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Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice

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Abstract

Pomegranate is known to contain estrogens (estradiol, estrone, and estriol) and show estrogenic activities in mice. In this study, we investigated whether pomegranate extract is effective on experimental menopausal syndrome in ovariectomized mice. Prolongation of the immobility time in forced swimming test, an index of depression, was measured 14 days after ovariectomy. The bone mineral density (BMD) of the tibia was measured by X-ray absorptiometry and the structure and metabolism of bone were also analyzed by bone histomorphometry. Administration of pomegranate extract (juice and seed extract) for 2 weeks to ovariectomized mice prevented the loss of uterus weight and shortened the immobility time compared with 5% glucose-dosed mice (control). In addition, ovariectomy-induced decrease of BMD was normalized by administration of the pomegranate extract. The bone volume and the trabecular number were significantly increased and the trabecular separation was decreased in the pomegranate-dosed group compared with the control group. Some histological bone formation/resorption parameters were significantly increased by ovariectomy but were normalized by administration of the pomegranate extract. These changes suggest that the pomegranate extract inhibits ovariectomy-stimulated bone turnover. It is thus conceivable that pomegranate is clinically effective on a depressive state and bone loss in menopausal syndrome in women.

Keywords: Pomegranate; Menopausal syndrome; Ovariectomy; Depression; Bone loss

1. Introduction

The pomegranate tree, which is said to have flourished in Eden, has been used extensively in the folk medicine for a number of therapeutic purposes (Langley, 2000). Pomegranate is a rich source of crude fibers, pectin, sugars, and several tannins (Gil et al., 2000). In addition, it has recently been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract (Schubert et al., 1999; Gil et al., 2000; Aviram et al., 2000; Aviram and Dornfeld, 2001; Kaplan et al., 2001; Halvorsen et al., 2002; Noda et al., 2002; Singh et al., 2002). Furthermore, the chemopreventive and adjuvant therapeutic applications of pomegranate to human breast cancer have been warranted recently (Kim et al., 2002). Owing to these significant biological activities, pomegranate juice is being increasingly popularized in Japan.

However, one of the most remarkable characteristics of pomegranate fruit is that its seeds are the richest plant source of estrogens. Pomegranate seeds are known to contain the estrogenic compounds, estrone and estradiol, that are chemically identical to those biosynthesized in human body (Heftmann et al., 1966), and coumesterol as well (Moneam et al., 1988). According to Kim et al. (2002), pomegranate seeds contain not only estrogens (estradiol, estrone, and estriol) but also other steroids such as testosterone and β-sitosterol, and coumesterol, whereas, anthocyanins and phenolic acids are the main ingredients of pomegranate juice. In our preliminary HPLC assay, isoflavone phytoestrogens such as genistein and daidzein were also detected in the pomegranate extract containing seeds used in this study. The estrogenic activities of pomegranate seed extract on the uterus weight and vaginal smears have already been investigated in ovariectomized animals (Sharaf and Nigm,

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1964). Then, the question raised is whether pomegranate can, as the richest plant source of estrogens, exhibit additional important biological actions on menopausal syndrome in women? This question prompted us to investigate the effect of "pomegranate extract" (see Section 2 for the property of "pomegranate extract") on the estrogen deficiency produced by bilateral ovariectomy of experimental animals.

A depressive state is a clinically significant mental profile of human menopausal syndrome (Gerdes et al., 1982). A forced swimming test is considered to be useful for experimentally investigating a depressive state in humans because the behavioral immobility of mice or rats which occurs during forced swimming has been reported to be able to reproduce some aspects of human depression (Porsolt et al., 1977; Yoshimura and Yamakawa, 2000). A depressive animal is liable to despair of escaping from forced swimming and become immobile. In fact, ovariectomized mice have been confirmed to show not only a prolonged immobility time in forced swimming test but also regional skin temperature changes alike a hot-flush symptom in women (Okada et al., 1997).

Another characteristic of menopausal syndrome is bone loss. Involutional bone loss in post-menopausal women has been suggested to occur in the two phases: an early rapid phase beginning at menopause and lasting for 6–10 years; and a subsequent slow phase lasting for the rest of a woman's life (Riggs et al., 1998). It is also known that estrogen replacement therapy can prevent the early phase of involutional bone loss from occurring and also restore the rate of bone resorption and formation to pre-menopausal levels in menopausal women (Barzel, 1988; Heshmati et al., 2002). Thus, it is clear that the menopausal bone loss is attributable to the cessation of ovarian function and tapering-off of estrogen secretion. On this basis, we also investigated the effects of the pomegranate extract on bone properties by measuring the mineral bone density and some histological parameters of bone turnover.

We report here that the pomegranate extract is pharmacologically effective on the menopausal symptoms produced in mice by bilateral ovariectomy and finally propose that the daily regular consumption of juice from whole pomegranate fruit can protect women from a variety of menopausal syndromes.

2. Materials and methods

2.1. The pomegranate extract used

The pomegranate extract used in this study was produced and blended under license in Iran and distributed in Japan by Perusha Zakuro Co. Ltd. (Tokyo, Japan), and contained both the seed and juice of the four Persian black and red pomegranate species, Siyah Daneh Dorosht Shiraz, Tough Gardan Shahvar, Malas Shirin Yazd, and Tabolarz Dorosht Shiraz. This pomegranate seed extract and juice (pomegranate extract, hereafter) had been confirmed to contain estrogens (estradiol, estrone, and estriol), genistein and daidzein by RIA and/or HPLC/ECD assays.

2.2. Animals and administration of pomegranate extract

The ICR albino mice obtained from CLEA Japan Inc. (Osaka, Japan) were used for experiments and were ovariectomized when they weighed 25–30 g. Thirty of them were bilaterally ovariectomized under sodium thiopental anesthesia on day 7 after purchasing, and forty of them including intact mice were individually housed in animal cages with wood shavings. Food and water were freely available. The animal room was conditioned at $23 \pm 1\,^{\circ}C$ and illuminated according to a 12 h light–dark cycle (light on at 07:00 and off at 19:00). The following experiments were conducted in accordance with the Guide for Animal Experimentation at Saitama Prefectural University following the NIH Guideline.

The pomegranate extract was diluted to 20 or 10% with distilled water, and these dilute extracts were stored frozen at $-20\,^{\circ}C$ for maximum 3 weeks until administration to animals. The extract (0.01 ml/g body weight) was given to each animal by forced oral administration at approximately 16:00–17:00 every day for 2 weeks starting on the next day of ovariectomy. To 10 control mice, 5% glucose aqueous solution was administered similarly. In addition, intact mice (not ovariectomized) were handled every day in a manner similar to mice in the control and pomegranate extract groups.

2.3. Behavioral tests

The forced swimming test was performed after administration of pomegranate extract following the conventional methods reported previously (Porsolt et al., 1977,1978; Yoshimura and Kan, 1998). Briefly, mice were placed individually into a vertical polycarbonate cylinder (40 cm in height and 15 cm in diameter) containing water at $25\,^{\circ}C$ at a depth of 15 cm. After 15 min free swimming, the animal was removed from the water and allowed to dry the body surface for about 15 min in a heated enclosure ($32\,^{\circ}C$) before being returned to its home cage. During this 15 min forced swimming, the mouse shows a state of immobility (floating passively) several times. The mouse is mildly hypothermic ($-3\,^{\circ}C$) on removal from the water, and then continues to be hypoactive for periods up to 30 min. After 24 h, the mice were replaced in the cylinder and the total duration of immobility was measured during a swimming time of 6 min. The mouse was judged to be immobile whenever it remained floating passively in water in a slightly hunched upright position with its head being just above the water surface.

2.4. Bone mineral densitometry (BMD)

The left tibia of each mouse was separated together with the periosteum from adherent muscles and connec-

tive tissues at sacrifice by over-anesthesia with sodium thiopental and ethylether, and subjected to the single energy X-ray absorptiometry using DCS-600R (Aloka, Tokyo, Japan). BMD values were obtained from the proximal two-fifth of tibia including the epimetaphyseal region.

2.5. Bone histomorphometry

The right tibia removed from each mouse was fixed with 70% ethanol and embedded in methyl methacrylate (MMA) without decalcification. The fixed tibia was sectioned (5 μm in thickness) serially and longitudinally using the microtome (Model 2050; Reichert Jung, Buffalo, NY), and the sections were then stained by Villanueva Goldner's trichrome method for discrimination between mineralized and unmineralized bones and also for identification of cellular components. Stained bone sections were analyzed at a magnification of 400× using the semi-automated histomorphometry system (Osteoplan II; Carl Zeiss, Thornwood, NY). The parameters measured for bone structures were the total bone volume per tissue volume (BV/TV, %) and the mineralized bone volume per tissue volume (Md. BV/TV, %), trabecular thickness (Tb. Th., μm), trabecular number (Tb. N., mm) and trabecular separation (Tb. Sp., μm).

The parameters obtained for the bone formation were the osteoid volume per bone volume (OV/BV, %), the osteoid surface per bone surface (OS/BS, %), and the osteoblast surface per bone surface (Ob. S/BS, %). The parameters measured for bone resorption were the eroded surface per bone surface (ES/BS, %), the number of osteoclasts per the bone perimeter (N. Oc./B. Pm./100 mm) and the osteoclast surface per bone surface (Oc. S/BS, %).

2.6. Statistical analysis

All data were expressed as the mean±S.D. The difference between the two data sets obtained from the intact, control, and pomegranate groups was analyzed by using Student's two-sided *t*-test (SPSS Statistical Methods, Version 10.0) taking $P < 0.05$ as statistically significant.

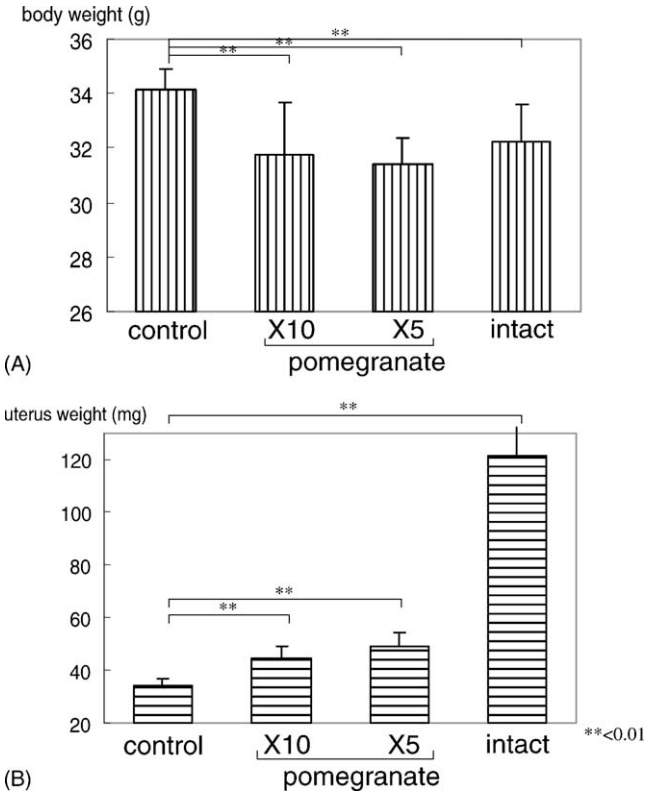

Fig. 1. Effects of pomegranate extract on the body and uterus weights. The increase of body weight (A) and the decrease of uterus weight (B) in ovariectomized (control) mice were significantly suppressed by administration of 10% (×10) and 20% (×5) dilute pomegranate extract.

3. Results

3.1. Effects on the body weight and uterus weight

The mean values of body weight and uterus weight of intact and ovariectomized mice ($n = 10$ for each) measured after the 2-week administration of 5% glucose solution or pomegranate extract are shown in Table 1 and Fig. 1. As shown in Table 1 and Fig. 1, the mean body weight was 34.1±0.8 g in 5% glucose-dosed ovariectomized mice (control group) which was significantly greater than that in intact mice, 32.2 ± 1.4 g ($P < 0.01$). However, this ovariectomy-induced increase in body weight was abolished

Table 1
Effects of pomegranate extract on the body and uterus weights and the immobility time in forced swimming

Group	Body weight (g)	Uterus weight (mg)	Immobility time (s)
Control	34.1 ± 0.8	34.42 ± 2.40	222.7 ± 11.9
Pomegranate (10% extract)	31.8 ± 1.9**	44.36 ± 4.84**	177.7 ± 24.5**
Pomegranate (20% extract)	31.4 ± 1.0**	48.91 ± 5 53**	169.9 ± 23.1**
Intact	32.2 ± 1.4**	121.4 ± 26.3**	190.5 ± 40.6*

Mean ± S.D., $n = 10$.
* $P < 0.05$, significantly different from control.
** $P < 0.01$, significantly different from control.

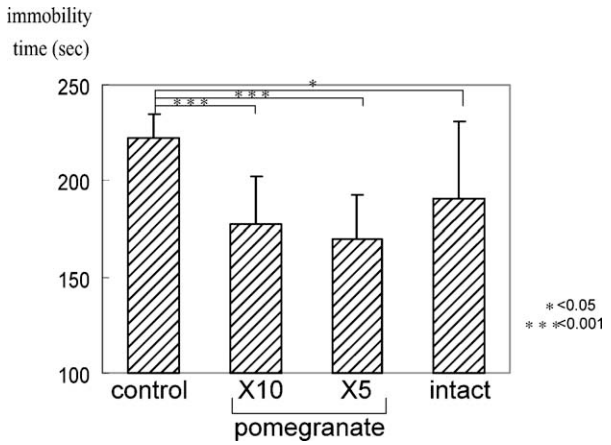

Fig. 2. Effects of pomegranate extract on the immobility time in forced swimming. Ovariectomy-induced prolongation of immobility time (control) was significantly shortened by administration of 10% (×10) and 20% (×5) dilute pomegranate extract.

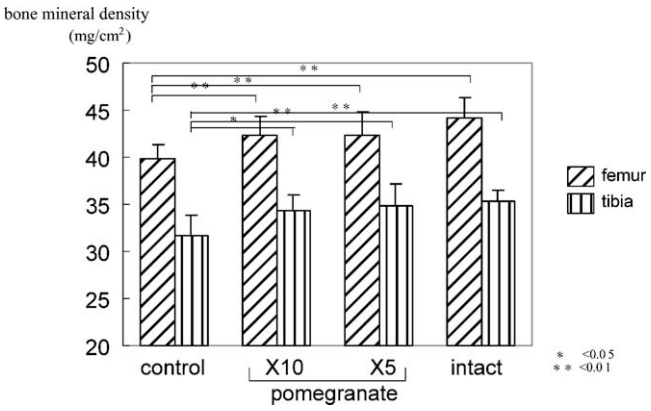

Fig. 3. Effects of pomegranate extract on the bone mineral density of the femur and the tibia. The decrease of the bone mineral density of the whole femur and that of the proximal two-fifth of tibia were completely reversed by administration of 10% (×10) and 20% (×5) dilute pomegranate extract.

by the administration of the pomegranate extract diluted to either 20 or 10% in water. Moreover, the mean body weight was lower in both pomegranate groups than in the intact or control group.

The mean uterus weight was dramatically decreased to a level of one-third by bilateral ovariectomy: 121.4 ± 26.3 mg for intact mice versus 34.42 ± 2.40 mg for control mice (Table 1 and Fig. 1). However, administration of pomegranate extract for 2 weeks after ovariectomy prevented the loss of uterus weight (44.36 ± 4.84 mg for 10% dilute extract and 48.91 ± 5.53 mg for 20% dilute extract) showing a significant difference ($P < 0.01$) between either one of pomegranate groups and the control group (Table 1 and Fig. 1).

3.2. Effects on the immobility time in forced swimming test

By bilateral ovariectomy, the mean immobility time in forced swimming test was significantly ($P < 0.05$) prolonged (222.7 ± 11.9 s for control mice versus 190.5 ± 40.6 s for intact mice) as shown in Table 1 and Fig. 2, in confirmation of our previous reports (Yoshimura and Yamakawa, 2000). This ovariectomy-induced prolongation of immobility time was significantly shortened by administration of pomegranate extract ($P < 0.001$ for 5 and 10 times diluted extract). Although, the mean immobility time appeared to be smaller in either one of pomegranate groups than in the intact group, the difference was not statistically significant. Thus, these results indicate that the pomegranate extract inhibited the prolongation of the immobility time produced by bilateral ovariectomy.

3.3. Effects on bone properties

The bone mineral density (BMD) of the whole femur and that of the proximal two-fifth of tibia were measured by X-ray absorptiometry, and bone histomorphometry of the metaphyseal secondary spongiosa was performed. The BMD was significantly decreased ($P < 0.01$) 2 weeks after ovariectomy in both, the whole femur and the proximal two-fifth of tibia but these ovariectomy-induced decreases in bone mineral density were completely reversed by administration of dilute pomegranate extract, as shown in Fig. 3. Since the pomegranate groups treated with 20 and 10% dilute extract showed practically identical BMD values, the group which received 20% dilute pomegranate extract was further analyzed to investigate the effect of pomegranate on bone properties.

In the longitudinal sections of trabecular bone with well-preserved morphology allowing observation of the secondary spongiosa, the trabecular bone volume decreased as expected in ovariectomized mice (Fig. 4A) as compared with intact mice (Fig. 4C), and the trabecular bone loss which was to be induced by ovariectomy was clearly prevented by administration of pomegranate juice to ovariectomized mice (Fig. 4B).

As shown in Table 2 and Fig. 5, histomorphometrical analysis of bone structures showed that the bone volume per tissue volume, the mineralized bone volume per tissue volume, and the trabecular thickness decreased in the 5% glucose control group compared with the intact group, though not significantly. Only the trabecular separation was significantly increased by ovariectomy ($P < 0.05$, Fig. 5D). All these parameters in pomegranate groups were close to those in the intact group. Some histological parameters for bone formation and resorption such as the osteoblast surface per bone surface, the eroded surface per bone surface, the number of osteoclasts per bone perimeter, and the osteoclast surface per bone surface significantly increased in the 5% glucose control group compared with the intact group ($P < 0.001$ to $P < 0.05$, Fig. 6), and these changes were completely restored to normal levels by administration of pomegranate extract (Fig. 6).

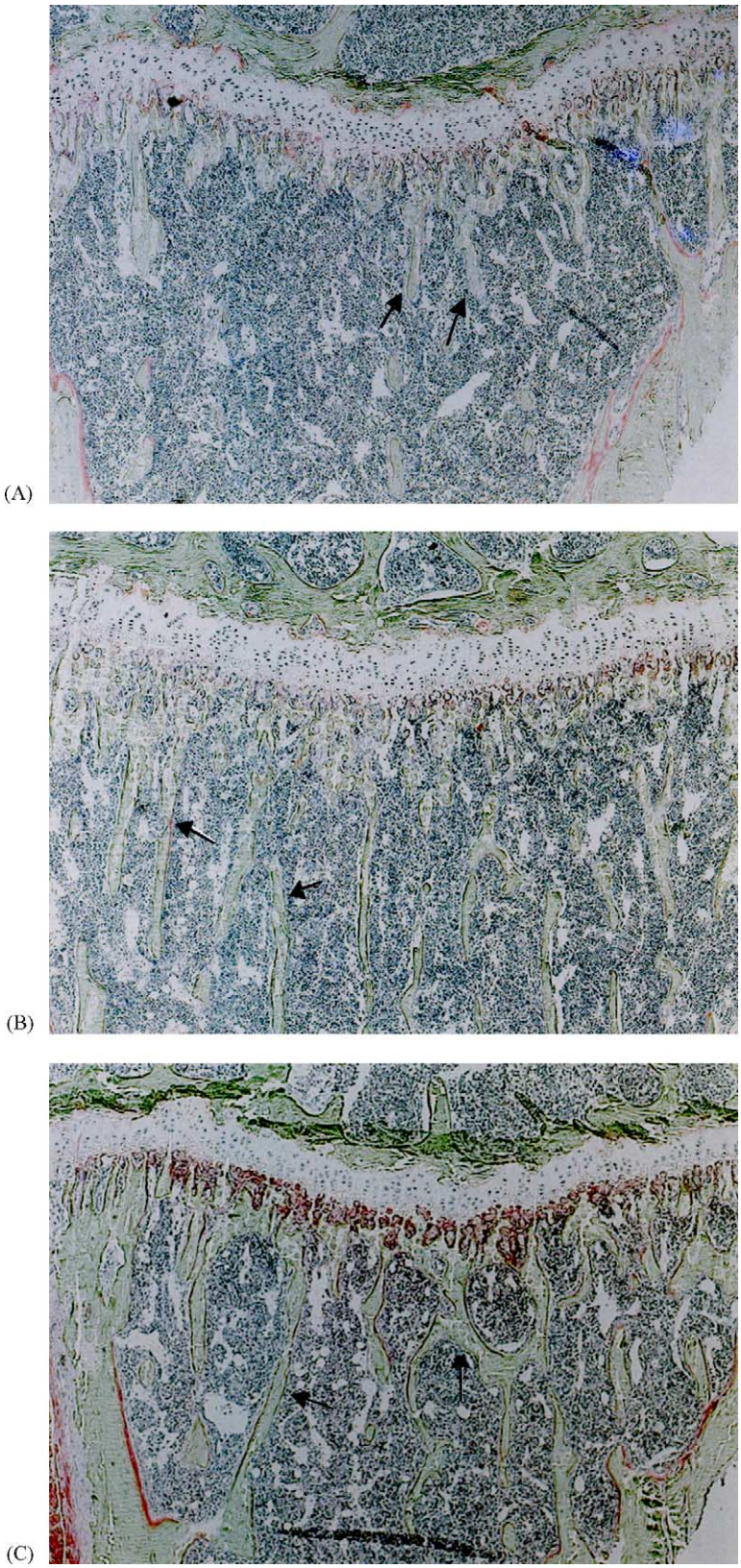

Fig. 4. Effect of pomegranate extract on bone structures. Trabecular bones (arrows) lost in ovariectomized mice (A) compared with intact mice (C) and the loss was recovered by administration of pomegranate extract (B).

Table 2
Effects of pomegranate extract on bone properties of ovariectomized mice

Parameters	Control	Pomegranate	Intact
Bone volume/tissue volume (BV/TV, %)	6.26 ± 3.34	7.64 ± 3.06	9.75 ± 1.60
Mineralized bone volume/Tissue volume (Md. BV/TV, %)	6.00 ± 3.44	7.17 ± 3.30	9.32 ± 2.70
Trabecular thickness (Tb. Th., μm)	27.94 ± 6.00	28.09 ± 4.42	34.57 ± 6.81
Trabecular number (Tb. N., mm)	2.22 ± 1.06	2.58 ± 0.81	2.79 ± 0.46
Trabecular separation (Tb. Sp., μm)	662.06 ± 402.27	400.38 ± 111.06	334.49 ± 73.30
Osteoid volume/bone volume (OV/BV, %)	6.45 ± 2.78	3.78 ± 1.91	4.50 ± 1.54
Osteoid surface/bone surface (OS/BS, %)	29.39 ± 9.99	21.70 ± 7.42	26.14 ± 6.36
Osteoblast surface/bone surface (Ob. S/BS, %)	14.67 ± 3.79	7.07 ± 4.67	5.67 ± 3.63
Eroded surface/bone surface (ES/BS, %)	21.30 ± 6.25	9.99 ± 3.15	10.54 ± 4.33
Number of osteoclasts/bone perimeter (N. Oc./B. Pm., 100 mm)	132.79 ± 62.73	77.50 ± 30.61	65.41 ± 25.02
Osteoclast surface/bone surface (Oc. S/BS, %)	3.14 ± 0.88	1.69 ± 0.67	1.54 ± 0.77

Mean ± S.D., $n = 10$.

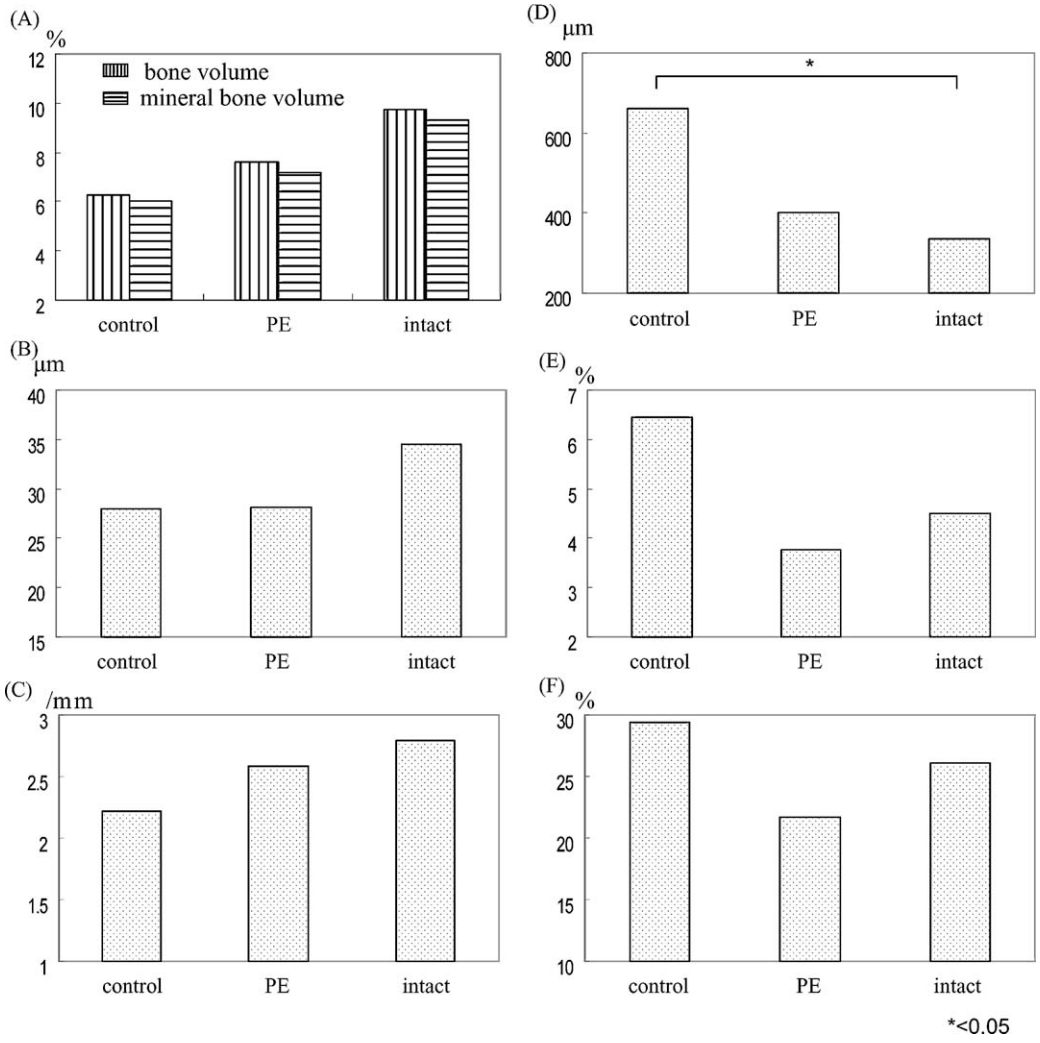

Fig. 5. Effects of pomegranate extract (PE) on bone properties. (A) Bone volume/tissue volume, and mineralized bone volume/tissue volume; (B) trabecular thickness; (C) trabecular number; (D) trabecular separation; (E) osteoid volume/bone volume; (F) osteoid surface/bone surface. All these parameters in pomegranate-dosed groups were close to those in the intact group except for the osteoid volume/bone volume and the osteoid volume/bone volume.

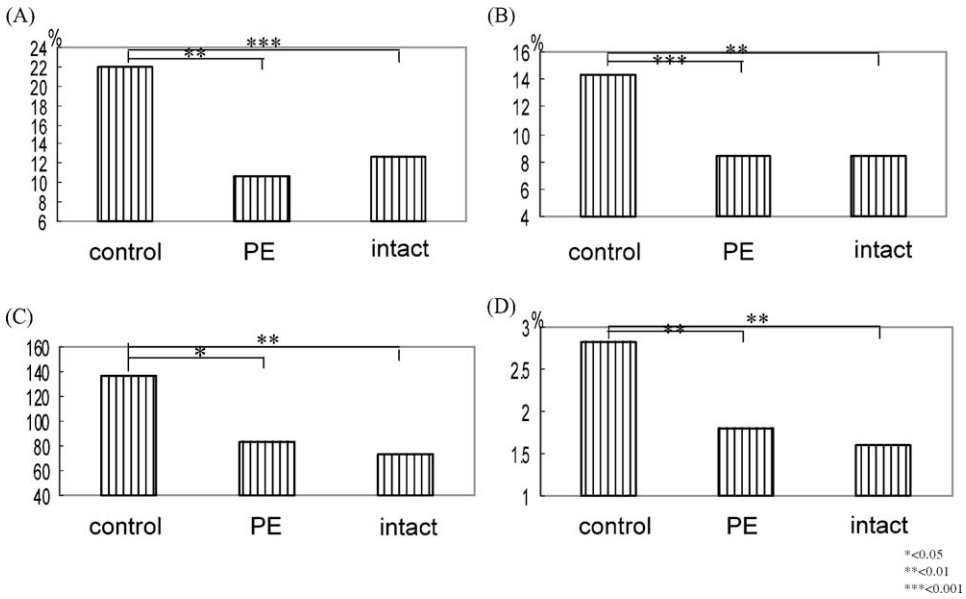

Fig. 6. Effects of pomegranate extract (PE) on bone properties. (A) Osteoblast surface/bone surface; (B) eroded surface/bone surface; (C) number of osteoclasts/bone perimeter; (D) osteoclast surface/bone surface. The histological parameters that were increased in the 5% glucose control group compared with the intact group were completely restored to normal levels by administration of pomegranate extract.

4. Discussion

It is well known that ovariectomy induces the increase of body weight (Wade, 1979; Yoshimura and Kan, 1998). Also in the present study, the body weight of mice increased after bilateral ovariectomy, and the increase was inhibited by chronic administration of pomegranate extract.

Regarding the role of estrogens in lipid metabolism, estrogen insufficiency is thought to be largely responsible for an increase in adiposity during menopause because postmenopausal women under estrogen replacement therapy do not display the characteristic pattern of abdominal weight gain usually associating with menopause (Gambacciani et al., 1997; Jones et al., 2000). Furthermore, it has been observed that the aromatase-knockout mice (an animal model of estrogen insufficiency) display progressive increases in adiposity as compared with wild type littermates (Heine et al., 2000). Thus, it is conceivable that the pomegranate extract administered as a rich plant source of estrogens is able to regulate the lipid metabolism instead of insufficient endogenous estrogens.

The mean uterus weight was dramatically decreased in ovariectomized control mice compared with intact mice, and administration of pomegranate extract for 2 weeks after ovariectomy prevented the ovariectomy-induced loss of uterus weight. A similar estrogenic activity has already been identified by injection of pomegranate seed oil to mice in 1964 (Sharaf and Nigm, 1964) and to rabbits in 1966 (Heftmann et al., 1966). In addition, Sharaf and Nigm have demonstrated that the keratinization of vaginal cells in vaginal smears is enhanced by administration of pomegranate seed oil in mice (Sharaf and Nigm, 1964). Based on these previous results, it may be said that our pomegranate extract is effective on the development and function of the uterus as well.

In the present study, forced swimming test was utilized as a model system for evaluating the effects of pomegranate extract on a depressive state in human menopausal syndrome. The summed immobility time during forced swimming test was significantly prolonged in ovariectomized mice, and this prolongation of the immobility time was significantly shortened by the administration of pomegranate extract to ovariectomized mice. Porsolt et al. have reported that the immobility observed during forced swimming test reflects the state of lowered activity in rats and selectively responds to treatment with antidepressants (Porsolt et al., 1977,1978).

Nishimura et al., on the other hand, have stated that the immobility does not necessarily imply “behavioral despair”, but rather implies an emotional reaction to an inescapable stressor (Nishimura et al., 1988). Recently, it was demonstrated that ovariectomized rats showed a prolonged immobility time in forced swimming test and this prolongation was shortened by 17β-estradiol treatment, and also that ovariectomy-induced elevation of tail skin temperature, like hot-flush phenomenon in human, was also suppressed by 17β-estradiol (Okada et al., 1997). Considering these effects of 17β-estradiol, it is very likely that the immobility in forced swimming test reflects a state of lowered mood in the rat. In the present study, we demonstrated that ovariectomy-induced prolongation of immobility time in mice was significantly shortened by the administration of pomegranate extract. Thus, we conclude that our pomegranate extract would be effective on the depressive state of menopausal syndrome in women.

It is now obvious that the depression and anxiety of menopausal syndrome in women are clinically improved

by estrogen replacement therapy (Gerdes et al., 1982). Regarding the anti-depressant effect of estrogens, it has been suggested that estrogens may enhance the function of central adrenergic systems through the increase of noradrenaline content and the decrease of monoamine oxidase activity (Klaiber et al., 1979; Wickelgren, 1997). In addition to this antidepressant effect, it has also been confirmed that estrogens can improve memory in both healthy women and female patients with Alzheimer's disease possibly through the stimulation of the sprouting of axons and dendrites of neurons (Regan and Guo, 1997; Wickelgren, 1997; Woolley et al., 1997). Thus, estrogens are very likely to positively activate the nervous systems participating in the processes of learning and memory. On this basis, it may be strongly suggested that the consumption of pomegranate extract containing an appropriate amount of estrogens would be effective not only in the improvement of menopause-related depression but also on age-related memory loss.

By histomorphometrical analysis of bone structures in the present study, the bone volume per tissue volume, the mineralized bone volume per tissue volume, and the trabecular thickness were found to decrease and the trabecular separation was increased in the 5% glucose control group. On the other hand, these ovariectomy-induced changes in bone parameters were prevented by pomegranate administration to levels close to those in the intact group. In addition, the histological bone formation parameters (the osteoid volume per bone volume, the osteoblast surface per bone surface, and osteoblast surface per bone surface) and the bone resorption parameters (the eroded surface per bone surface, the number of osteoclasts per bone perimeter, and the osteoclast surface per bone surface) significantly increased in the 5% glucose control group and these changes were completely restored to normal levels by pomegranate. Thus, these changes of parameters concerning the bone formation and resorption indicate that our pomegranate extract could inhibit ovariectomy-stimulated bone turnover. It is thus conceivable that pomegranate is clinically useful and effective in bone loss in menopausal syndrome in women.

It is well-known that bone loss is characteristic of menopausal syndrome and is attributable to the cessation of ovarian function and tapering-off of estrogen secretion. Furthermore, estrogen replacement therapy can prevent the early phase of involutional bone loss and also restore the rate of bone resorption and formation to pre-menopausal levels in menopausal women (Barzel, 1988; Heshmati et al., 2002). However, Heshmati et al. have shown that a low serum estrogen level, even an undetectable level, can regulate the bone resorption without inducing bone formation in menopausal women (Heshmati et al., 2002). Thus, it is not surprising that the pomegranate extract used here could restrain the bone turnover because it contains estrogens derived from seeds at concentrations ranging from 100 to 200 pg/ml. Since genistein and daidzein are contained in our pomegranate juice, and anthocyanins and phenolic acids are reported as the ingredients of pomegranate juice (Kim et al., 2002), the effects of these substances on menopausal syndrome due to estrogen insufficiency remain to be investigated. In addition, the effects of testosterone and coumesterol in pomegranate seeds (Kim et al., 2002) are to be explored. In any case, the daily regular consumption of pomegranate juice containing the seed oil is expected to protect women from not only bone loss but also a variety of menopausal symptoms.

In conclusion, the pomegranate extract was pharmacologically effective on the menopausal symptoms produced experimentally in mice by bilateral ovariectomy. It is thus conceivable that pomegranate is effective on a depressive state and bone loss in menopausal syndrome in women. We finally propose that the daily regular consumption of pomegranate extract (juice and seeds) can protect women from a variety of menopausal symptoms.

Acknowledgements

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