

Original Article

Royal Jelly Prevents Osteoporosis in Rats: Beneficial Effects in Ovariectomy Model and in Bone Tissue Culture Model

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Royal jelly (RJ) has been used worldwide for many years as medical products, health foods and cosmetics. Since RJ contains testosterone and has steroid hormone-type activities, we hypothesized that it may have beneficial effects on osteoporosis. We used both an ovariectomized rat model and a tissue culture model. Rats were divided into eight groups as follows: sham-operated (Sham), ovariectomized (OVX), OVX given 0.5% (w/w) raw RJ, OVX given 2.0% (w/w) RJ, OVX given 0.5% (w/w) protease-treated RJ (pRJ), OVX given 2.0% (w/w) pRJ, OVX given 17 β -estradiol and OVX given its vehicle, respectively. The Ovariectomy decreased tibial bone mineral density (BMD) by 24%. Administration of 17 β -estradiol to OVX rats recovered the tibial BMD decrease by 100%. Administration of 2.0% (w/w) RJ and 0.5–2.0% (w/w) pRJ to OVX rats recovered it by 85% or more. These results indicate that both RJ and pRJ are almost as effective as 17 β -estradiol in preventing the development of bone loss induced by ovariectomy in rats. In tissue culture models, both RJ and pRJ increased calcium contents in femoral-diaphyseal and femoral-metaphyseal tissue cultures obtained from normal male rats. However, in a mouse marrow culture model, they neither inhibited the parathyroid hormone (PTH)-induced calcium loss nor affected the formation of osteoclast-like cells induced by PTH in mouse marrow culture system. Therefore, our results suggest that both RJ and pRJ may prevent osteoporosis by enhancing intestinal calcium absorption, but not by directly antagonizing the action of PTH.

Keywords: bone mineral density – bone tissue culture – ovariectomized rats – post-menopausal osteoporosis model – protease-treated RJ – royal jelly

Introduction

With the growing aged population, we urgently need to develop new and effective treatments for chronic and protracted diseases, e.g. chronic pain, diabetes, arthritis, osteoporosis, cancer as well as cardiovascular and brain ailments. Recently, more and more unconventional therapies have been used for treating these diseases (1–3).

The peak bone mass of women is less than that of men because of the decrease in bone mass owing to menopause. Therefore, women are vulnerable to bone loss known as post-menopausal osteoporosis (4,5). The primary cause for this symptom is an estrogen deficiency. An ovariectomized rat model, which artificially produces estrogen deficiency, has been used for the study of post-menopausal osteoporosis. Both aged and mature rats have been employed as animal models for experimentally induced osteoporosis. The mature rat model has characteristics which are seen in early post-menopausal bone loss (6). Using this model, several compounds were screened and found to be effective for the

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therapeutic management of osteoporosis; namely, estrogen (5,6), bisphosphonate (6,7), calcitonin (6,8), calcium products (6,9) and anabolic steroids (10). Recently, efficacy of traditional Chinese (Kampo) medicines has been reevaluated by clinicians (11). Kampo medicines have less side effects, and therefore, seem to be more suitable for a long-term medication. We reported their inhibitory effects on both the progression of tibial bone loss and the alteration of periodontium induced by ovariectomy in rats (12–15).

Royal jelly (RJ) is the principal food for the honeybee queen. This is produced in the hypopharyngeal and mandibular glands of worker honeybees and has been demonstrated to possess several pharmacological activities, such as life-span-elongating (16), antifatigue (17), antiallergic (18), antitumor (19), antihypercholesterolemic (20), antihypertensive (21) and anti-inflammatory effects (22). Indeed, RJ has been used worldwide as commercial medical products, health foods and cosmetics. The chemical composition of RJ was reported to be proteins, sugars, lipids and vitamins (23). It contains many bioactive substances such as 10-hydroxy-2-decenoic acid (23), proteins mentioned above (17,18) and antibacterial protein (24). RJ contains testosterone and has activities similar to other steroid hormones (20,25,26). Therefore, RJ may be beneficial for osteoporosis. Although RJ has been traditionally known to improve post-menopausal symptoms, to our knowledge, no one has studied whether or not it prevents the decrease of bone mass, which is one of the deleterious symptoms for post-menopausal women.

Therefore, we undertook this study to evaluate the efficacy of RJ and protease-treated RJ (pRJ) in preventing osteoporosis using the mature rat model. We included pRJ because this is known to enhance intestinal absorption of calcium (27). In order to analyze the mechanism of action of these compounds, their *in vitro* effects on the bone formation were also investigated using a bone tissue culture model. We also examined whether these compounds could antagonize the osteoporotic actions of parathyroid hormone (PTH) in *in vitro* experiments.

Materials and Methods

In Vivo Experiments

RJ and Chemicals

Both dried powders of raw RJ and pRJ were supplied by Yamada Apiculture Center, Inc., (Okayama Prefecture, Japan). The constituents and contents of both RJ and pRJ used in this study are shown in Table 1. 17 β -Estradiol was purchased from Sigma–Aldrich Co. (St Louis, MO, USA). All other reagents were purchased from ABIOZ Co. Ltd (Osaka, Japan).

Ovariectomy and Administration of RJ

Forty-eight female Sprague–Dawley rats, aged 9 weeks, were purchased from Seac Yoshitomi Ltd (Fukuoka Prefecture).

Table 1. Constituents and contents in royal jelly and protease-treated royal jelly

Constituents	Contents	Royal jelly	Protease-treated royal jelly
Water	% (w/w)	1.5	4.0
Proteins	% (w/w)	40.4	37.7
Carbohydrates	% (w/w)	47.1	45.3
Lipids	% (w/w)	8.0	4.4
Ashes	% (w/w)	3.0	8.6
Minerals			
Sodium	mg per 100 g	38.7	3000
Phosphorus	mg per 100 g	661	619
Calcium	mg per 100 g	18.7	30.5
Magnesium	mg per 100 g	91.7	83.9
Zinc	mg per 100 g	6.3	7.6
Iron	mg per 100 g	3.0	3.1
Free amino acids	mg per 100 g	1569	4780

Twenty-seven days later [90-days-old; a mature rat model (6) was used], six rats were given a sham operation (control rats; first group) and 42 rats were bilaterally ovariectomized under nembutal (pentobarbital sodium; 50 mg kg⁻¹ body weight; Abbott Lab., IL, USA) anesthesia. They were also equally divided into the second to the eighth groups. The second group received orally MF powdered pellets (Oriental Yeast Co. Ltd, Tokyo, Japan), which are designed for feeding rodents. Their contents are 24% (w/w) proteins, 5.1% (w/w) lipids, 2.9% (w/w) fibers and 6.1% (w/w) ashes. The third and fourth groups orally received MF powdered pellets containing 0.5% (w/w) (0.5 g RJ was mixed with 100 g MF powdered pellets; RJ-1) and 2.0% (w/w) RJ (RJ-2). The fifth and sixth groups orally received MF powdered pellets containing 0.5% (w/w) (pRJ-1) and 2.0% (w/w) pRJ-2. Each ovariectomized rat in the seventh group was injected subcutaneously with 17 β -estradiol [in 20% (v/v) polyethylene glycol 400] 5 days per week at a dose of 10 μ g kg⁻¹ body weight (12,14). Rats in the eighth group were injected with 20% (v/v) polyethylene glycol 400 (vehicle) in the same manner as the seventh group.

To develop bone loss in ovariectomized rats, all animals were maintained for 7 weeks under regulated 12 h/12 h light/dark illumination cycles at constant temperature (24 \pm 0.5°C) and humidity (45–50%). Food and drinking water (tap water) were supplied *ad libitum*.

The time for measuring daily food consumption and body weight was the same during the entire period. After euthanizing the rat with cervical dislocation under chloroform anesthesia, the right leg was dissected from each animal. The uterus of each rat was dissected and weighed (12,14).

Measurement of Bone Mineral Density

After removing adherent soft tissues, longitudinal section of right tibiae was prepared by manual grinding with whetstones (#600).

All longitudinal specimens were dehydrated by a step-wise application of 70 and 99.9% (v/v) ethanol solutions. These samples were used for both bone mineral density (BMD) measurements and scanning electron microscope (SEM) observations.

The BMD was quantitatively determined by the computed X-ray densitometry (CXD) method (Bonalyzer; Teijin Company, Tokyo, Japan) (12,14). The radiographs of the longitudinal sections of tibiae were taken along with an aluminum step-wedge using an X-ray apparatus (Model CSMW-2 Specific; Softex Co., Tokyo, Japan) set at 5 mA, 40 cm, 30 s, 50 V and 55 kV_p. The densitometric pattern of the proximal tibia on an X-ray picture was read by a personal computer (PC-9801; NEC, Tokyo, Japan) using a software program for rat bone density (Version 2.10A-M; Teijin Company). Σ GS (mm Al) and Σ GS/*D* were used as the indices of bone mineral content (BMC) and BMD, respectively. Here, the value for Σ GS was obtained by integrating the pattern area, which was obtained optically and was converted into a number of steps in an aluminum step-wedge. *D* represents the bone width. The value for Σ GS/*D* was calculated for the areas that cover the epiphysis and a part of metaphysis in the proximal tibia. The determination of the area and the precision of the measurements were described by Hidaka *et al.* (12).

Electron Microscopic Observations

Longitudinal sections of tibiae were shadowed with carbon before examination in a SEM (JSM-6330F; Nihon Denshi, Tokyo, Japan) at 10 kV.

In Vitro Experiments

Chemicals

Dulbecco's modified Eagle's medium (DMEM) (high glucose, 4.5 g dl⁻¹) and a penicillin-streptomycin solution (penicillin 5000 U mg⁻¹; streptomycin 5000 μ g ml⁻¹) were purchased from Gibco Laboratories (Grand Island, NY, USA). PTH [synthetic human PTH (1-34)] was purchased from Sigma-Aldrich Co. All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Male Rats or Mice for Bone Tissue Cultures

Male Wistar rats weighing 90-100 g (4 weeks old) or male mice (ddY strain; 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed with a commercial laboratory chow containing 1.1% calcium and 1.1% phosphorus at a room temperature of 25°C, with free access to distilled water.

Bone Culture Experiments

Under ether anesthesia with cervical dislocation, the femurs of male rats were removed aseptically after bleeding and were then soaked in ice-cold 0.25 M sucrose solution. The soft tissue and marrow were cleaned-off from the femur, and the

diaphysis and metaphysis (not containing epiphyseal tissue) were separated by a morphological tool. The femoral-diaphyseal and femoral-metaphyseal tissues were cut into small pieces (the size of about 2 × 2 mm) by a pair of scissors. Diaphyseal or metaphyseal fragments (pieces of 3 or 4) were cultured for 48 h in a 35 mm dish in 2.0 ml of medium consisting of Minimum essential medium (MEM) (high glucose, 4.5 g dl⁻¹) supplemented with antibiotics (penicillin 100 units and streptomycin 100 μ g ml⁻¹) (28). In order to determine the effects of RJ and pRJ on bone calcium content, bone tissues were cultured in a medium containing either 10-100 μ g ml⁻¹ RJ or 50-100 μ g ml⁻¹ pRJ in the presence or absence of 10⁻⁷ M PTH. Both RJ and pRJ were soluble in the culture medium.

Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air. After culture, the diaphyseal or metaphyseal tissues were removed, washed with ice-cold 0.25 M sucrose solution and dried for 16 h at 110°C. The calcium content was determined by atomic absorption spectrophotometry (28,29). The concentration of calcium in the medium containing RJ or pRJ was in the range of 0.05-1.0 μ g ml⁻¹ of medium. The calcium content in the bone tissue was expressed as mg g of dry bone. All experimental data are mean \pm SE for six animals.

Marrow Culture Experiments

Bone marrow cells isolated from male mice were used. Briefly, both bone ends of the femur were cut-off, and the marrow cavity was flushed with 1 ml of α -MEM. The marrow cells obtained from six animals were washed with α -MEM and cultured in the same medium containing 10% heat-inactivated fetal bovine serum at 1.0 × 10⁷ cells per ml in 24-well plates (0.5 ml per well) in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37°C. The cells were cultured for 3 days, then 0.2 ml of the old medium was replaced with fresh medium and the cultures maintained for an additional 4 days (30,31). RJ or pRJ was added to the culture medium containing either the vehicle or PTH (10⁻⁷ M) (or the vehicle) at the beginning of the culture and at the time of medium change.

Enzyme Histochemistry

After being cultured for 7 days, cells which adhered to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts (32,33). Briefly, cells were washed with the Hank's balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone (32). The fixed cells were incubated for 12 min at room temperature (25°C) in an acetate buffer (pH 5.0) containing 10 mM sodium tartrate and naphthol AS-MX phosphate (Sigma) as a substrate, and red violet LB salt (Sigma) as a stain for the reaction product. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Ethics

The ovariectomy analyses were performed at the Fukuoka Dental College. The tissue culture experiments were carried out at the University of Shizuoka. The study was approved by the Institutional Use and Care of Animal Committee of the respective university. All procedures were in accordance with the Guidelines on Animal Experiments in either Fukuoka Dental College or University of Shizuoka of Japan, and performed following the Government Law Concerning the Protection and Control of Animals (No. 221) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

Statistics

Data were obtained from three measurements and expressed as means \pm standard deviations. Data of tissue cultures were expressed as means \pm standard errors. Statistical comparisons were made by ANOVA with Fisher's Protected Least Significant Difference test using a statistical software program. The difference was considered significant when $P < 0.05$.

Results

Food Consumption and Body Weight

As shown in Fig. 1, the mean daily food consumption of the Sham, OVX + Est and OVX + pRJ-2 rats increased and reached a plateau at Week 2 after surgery, while that of food consumption of the remaining five groups increased until Week 3 after surgery and then leveled off (data of OVX + RJ-1, + pRJ-1 and + Peg rats were not shown).

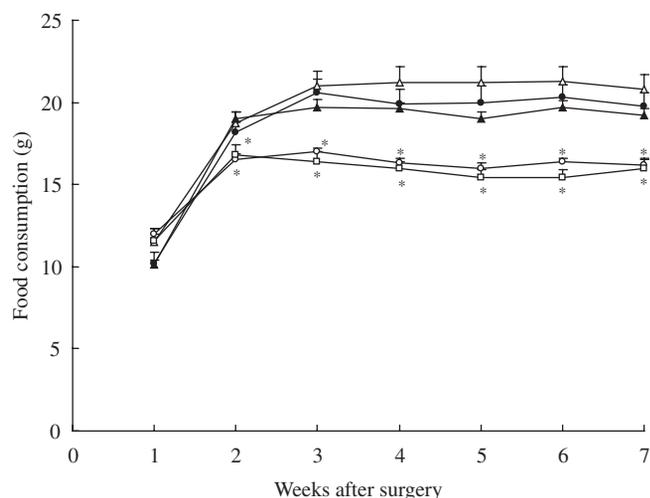


Figure 1. Changes in the food consumption of rats. Rats were sham-operated (open circles), ovariectomized (OVX) (closed circles); ovariectomized and administered with 2.0% (w/w) RJ (open triangles), 2.0% (w/w) protease-treated RJ (closed triangles) and 17 β -estradiol (open squares). Data of OVX administered 0.5% (w/w) RJ, 0.5% (w/w) protease-treated RJ and 20% (v/v) polyethylene glycol 400 were not shown. Each point represents the mean \pm SD ($n = 6$). * $P < 0.05$, significant difference from the ovariectomized group at the respective time.

Since food consumption in Week 1 was particularly smaller than that in the following weeks, two values (Week 1 and Weeks 2–7) were examined separately. As shown in Fig. 2, the mean daily food consumption of the OVX rats in Week 1 was comparable with all other groups. However, that of OVX rats in Weeks 2–7 was higher than that of Sham rats by 21%.

The daily food consumption for OVX + RJ-2 rats in Weeks 2–7 was 20.7 g (Fig. 2). The food for this group contains 2.0 g RJ per 100 g [2.0% (w/w)]. Therefore, the daily consumption of RJ was 0.41 g. Since the body weight of a rat varied from 280 to 378 g (Figs 3 and 4A), the daily dose of RJ was between 1.08 and 1.46 g kg⁻¹ (an average 1.27 g kg⁻¹) body weight. The daily dose taken by RJ-1 rats was about one-fourth (0.32 g kg⁻¹) of that in RJ-2 rats. Similarly, the daily food consumption for OVX + pRJ-2 rats in Weeks 2–7 was 19.4 g (Fig. 2). The food for this group contains 2.0 g pRJ per 100 g [2.0% (w/w)]. The daily consumption of pRJ was 0.39 g. Since the body weight of a rat varied from 280 to 370 g (Figs 3 and 4A), the daily dose of pRJ was between 1.05 and 1.39 g kg⁻¹ (an average 1.22 g kg⁻¹) body weight. The daily dose taken by pRJ-1 rats was about one-fourth (0.31 g kg⁻¹) of that in pRJ-2.

The increase of body weight in ovariectomized (OVX) rats was significantly higher than that in sham control (Sham) rats (Fig. 3). After Week 7, the mean body weight \pm SD in Sham and OVX rats were 315 \pm 11 ($n = 6$) and 375 \pm 13 ($n = 6$), respectively (Fig. 4A). The increases in the body weight of OVX + RJ-1, + RJ-2, + pRJ-1, + pRJ-2 and + Peg rats were similar to that in OVX rats (data of OVX + RJ-1, + pRJ-1 and + Peg were not shown). With the administration of the 17 β -estradiol to OVX rats (OVX + Est), the increase was reduced to almost the same level as that for Sham rats (Figs 3 and 4A).

Uterine Weight

As shown on the right side of Fig. 4A, the uterine weight of OVX rats decreased significantly from that of the Shams ($P < 0.01$). The weight in the OVX + RJ-1, + RJ-2, + pRJ-1 or + pRJ-2 group was almost the same as that in the OVX group. The weight of the group injected with 17 β -estradiol was similar to that of the Sham rats. The weight of the group injected with the vehicle was almost as high as that of the OVX rats.

Bone Mineral Density

As shown in Fig. 4B, the tibial BMD of OVX rats (0.50 \pm 0.01) was 24% lower than that of Sham (0.66 \pm 0.01). The administration of the 2.0% (w/w) RJ and 0.5–2.0% (w/w) pRJ to the rats significantly recovered the BMD. However, the administration of 0.5% (w/w) RJ to OVX rats did not recover the BMD. The recoveries with 2.0% (w/w) RJ, 0.5% (w/w) pRJ and 2.0% (w/w) pRJ were 85, 87 and 93% of that with 17 β -estradiol. The injection of 17 β -estradiol to OVX rats restored BMD almost completely to the level of the Sham rats. The vehicle had no effect.

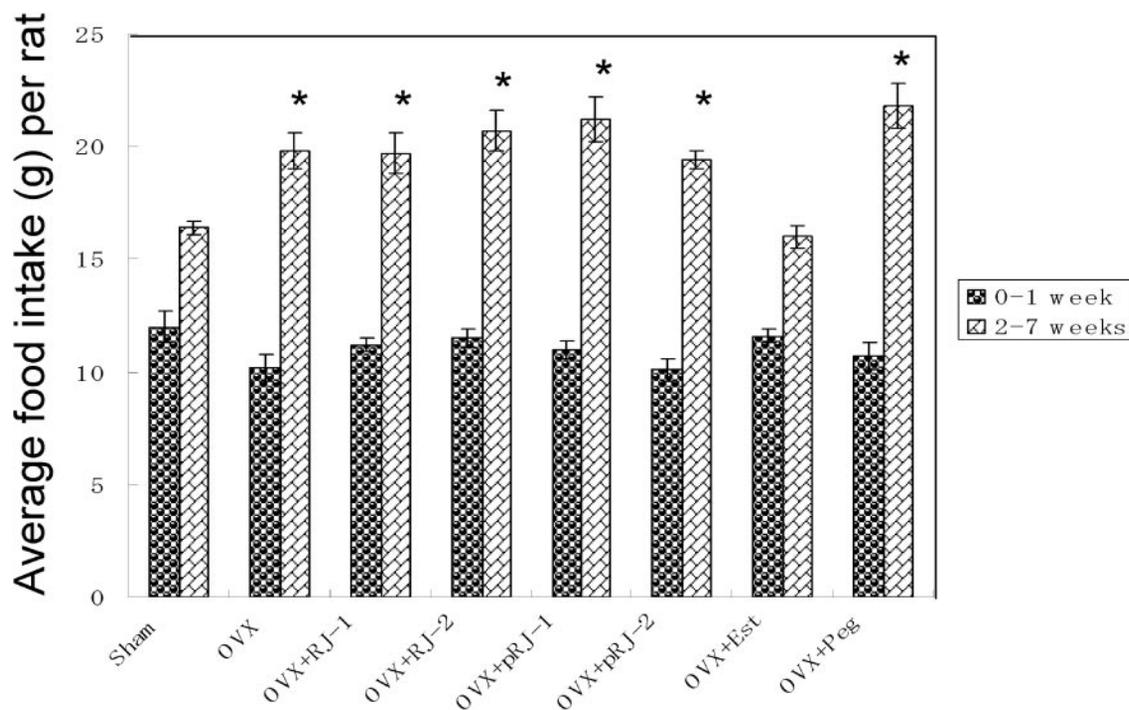


Figure 2. Mean daily food consumption in sham-operated and ovariectomized rats treated with various compounds for 7 weeks. Rats were divided into eight groups, sham-operated (Sham), ovariectomized (OVX), OVX given 0.5% (w/w) RJ (OVX + RJ-1), OVX given 2.0% (w/w) RJ (OVX + RJ-2), OVX given 0.5% (w/w) pRJ (OVX + pRJ-1), OVX given 2.0% (w/w) pRJ (OVX + pRJ-2), OVX given 17 β -estradiol (OVX + Est) and OVX given polyethylene glycol 400 (OVX + Peg), each having six rats. *Significant difference ($P < 0.05$) when compared with the Sham rats.

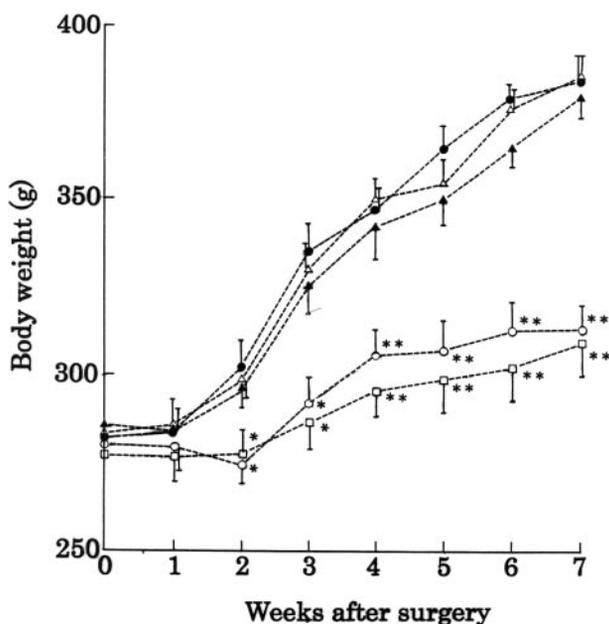


Figure 3. Changes in the body weight of rats. Rats were sham-operated (open circles), ovariectomized (OVX) (closed circles); ovariectomized and administered with 2.0% (w/w) RJ (open triangles), 2.0% (w/w) pRJ (closed triangles) and 17 β -estradiol (open squares). Data of OVX administered 0.5% (w/w) RJ, 0.5% (w/w) pRJ and 20% (v/v) polyethylene glycol 400 were not shown. Each point represents the mean \pm SD ($n = 6$). * $P < 0.05$ and ** $P < 0.01$, significant difference from the ovariectomized group at the respective time.

Scanning Electron Microscopic Analyses

The representative scanning electron micrograph of the proximal tibiae taken at 7 weeks after the ovariectomy is shown in Fig. 5B. Compared to that of Sham rats (Fig. 5A), the connectivity of cancellous bone in the epiphysis and that of trabecular bone in the metaphysis exhibited greater loss. The administration of 17 β -estradiol restored the connectivity in the metaphysis to those of the Sham rats, whereas the vehicle had no effect (Fig. 5C and D). The administration of 2.0% (w/w) RJ and 0.5% (w/w) pRJ slightly restored the connectivity (Fig. 6B and C), but 0.5% (w/w) RJ did not (Fig. 6A). The administration of 2.0% (w/w) pRJ (Fig. 6D) restored the connectivity in the metaphysis to the level of those of 17 β -estradiol rats.

Calcium Contents in Bone Tissues

As shown in Fig. 7, the calcium content in the diaphyseal or metaphyseal tissues significantly increased when the bone tissues were cultured in the presence of 50–100 $\mu\text{g ml}^{-1}$ RJ or 50–100 $\mu\text{g ml}^{-1}$ pRJ. However, as shown in Fig. 8, the presence of PTH (10^{-7} M) induced a significant decrease in calcium content in the diaphyseal or metaphyseal tissues. This decrease was not significantly prevented in the presence of RJ (50–100 $\mu\text{g ml}^{-1}$) or pRJ (50–100 $\mu\text{g ml}^{-1}$).

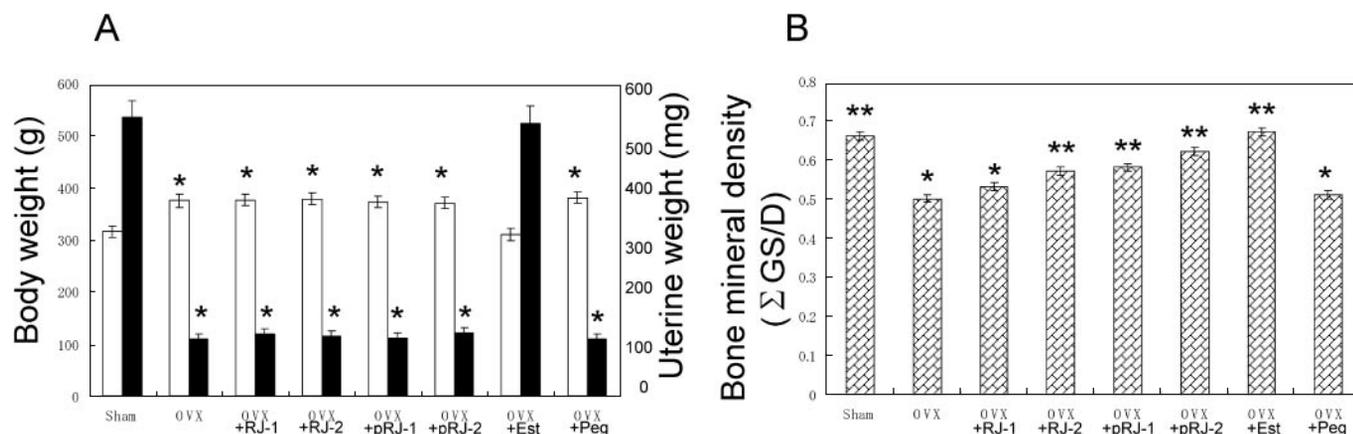


Figure 4. (A) Either body weight (white bar) and uterine weight (black bar) or (B) tibial BMD of sham-operated rats and those of ovariectomized rats treated with various compounds for 7 weeks. Abbreviations were the same as those shown in Fig. 2. *Significant difference ($P < 0.05$) when compared with Sham rats. **Significant difference ($P < 0.05$) when compared with OVX rats.

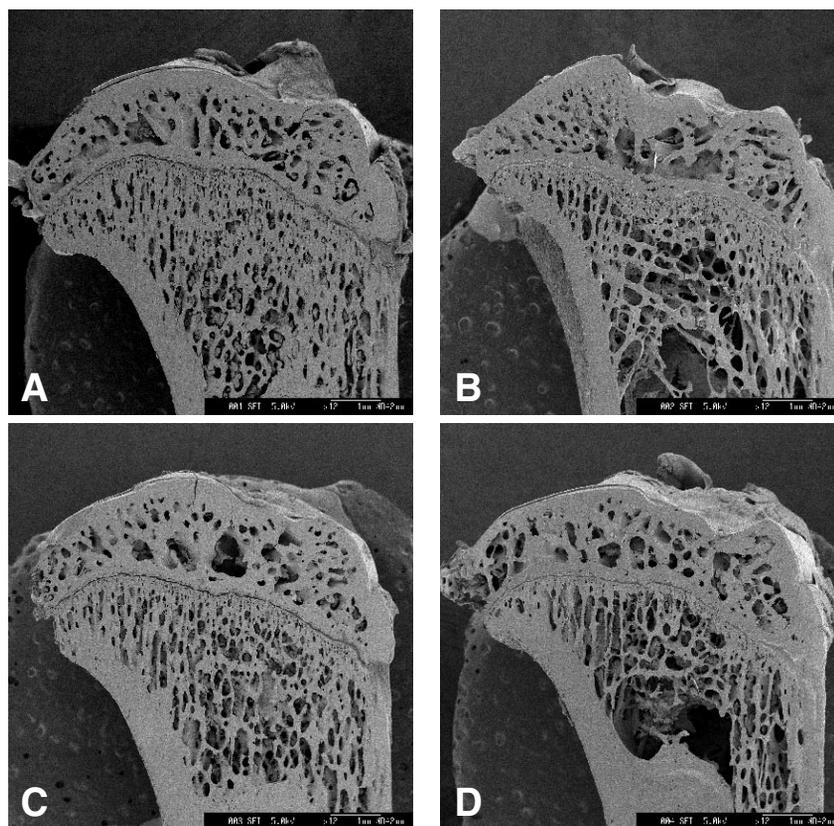


Figure 5. Representative scanning electron micrographs of the longitudinal sections of the proximal tibia sampled at Week 7 after ovariectomy from (A) sham-operated, (B) ovariectomized (OVX), (C) OVX given 17β-estradiol and (D) OVX given the vehicle.

Osteoclast-like Cell Formation

As shown in Fig. 9, the presence of PTH caused a significant increase in osteoclast-like cell formation in mouse marrow culture system *in vitro*. This increase was not significantly suppressed by either RJ ($1-100 \mu\text{g ml}^{-1}$) or pRJ ($1-100 \mu\text{g ml}^{-1}$).

Discussion

It has been reported that ovariectomy (OVX) increased food intake and body weight and these changes could be inhibited or reversed by treatment with estradiol (12,14,34). In fact, the mean daily food consumption of OVX rats in

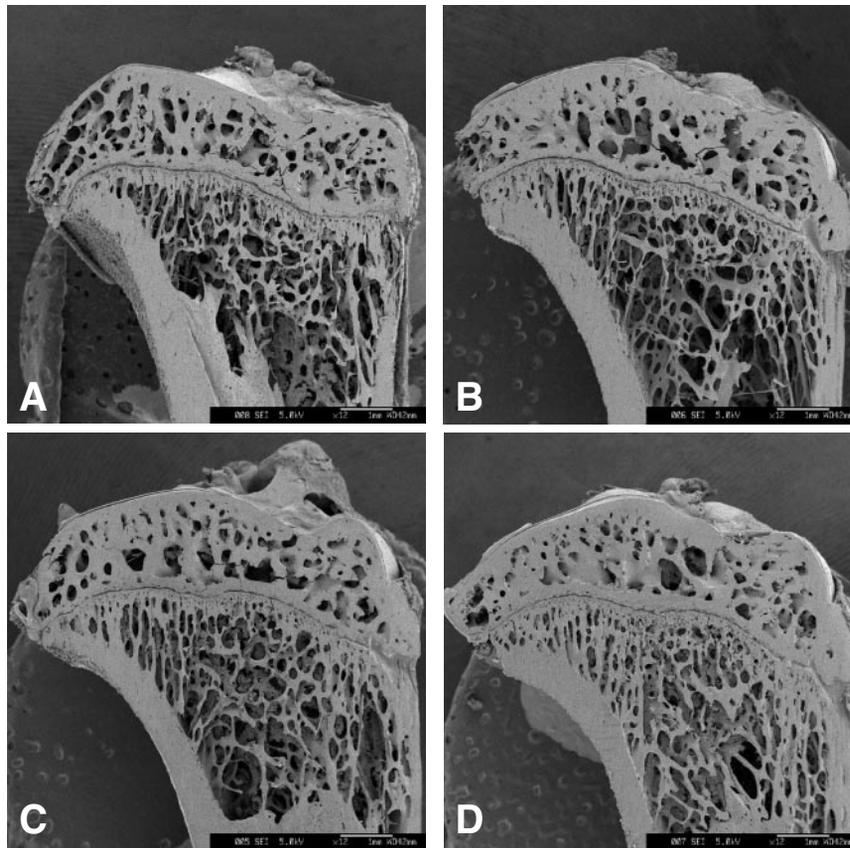


Figure 6. Representative scanning electron micrographs of the longitudinal sections of the proximal tibia sampled at Week 7 after ovariectomy of rats. (A) Ovariectomized (OVX) given 0.5% (w/w) RJ, (B) OVX given 2.0% (w/w) RJ, (C) OVX given 0.5% (w/w) pRJ and (D) OVX given 2.0% (w/w) pRJ.

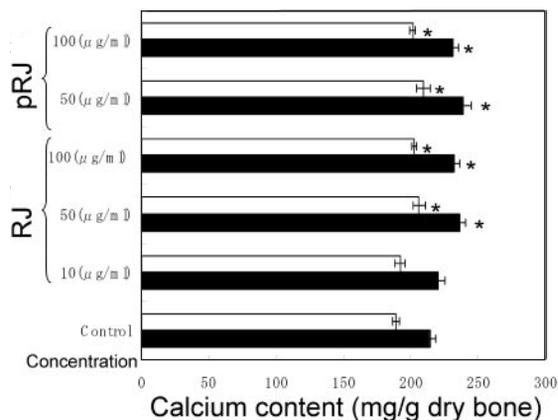


Figure 7. RJ and pRJ on the calcium contents in the femoral-diaphyseal and femoral-metaphyseal tissues obtained from normal rats *in vitro*. Bone tissues were cultured for 48 h in a medium containing vehicle, RJ or pRJ. Each value is the mean \pm SE for six rats. RJ, royal jelly; pRJ, protease-treated royal jelly. *Significant difference ($P < 0.05$) when compared with the control.

2–7 weeks was higher than that of Sham rats by 21% (Fig. 2). The administration of 17β -estradiol preserved the level of normal food consumption (Figs 1 and 2). Since neither RJ nor pRJ caused a decrease in food consumption in ovariectomized rats (during Weeks 2–7), their effect seems to be

different from that of estrogen. However, it is noticed that 2% (w/w) pRJ seems to have a greater effect than its lower concentration [0.5% (w/w)] or both RJ's concentrations. Both values of the food intake and the weight gain of OVX + pRJ-2 rats were below those for OVX rats (Figs 1 and 3). Their food consumption reached a plateau at 2 weeks similar to that of the Sham rats.

OVX also caused an increase in body weight (Figs 2 and 4A), which had been postulated not only to be associated with regulatory effect of estrogen on the adipose tissue (35) but also to provide a partial protection against the development of osteoporosis (36). The increase in food consumption in ovariectomized rats (21% increase as described above) may in part contribute to the increase in body weight (12,14). However, OVX rats gained more weight than Sham control rats in the pair-fed condition (37,38). Therefore, we conclude that OVX rats seem to make more efficient use of their food (39).

A marked atrophy of the uterus has been used as evidence of the success of ovariectomy, because estrogen directly influences uterine weight (12). Indeed, ovariectomy also resulted in a dramatic decrease in uterine weight (Fig. 4B). Since both RJ and pRJ did not increase uterine weight, it is suggested that these agents do not influence the actions of estrogen or its receptor (40).

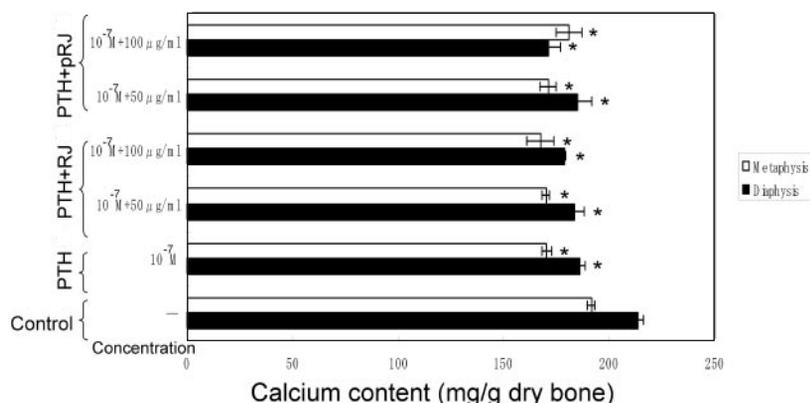


Figure 8. RJ and pRJ on the PTH-induced decrease in calcium content in the femoral-diaphyseal or femoral-metaphyseal tissues of the rat obtained from normal rats *in vitro*. Bone tissues were cultured for 48 h in a medium containing vehicle, RJ or pRJ in the presence or absence of PTH (10^{-7} M). Each value is the mean \pm SE for six rats. PTH, parathyroid hormone; RJ, royal jelly; pRJ, protease-treated royal jelly. *Significant difference ($P < 0.05$) when compared with the control.

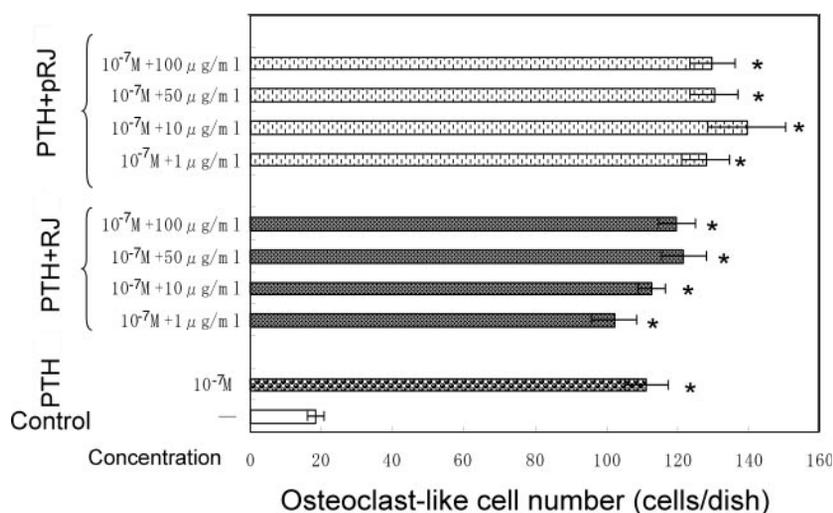


Figure 9. RJ and pRJ on the PTH-induced osteoclast-like cell formation in mouse marrow cultures *in vitro*. Mouse marrow cells were cultured for 7 days in a medium containing vehicle, RJ or pRJ in the presence or absence of PTH (10^{-7} M). Each value is the mean \pm SE for four wells. PTH, parathyroid hormone; RJ, royal jelly; pRJ, protease-treated royal jelly. *Significant difference ($P < 0.01$) when compared with the control.

The primary proof for an effective antiosteoporotic agent is believed to be a prominent inhibitory effect against bone loss. The administration of 2.0% (w/w) RJ, 0.5% (w/w) pRJ and 2.0% (w/w) pRJ to the OVX rats recovered BMD by 14, 16 and 24%, after 7 weeks, while that of 17β -estradiol recovered BMD by 34%. The efficacy of the inhibition of bone loss by 2.0% (w/w) RJ, 0.5% (w/w) pRJ and 2.0% (w/w) pRJ was 41, 47 and 71% of that of 17β -estradiol. The efficacy of pRJ on the tibial BMD was greater than that of RJ.

Ovariectomy resulted in a decrease in the connectivity of cancellous bones in the epiphysis and that of the trabecular bones in the metaphysis (Fig. 5A and B). Our data show that pRJ was more effective than RJ in inhibiting the elution of bone calcium, because the connectivity in OVX rats was restored by the administration of 2.0% (w/w) pRJ. The appearance was comparable with that of the Sham rats (Figs 5A and 6D).

After the protease treatment of RJ, the contents of sodium, calcium and free amino acids increased by 7.8, 1.6 and 3.0 times (Table 1). Their increased content of sodium was derived from the addition of NaOH, which was used to adjust pH during the protease treatment. The reason for the increase of calcium content is not known. However, the effects of these increases may be small because food itself contains large amounts of sodium and calcium, and because the percentages of RJ and pRJ in the food are very small.

Nakasa *et al.* (27) reported that pRJ significantly promoted calcium absorption in the intestine as compared to that of raw RJ. Therefore, it is conceivable that the greater effect of pRJ may be caused by the enhanced digestibility (or utility) and by its promotion of calcium absorption in the intestine. The protease treatment of RJ eliminates the protein fractions above 10 000 molecular weight from RJ, leaving the peptides of 500–3000 molecular weight (MW) (27). Phosphoserine or

acidic amino acids contained in peptides in pRJ could selectively bind calcium and facilitate calcium transport across the intestinal membranes.

As opposed to the *in vivo* models, both RJ and pRJ were similarly effective in increasing the calcium contents in the rat femoral-diaphyseal and femoral-metaphyseal tissue culture models (Fig. 7). However, they did not inhibit the PTH-induced calcium loss in the femoral tissues and PTH-induced formation of osteoclast-like cells in mouse marrow culture system *in vitro* (Figs 8 and 9). These observations demonstrated that both RJ and pRJ have a stimulatory effect on bone formation, but that they do not have an inhibitory effect on bone resorption.

Combining these *in vivo* and *in vitro* results, we speculate that the mechanism of action of RJ or pRJ is to stimulate calcium absorption of bone. The greater efficacy of pRJ in the animal model may be derived from the enhancement of intestinal calcium absorption by pRJ. However, we cannot exclude the possibility that other factors, such as calcium-regulatory hormones or cytokines, are also involved.

Recently, estrogen replacement therapy (ERT) has raised serious concerns about its safety because of tumor-inducing side effects (41). Our initial study shows that the RJ products may be used as alternative agents for the treatment of osteoporosis without such side effects, may be different from that for Kampo medicines or isoflavones (12,14). Since RJ contains a male hormone testosterone (25), this agent may also be effective in men's osteoporosis that could be induced by a decrease of androgen (42).

In summary, both RJ and pRJ inhibited the progression of bone loss induced by ovariectomy in rats to a similar degree to that of 17 β -estradiol. The results of *in vitro* tissue culture suggested that both nutrients increased calcium content of the femoral tissues. Our data indicated that pRJ seems to be more efficacious. We believe that pRJ may be useful in humans for the prevention of not only post-menopausal osteoporosis but also osteoporosis in general.

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