and OVXinf groups, with the highest level of OP have been found. The serum OP level was ameliorated by the PG treatment. These findings suggest that PG shifted the bone formation/resorption balance in a positive direction in terms of osteblastic activity.

The pathogenesis of osteoporosis is multifactorial, with many proinflammatory cytokines such as IL-1 and TNF released under osteoporotic conditions where they are involved in stimulating osteoclastic activity and regulation of bone resorption. These cytokines are thought to make an important contribution to stimulating bone resorption and suppressing bone formation (35, 59, 61).

We investigated the role of PG in the pathogenesis of inflammation-induced bone loss in an animal model of inflammation-induced osteoporosis. Bellido et al. reported that IL-6–deficient mice are protected from gonadectomy-induced osteopenia (58). Another study reported that the proinflammatory cytokine TNF affected bone inflammation and that direct administration of an anti-TNFR antibody neutralized the bone inflammation (15). IL-1β was reported to play a critical role in the activation and survival of osteoclasts (47). Lorenza et al. reported that transgenic mice lacking the IL-1β receptor are resistant to ovariectomy-induced bone loss (61). They also reported that aging-linked estrogen deficiency led to an uncontrolled chronic inflammatory process by increasing the level of local cytokines in bone tissue. Moreover, considerable evidence has demonstrated that proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 are involved in the regulation of bone turnover and that these are correlated negatively with changes in BMD (58, 60). Proinflammatory cytokines have also been implicated in the release of free radicals (71). The pathways by which they exert their effects may involve inhibiting osteoblastic recruitment and the activity of mature cells (55) and increasing osteoclastic resorption (75). We demonstrated in this study that administration of PG decreased serum levels of IL-1β, TNF-α, and IL-6 that are known to be produced by osteoblasts and induce bone resorption. Due to the antioxidant/anti-inflammatory effect of PG on the serum levels of BMD, the inhibition of OC and OP was more significant in the OVXinf + PG group compared with the OVX + PG only group. PG has been found to be a potent antioxidant in vivo (34, 56). In fact, the production of oxide-derived free radicals is known to exaggerate bone resorption (23) and to inhibit osteoblastic activity (55) associated with inflammation, leading to the generation of free radicals, which trigger additional inflammation (29). As a result, some studies have focused on antioxidant nutrients to restore bone formation and decrease the production of free radicals that contribute to bone resorption (68). This effect may also be related to anti-inflammatory properties. PG could offer an approach to prevent accelerated bone loss caused by antioxidant/anti-inflammatory activity (1, 11, 52). In the present study, PG prevented the increase in serum inflammatory cytokines in the serum after inflammation.

Various drugs such as bisphosphonates, parathyroid hormone, estrogen, selective estrogen receptor modulators, calcitonin, vitamin D, and calcium channel blockers are available to prevent or treat osteoporosis; they work by targeting the resorptive pathway and bone loss or (28, 37) by amplifying or mimicking steps in the anabolic pathway to build new and improved skeletons (22, 24, 27, 50, 69, 70).

However, whether these drugs are effective in helping patients with osteoporosis or osteopenia is unclear. PG may prove helpful in this regard, with several previous studies showing that it has many beneficial properties such as anti-oxidant and anti-inflammatory effects (36, 38, 56).

In conclusion, our study showed that inflammation via magnesium silicate aggravated osteoporosis in an OVX rat model. PG protected against bone loss in this rat model. The preventive effect of PG in the OVX model and in inflammation-induced bone loss depended on decreasing levels of OC, OP, IL-1β, IL-6, and TNF- α in the serum. PG reduced the risk of inflammation-induced osteoporosis in the osteoporotic rat by its antioxidant activity.

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