Combined intervention of exercise and genistein prevented androgen deficiency-induced bone loss in mice

JIAN WU,¹ XIN XIANG WANG,¹ HIROSHIGE CHIBA,¹ MITSURU HIGUCHI,² MISAO TAKASAKI,³ ATSUTANE OHTA,³ AND YOSHIKO ISHIMI¹

¹Division of Food Science, and ²Division of Health Promotion, National Institute of Health and Nutrition, Tokyo 162–8636, Japan; and ³Nutritional Science Center, Bioscience Laboratories, Meiji Seika Kaisha, Saitama 350-0298, Japan

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Wu, Jian, Xin Xiang Wang, Hiroshi Ge Chiba, Mitsuru Higuchi, Misao Takasaki, Atsutane Ohta, and Yoshiko Ishimi. Combined intervention of exercise and genistein prevented androgen deficiency-induced bone loss in mice. J Appl Physiol 94: 335–342, 2003; 10.1152/japplphysiol.00498.2002.—There is evidence that estrogen plays an important role in skeletal tissue in males as well as females. We have reported that phytoestrogens, such as genistein, selectively act on bone and exhibit cooperative effects on bone mass when combined with exercise in ovariectomized mice. In this study, we examined whether both interventions exhibit cooperative effects on bone loss in androgen-deficient mice similar to those in estrogen-deficient mice. Male mice aged 7 wk were either sham operated or orchidectomized (ORX) and divided into six groups: 1) sham; 2) ORX; 3) ORX and treated with genistein (0.4 mg/day) subcutaneously; 4) ORX, exercised on a treadmill daily for 30 min/day at 12 m/min; 5) ORX, given genistein, and exercised (ORX + ExG); and 6) ORX and treated with 17β-estradiol (E2). Four weeks after the intervention, seminal vesicle weight strikingly decreased in ORX mice, and it was not affected by administration of genistein or E2. Bone mineral density of whole femur increased in ORX mice, and it was not affected by administration after the intervention, seminal vesicle weight strikingly decreased by 10.2% in ORX mice compared with the gonadal hormones-deficient state. In fact, we reported that the combined intervention of moderate exercise and a low dose of genistein administration shows an additive effect in preventing bone loss in ORX mice similar to that in ovariectomized mice.

androgen deficiency; phytoestrogens; genistein; exercise; osteoporosis

OSTEOPOROSIS IN MEN IS A SUBSTANTIAL PUBLIC HEALTH PROBLEM (25). Although attention has traditionally focused on the role of androgens in bone metabolism in men, reports of severe osteopenia in men with a mutated gene of the estrogen receptor or aromatase deficiency have raised the question of the role of estrogens in the male skeleton (5, 21, 32). Recently, results from clinical investigations indicated that bone loss in elderly men is more significantly correlated with declining estrogen levels than with androgen levels (17, 31). Furthermore, a study was designed to examine the relative contributions of androgen vs. estrogen in regulating bone turnover in normal elderly men, which demonstrated that estrogen was the dominant sex steroid regulating bone resorption (7). These reports suggest that estrogen plays an important role in regulating the male skeleton.

Nonsteroidal estrogen-like plant compounds called phytoestrogens are presently being investigated as alternatives to hormone replacement therapy for the prevention and treatment of postmenopausal osteoporosis in women (1, 3, 4). These compounds have been shown to maintain estrogen’s positive bone and cardiovascular effects while minimizing several undesirable side-effects of estrogen. We found that genistein, one of the phytoestrogens, prevented bone loss caused by estrogen or androgen deficiency without substantial effects on the reproductive organs in both male and female osteoporotic animal models (12–14).

There are also numerous positive reports on the effects of exercise training on bone growth and bone formation in young animals (16, 22). However, in gonadectomized animals, there have been conflicting results concerning the efficacy of exercise training on bone mass (11, 35). Frost (8) suggested that estrogen deficiency increases the “set point” for the skeleton to detect loads, which causes the skeleton to be less sensitive to mechanical force. In accord with this hypothesis, the same loading force would have more favorable effects on the skeleton in the gonadal hormones-sufficient compared with the gonadal hormones-deficient state. In fact, we reported that the combined intervention of a moderate intensity of running exercise and a submaximal dose of genistein expressed more advantageous effects on the prevention of bone loss in ovariectomized (OVX) mice than exercise alone (35). In this study, we investigated the effects of running exercise.

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and genistein administration on the bone mass in androgen-deficient mice to examine whether both treatments exhibit cooperative effects similar to those in estrogen-deficient mice.

MATERIALS AND METHODS

Animal and intervention. Seven-week-old male mice of the ddY strain were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and fed an AIN-93G diet with corn oil instead of soybean oil (Funabashi Farm, Chiba, Japan) (30). The mice were individually housed in 24 × 15 × 15-cm cages under a 12:12-h light-dark cycle at 22°C and were allowed free access to water and diet. Both genistein (Fujicco, Kobe, Japan) and 17β-estradiol (E2; Sigma Chemical, St. Louis, MO) were dissolved in 20% dimethylsulfoxide (DMSO) in polyethylene glycol-300 and were administered to the mice subcutaneously by using a minisomatic pump (model 2002; Alza, Palo Alto, CA) immediately after surgery. Before the combined intervention of genistein and moderate exercise on bone mass in orchidectomized (ORX) mice were studied, the dose-dependent effect of genistein on bone was examined.

To examine the dose-dependent effect of genistein on bone mass in ORX mice, the mice were randomly divided into five groups: ORX (ORX, n = 8); genistein administration (ORX + G, n = 8); exercise training (ORX + Ex, n = 8); combined genistein and exercise (ORX + ExG, n = 8); and E2 administration (ORX + E2, n = 8). Four weeks after the start of intervention, the mice were killed, and the femora were removed to analyze bone mineral density (BMD) and structure. Genistein was given at a low dose (0.4 mg/day), and E2 was given at 0.03 μg/day by using the minisomatic pump mentioned above. The exercise regimen consisted of daily running on a treadmill (Natsume, Tokyo, Japan) for 30 min/day at 12 m/min at a 10° upward slope. All procedures were performed in accordance with the National Institutes of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

Radiographic analysis. Radiographic analysis of the femora was performed by using a soft X-ray system (model DCS-600R; Aloka, Tokyo, Japan). BMC of the mouse femora was closely correlated with the ash weight (r = 0.978) (19). BMD was calculated by BMC of the measured area. The scanned area of the femur was equally divided into three regions (proximal femur, midshaft, and distal femur; 5.3 mm each) to assess the regional differences in the femur.

Peripheral quantitative computed tomography analysis. The femora were scanned with a peripheral quantitative computed tomography (pQCT) system XCT Research SA combined with µScope (Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). The voxel size was 0.08 mm, the slice thickness was 0.5 mm, and the cortical threshold was 464 mg/cm³. We employed the Peel mode 20 measuring mode for dividing into cortical bone or trabecular bone. This mode can detect the inner threshold automatically. One cross-sectional slice from each femora was scanned at the midshaft, 8 mm from the distal end, which was determined from a scout view of the pQCT device. Cortical mineral content and density at the midshaft were analyzed for each slice. Total cross-sectional area (CSA) and periosteal perimeter (Peri) were also evaluated by the scanning.

Histomorphometric analysis. The left femora was fixed in 70% ethanol, and the undecalciﬁed bone was embedded in glycol methacrylate. A 3-μm-thick section was cut longitudinally in the distal region of the femora and was stained for tartrate-resistant acid phosphatase. Histomorphometry was performed with the semiautomatic image analyzing system (Osteoplan; Carl Zeiss, Thornwood, NY) linked to a light microscope (18). The histomorphometric measurements were made at ×200 magniﬁcation by using a minimum of 10 optical fields in the secondary spongiosa area from the growth plate-metaphysal junction. All sections were examined blind. Nomenclature, symbols, and units that were used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (27).

Statistical analysis. Data are presented as means ± SE. Signiﬁcance of differences was determined by one-way ANOVA followed by Fisher’s protected least-significant difference test. The effect of genistein administration, running exercises, and interaction of both interventions were analyzed by two-way ANOVA. Signiﬁcance of differences was determined by Fisher’s protected least-significant difference test. Differences were considered signiﬁcant at the level of P < 0.05.

RESULTS

Dose-response of genistein on BMD in ORX mice. To examine the dose-dependent effect of genistein on BMD, ORX mice were treated for 3 wk with 0.4 or 0.8 mg/day of genistein, and BMD of the femora were measured. Distal and whole femoral BMD were signiﬁcantly reduced by ORX, and administration of genistein signiﬁcantly suppressed this reduction in a dose-dependent manner (Table 1). Genistein at 0.4 mg/day has been deﬁned as a minimal dose sufﬁcient for a bone-protective effect in ORX mice.

Cooperative effects of genistein and exercise on bone mass in ORX mice. There was no signiﬁcant difference in body weight among sham-operated mice, ORX mice, and ORX mice treated with genistein with or without exercise intervention during a 4-wk experimental period. The lower body weight in the ORX groups com-

<table>
<thead>
<tr>
<th>Group</th>
<th>BMD, mg/cm²</th>
<th>Distal</th>
<th>Whole</th>
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<tbody>
<tr>
<td>Sham</td>
<td>40.8 ± 0.5</td>
<td>38.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>ORX-Cont</td>
<td>33.9 ± 0.6*</td>
<td>34.3 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td>ORX-0.4G</td>
<td>36.2 ± 0.7*</td>
<td>34.9 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>ORX-0.8G</td>
<td>38.0 ± 0.4†</td>
<td>36.5 ± 0.4†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. Bone mineral density (BMD) of the distal and whole femora were measured at 3 wk after the operation by dual energy X-ray absorptiometry. Mice were sham operated (Sham) or orchidectomized (ORX), and ORX mice were treated with 0.4 mg/day (ORX-0.4G) or 0.8 mg/day (ORX-0.8G) of genistein. *Signiﬁcantly different from Sham group. †Signiﬁcantly different from ORX-Cont (ORX + treated with vehicle solution) group. All differences were analyzed by multiple comparison with one-way ANOVA (P < 0.05).
pared with the Sham group might be due to extirpation of the testicles (Fig. 1A). Seminal vesicle weight strikingly decreased in ORX mice, which indicated that the mice were androgen deficient. Administration of genistein for 4 wk at 0.4 mg/day did not affect seminal vesicle weight in ORX mice. The combination of genistein administration and running exercise or \( E_2 \) treatment also did not affect seminal vesicle weight (Fig. 1B).

**Bone mass and structural properties.** Figure 2A shows radiograms of the femora collected from mice in each group. X-ray analysis revealed that the mineralized cancellous bone mass in ORX mice had significantly decreased, especially in the distal metaphysis of the femur. Combined intervention of genistein administration and running exercise or \( E_2 \) administration markedly prevented bone loss.

During DXA analysis, whole femoral BMD was significantly reduced by ORX. The decrease in BMD was significantly inhibited by the combined intervention of exercise and 0.4 mg/day of genistein administration, although it was not affected by either intervention alone (Fig. 2B). The bone area of the whole femur in the ORX + Ex and ORX + ExG groups was significantly larger than that in the other groups (Fig. 2C). To evaluate a site-specific effect of genistein and/or exercise intervention, femoral BMD and area were further analyzed at the proximal, middle, and distal regions of the femur (Table 2). ORX reduced BMD in the proximal, middle, and distal regions of the Sham mice by 5.7, 9.4, and 8.6%, respectively. The combined intervention of genistein and exercise completely prevented bone loss at the proximal and distal regions in ORX mice. However, BMD in the middle region was not affected by the combined intervention. Exercise partially inhibited ORX-induced bone loss in the distal region. \( E_2 \) treatment completely prevented the decrease in BMD in all three regions. Bone area was markedly higher in both the exercise and combined intervention groups compared with those in the other ORX and Sham groups at the distal region.

The results of densitometric evaluation by pQCT are shown in Fig. 3. Both cortical BMD and BMC at the femoral midshaft, 8 mm from the distal end, were reduced in the ORX mice compared with Sham mice. Either genistein or exercise alone and combined intervention did not affect BMD and BMC in ORX mice. Neither CSA nor Peri at the midshaft was affected by either treatment alone or by the combined intervention in ORX mice. These results in the analysis by pQCT were well correlated to those evaluated by DXA in the middle region of the femur.

**Histological analysis.** To define the effects of genistein administration and running exercise on the trabecular bone, histological sections of distal femoral metaphysis were prepared, and bone volume-to-tissue volume ratio (BV/TV), Tb.Th, and Tb.Sp were evaluated (Fig. 4). BV/TV and Tb.Th were markedly decreased by ORX, however, these were significantly recovered by combined intervention. Although either genistein administration or running exercise partially prevented decreased BV/TV and Tb.Th, a two-factor ANOVA showed the effects of exercise and genistein intervention, and their interactions on histological parameters were not significant. Tb.Sp was dramatically increased in ORX mice, whereas combined intervention completely protected the separation, as did \( E_2 \).

**DISCUSSION**

The present study demonstrates that the combined intervention of moderate exercise and low-dose genistein administration shows an additive effect in preventing bone loss, especially in the femoral cancellous bone by inhibiting bone resorption in ORX mice. These results are consistent with our previous findings showing the protective effects of the combination of genistein administration with running exercise on bone loss in OVX mice (35). However, unlike OVX mice, cortical BMD, the periosteum perimeter and CSA at
the midshaft of the femur, was not affected by exercise or combined intervention in ORX mice.

Clinical hypogonadism is a well-established cause of osteoporosis in men. It has been reported that an androgen deficiency caused by ORX results in bone loss as a result of stimulating bone resorption in experimental animals (9, 23). Erben et al. (6) have reported that ORX not only reduces the serum levels of total and free testosterone but also the level of E2. They also showed that E2 was the only significant predictor of histomorphometric indexes of bone formation and resorption in ORX rats. Furthermore, because it has been reported that a mutated estrogen receptor or aromatase deficiency in men can result in osteoporosis, the role of estrogen in the male skeleton has received attention (5, 21, 32). In fact, in this study, bone loss induced by castration was prevented by estrogen treatment, as previously reported (23, 24). On the basis of these findings, natural dietary plant estrogens such as isoflavones, particularly those found in soy products, seem to be potential alternatives to estrogens (2, 12, 13). Recently, we reported that administration of 0.7–0.8 mg/day of genistein markedly prevented bone loss caused by either estrogen or androgen deficiency without substantial effects on the reproductive tissues in osteoporotic animal models (12–14). We also found that administration of a submaximal dose of genistein (0.4 mg/day) partially but significantly prevented the decrease in BMD of the femora in OVX mice (13, 35). Although the decrease in femoral BMD caused by ORX was not significantly affected by administration of a low dose of genistein (0.4 mg/day), the high

Table 2. Effects of exercise, genistein, and combined interventions on BMD and bone area of femora in ORX mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMD, mg/cm²</th>
<th>Area, cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Midshaft</td>
</tr>
<tr>
<td>Sham</td>
<td>43.8 ± 0.4</td>
<td>37.1 ± 0.6</td>
</tr>
<tr>
<td>ORX</td>
<td>41.3 ± 0.6*</td>
<td>33.6 ± 0.9*</td>
</tr>
<tr>
<td>ORX + G</td>
<td>41.2 ± 0.7*</td>
<td>33.7 ± 1.0*</td>
</tr>
<tr>
<td>ORX + Ex</td>
<td>43.0 ± 0.4*</td>
<td>33.1 ± 0.8*</td>
</tr>
<tr>
<td>ORX + ExG</td>
<td>44.2 ± 0.7b,c</td>
<td>34.1 ± 0.3a</td>
</tr>
<tr>
<td>ORX + E2</td>
<td>44.5 ± 1.3b,c</td>
<td>40.1 ± 1.3b,c,d,e</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. BMD and bone area at the proximal region, midshaft, and distal region of the excised femora were measured by dual X-ray absorptiometry 4 wk after the operation. Mice were sham operated or ORX, and some ORX mice were treated with 0.4 mg/day of genistein (ORX + G), trained to exercise with (ORX + ExG) or without genistein (ORX + Ex), or treated with 0.03 µg/day of 17β-estradiol (ORX + E2). Significantly different from Sham group. Significantly different from ORX group. Significantly different from ORX + G group. Significantly different from ORX + Ex group. Significantly different from ORX + ExG group. All differences were analyzed by multiple comparison with one-way ANOVA (P < 0.05).
dose at 0.8 mg/day significantly protected bone loss in ORX mice. These results suggest that intake of isoflavones would be beneficial to maintain bone mass in males with hypogonadism. Exercise has been reported to prevent bone loss induced by gonadectomy in both sexes (15, 28, 36–38). We reported that moderate running exercise significantly prevented bone loss and increased the bone area.
of the femur in OVX mice (35). In the present study, we found that running exercise, at the same intensity as in trained OVX mice, significantly increased the bone area and partially prevented bone loss at the distal femur in ORX mice (Table 2). We also reported that running exercise improved the structural parameters and bone formation rate at the midshaft of the femur in OVX mice. However, in ORX mice, the running exercise had no effect on BMD, CSA, and Peri at the midfemur (Fig. 3). These findings are consistent with the observation that running exercise had no significant effect on diaphyseal BMD (mainly cortical), whereas it increased metaphyseal BMD (predominantly cancellous) in young ORX rats (10). Therefore, it is considered that running exercise prevents bone loss mainly by the inhibition of bone resorption but not by the stimulation of bone formation under androgen-deficient conditions, especially in growing and young animals. In other words, androgen might be necessary to respond to the mechanical stress at the cortical bone in male mice. This hypothesis is also supported by the data of Horcajada-Molteni et al. (11). They reported that treadmill running exercise was able to prevent bone loss induced by ORX, mainly by inhibiting bone resorption without affecting osteoblastic activity.

It has been suggested that the effect of running exercise on augmentation of bone was site specific. In this study, we found that the running exercise increased bone area in the distal region, which may lead to an increase in bone area of whole femur, but not in proximal and middle regions evaluated by DXA. We also found that CSA and Peri at the femoral midshaft analyzed by pQCT did not differ between exercised and nonexercised animals. Iwamoto et al. (16) reported that growing rats subjected to treadmill exercise showed greater increases in bone volume at the distal tibia compared with the proximal tibia. One putative explanation is that the most distal aspects of the limb are in closer contact with the ground and are thus loaded more directly. A second compelling explanation is that interstitial fluid pressure, which is created by gravitational force from top to bottom of the body, regulates bone mass (33). This would suggest that an exercise of the lower extremities done while standing upright, such as erect bipedal standing or jumping, will be more effective in augmenting bone mass than exercise done while running in a four-footed animal. Yao et al. (36) have reported that exercise by making ORX rats rise to an erect bipedal stance partially prevented cancellous and cortical bone loss in tibiae induced by ORX. These results differ somewhat from the present study and previous reports that found that treadmill exercise had a beneficial effect on cancellous bone but not on cortical bone. Consequently, it appears that mechanical loading was greater in bipedal resistance stance than in running exercise by all four limbs. In fact, the magnitude of loading is more effective than the number of cycles for stimulating bone formation reported by human and animal studies (34).

When ORX mice were treated with the combination of genistein administration and running exercise, the decrease in BMD was completely prevented at the proximal and distal femoral regions by analyzing DXA measurements (Table 2). Similar to effects seen in OVX mice, the combined intervention also exhibited significant effects on BV/TV and Tb.Sp in the distal femoral metaphysis in the histomorphometric analysis in ORX mice. Two-way ANOVA revealed that there was no synergistic interaction between genistein administration and exercise, and this supports the idea that the beneficial effects were the result of the additive effects of the two independent factors. On the other hand, we did not find the efficacy of the combined intervention on the bone mass, CSA, and Peri at the midfemur measured by DXA and pQCT in ORX mice (Fig. 3). In fact, there were no differences in these parameters between the ORX group and any of the treatment groups alone. However, in OVX mice, we found that this combined intervention not only completely restored the bone mass to the sham level, but also increased the bone area, Peri, and bone formation rate at the diaphysis of the midfemur (35). ORX reduced periosteal appositional growth in cortical bone in growing male rats and can be reversed by androgen replacement (29). Thus this evidence suggests that androgens are important for bone formation on cortical bone in male animals. This may explain the different responses of the cortical bone at the midfemur to combined intervention in ORX and OVX mice.

The mechanism of interaction between running exercise and genistein administration in bone metabolism under androgen-deficient conditions is not clear. It has been suggested that androgen regulates bone metabolism after conversion to estrogen by aromatase in males (5, 21). In fact, in the aromatase-deficient mice, a marked loss of cancellous bone due to increased bone resorption was observed not only in females but also in young males (20, 26). These findings indicate that estrogen has crucial roles in bone metabolism in males. It has been suggested that estrogen may tend to lower the set points of bone modeling and remodeling thresholds in females (8). In the present study, we found that combined intervention exhibited a beneficial effect on trabecular bone, but not cortical bone, in ORX mice. Therefore, it is likely that the signals of phytoestrogen influences the set point in trabecular bone that is regulated mainly by estrogen even in ORX mice. Further studies are necessary to explain this point. Furthermore, it is important to examine whether the combined intervention could prevent bone loss attributed to androgen deficiency in men. If so, it may be useful to establish a clinical treatment regimen for the prevention of osteoporosis.

In conclusion, we demonstrated that the combined intervention of moderate running exercise and an administration of low-dose genistein showed an additive effect in preventing bone loss, especially at the trabecular bone of the distal metaphysis in ORX mice similar to that in OVX mice. These results suggested that combined dietary natural phytoestrogens such as genistein with moderate exercise might be useful for the prevention of
osteoarthritis not only in postmenopausal women but also in elderly men with hypogonadism.

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