Suppressive Effects of Genistein Dosage and Resistance Exercise on Bone Loss in Ovariectomized Rats

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Abstract This study was designed to determine whether combined treatments with genistein dosage and moderate resistance exercise would exhibit synergistically preventive effects on bone loss following the onset of menopause. Forty-one 12 wk-old female SD rats were assigned to five groups: 1) Sham operated (Sham); 2) ovariectomized (OVX-Cont); 3) OVX received genistein (OVX-GEN); 4) OVX exercised (OVX-EXE); and 5) OVX treated with both genistein and exercise (OVX-GEN-EXE). All rats were fed a low Ca (0.1%) diet ad libitum. Daily genistein dosage was 12 mg/kg body weight. Exercising rats took 40 sets of 1-min run interspersed with 1-min rest with a 100 g weight on the back on an uphill treadmill at 20 m/min. The experimental duration consisted of the adaptation and treatment periods of 4 weeks each. Uterine weight in OVX-Cont, OVX-GEN, OVX-EXE and OVX-GEN-EXE decreased to about 15% of that in Sham (p<0.001). The femoral BMD (mg/cm²; mean ± SE), assessed by DEXA (Lunar), of OVX-Cont was significantly lowered to 206 ± 5 by –9%, as compared to 226 ± 2 of Sham (p<0.001). The BMD of OVX-GEN, OVX-EXE and OVX-GEN-EXE were 217 ± 2, 217 ± 2 and 222 ± 2, respectively, and genistein dosage and resistance exercise equally increased the BMD of OVX rats by 5% (p<0.01). Combined treatment of genistein and exercise more successfully recovered their decreased BMD by 8% (p<0.001). BMD of the fourth lumbar vertebrae in OVX-Cont was declined to 191 ± 7 by −15%, as compared to 225 ± 4 in Sham (p<0.001). OVX-EXE and OVX-GEN-EXE gained the BMD by 6% to 205 ± 4 and 203 ± 3, respectively, as compared to that of OVX-Cont (p<0.01). These results suggest the possibility that the combined treatment of genistein dosage and resistance exercise have more beneficial effects by acting rather independently than their separate trials on the prevention of ovx-induced bone loss in femurs. J Physiol Anthropol 20 (5): 285-291, 2001 http://www.jstage.jst.go.jp/en/

Keywords: bone mineral density (BMD), femur and vertebrae, genistein, ovariectomized rat, resistance exercise

Introduction

Estrogen deficiency as in postmenopause and ovariectomy accelerates bone resorption and results in rapid bone loss with high bone metabolic turnover, increasingly developing to osteoporosis. The postmenopausal bone loss is prevented by estrogen administration (Wronska et al., 1988), which is clinically practiced and known as hormone replacement therapy (HRT) (Termine and Wong, 1998), but it is often accompanied with side effects such as breast cancer (Jacobs, 2000). It is important to find out harmless estrogen alternatives for postmenopausal women (Kessel, 1998). Dietary soybean products have been experimentally shown to suppress bone loss in ovariectomized animals, and this bone-sparing effect is now proven to be mediated by the isoflavones in soybeans (Arjmandi et al., 1998). Genistein known as phytoestrogen is a type of isoflavones that possesses a structural similarity in parts to estrogen and binds to estrogen receptor (ER) in several tissues such as uterine and skeletal bone in the body (Santell et al., 1997), and have the potential beneficial effects to improve health by preventing several postmenopausal common diseases including osteoporosis (Anderson and Garner, 1998). There are several reports that genistein operates on the suppression of bone loss in rats (Fanti et al., 1998; Anderson et al., 1998), but its efficacy is not yet well defined.

On the other hand, loading mechanical stresses are well elucidated to restrain bone metabolic turnover and result in maintaining bone mass (Frost, 1992). According to
this hypothesis, repeated strong stimulus to a bone can counterbalance estrogen depletion and prevent postmenopausal bone loss through the inhibition of increased bone resorption. Various exercises including jump (Umemura et al., 1995), running (Barentgols et al., 1993), squat exercises training (Westerlind et al., 1998), have been examined for the influences on bones and proved to improve their status effectively. Resistant exercise training directly stimulates bones and is assumed to act more effectively for increasing bone masses as compared to endurance training (Bennell et al., 1997). However, the details of effects of regular resistance exercise on the prevention of bone loss in estrogen deficiency remains to be solved.

Several authors have reported that the combined treatments with exercise and estrogen dosage cooperatively suppress the bone loss caused by estrogen deficiency in human (Kohrt et al., 1995) and rats (Yeh et al., 1994). In addition, the combined practices of exercise and some drugs such as bisphosphonate (Grigorev et al., 1992) and etidronate (Tamaki et al., 1998) were proved to inhibit synergistically bone loss. These results suggest that exercise and curatives would operate independently for the suppression of bone loss. In order to enhance the efficacy of genistein for practical use, genistein dosage should be combined with another treatment such as exercise. On the other hand, the combined suppressive effects on bone loss of genistein administration and physical exercise have not yet been fully investigated to be elucidated.

This study was designed to examine if the inhibitory effects of genistein dosage in combination with resistance exercise on OVX-induced bone loss would be cooperatively improved, compared with the separate effect of daily soy isoflavone intakes or regular weight bearing workouts.

Materials and Methods

Animal care

Forty-one female Sprague-Dawley rats aged 12 weeks, weighing 240–290 g, were purchased from Japan Charles River Co., Ltd. The animals were individually housed in a room maintained at 24°C and 50% humidity with 14 h/10 h light-dark cycle. They were sham or ovariectomy operated within several days after arrival, and randomly assigned to five groups on the basis of body weight: (A) sham-operated (Sham; n=8), (B) ovariectomized (OVX-Cont; n=8), (C) OVX genistein dosed with (OVX-GEN; n=8), (D) OVX exercised (OVX-EXE; n=8), and (E) OVX dosed with genistein in combination with exercise (OVX-GEN-EXE; n=9). The operative procedure for ovariectomy was as follows. The hair of the operative area of an anesthetized rat with diethyl ether was shaved and the area was painted with 70% ethanol as a skin disinfectant. A dorsal midline incision was made through the skin at the level of the kidneys. The exposed ovaries through the thin muscle wall by retracting the skin laterally toward either side were pulled into the incision and excised after the ligation of the upper horn of the uterus. The wounds were closed with surgical clips and painted with a tincture of iodine. The sham operation was performed in the same procedure as the ovariectomy except the ligation of the upper horn of the uterus and the excision of the ovaries were left undone before the closure of the incision. Rats were fed on a low calcium (0.1%) powdered diet prepared according to the AIN-93M prescription for eight weeks including initial four weeks of the adaptation period. After four weeks of adaptation after surgery, OVX-GEN and OVX-GEN-EXE groups received daily subcutaneous injections of 12 mg genistein per kg body weight, while the remaining groups were injected with vehicle. The dosage level of genistein was chosen according to the results obtained by Anderson et al. (1998) and Fanti et al. (1998), who showed that daily genistein dose of approximately 5 mg per rat could prevent bone loss induced by estrogen deficiency in OVX rats most effectively. Genistein (Fujicco Co., Ltd. Japan) was dissolved in 20% dimethylsulfoxide in polyethyleneglycol-300 (Ishimi et al., 1999). The body weight and food intakes were recorded once a week. At the end of experimental period, the rats were anesthetized with pentobarbital and exsanguinated to death. The femur and fourth lumbar vertebrae (L4) were removed and the bone status was measured. Uterus, spleen, abdominal fat depot and several other organs were weighed.

Exercise training

Rats in OVX-EXE and OVX-GEN-EXE groups were accustomed to run on a motor-driven treadmill (KN-73, Natsume Co., Ltd. Japan) with an electric grid at the rear of each compartment. The training started from the third week after ovariectomy. During the first week of the training, the rats practiced to run at a speed of 6 m/minute without the inclination of the treadmill for 10 minutes/day. The rats were trained to be able to run at 20 m/min with 11% uphill grade by a gradual increase of the speed and grade of the treadmill. After 2 weeks of practicing, the exercising rats were regularly submitted to 40 sets of intermittent 1-min run interspersed with 1-min rest, loading 100 g weight on the back for 5 days a week for a further four weeks (Fig. 1). All procedures were in accordance with the Waseda University Guidelines for the Care and Use of Laboratory Animals.

Bone status measurement

The femur and L4 were freed of soft tissue. Bone mineral density (BMD) and contents (BMC) were estimated using dual energy X-ray absorptiometry (DEXA, Lunar DPX-L, USA, Software version 1.0C). The
ultimate strength of a femoral shaft was measured with three point-bending methods (AGS-100D, Shimadzu Co., Ltd. Japan). The distance between bottom supports was set at 1.2 cm and the loading was kept on the middle shaft of a femur until it begins to fracture. The measurement was carried out with a cross head speed of 5 mm/min and the load range of 100 kg weight on the back.

Statistical analysis
Data were expressed as means ± SE. The significance of the differences was determined by ANOVA and Fisher’s protected least-significant-difference (PLSD) test. Differences were considered significant at the level of p<0.05.

Results

Change of body weight and organ weights
When the adaptation period started at 12 week old, the average body weights of all the groups were practically the same. When the experimental treatment with genistein dosage and/or regular exercise were initiated, at 16 week old, the body weight of OVX-Cont, OVX-GEN, OVX-EXE and OVX-GEN-EXE groups was approximately 10% higher than that of Sham (p<0.01) (Fig. 2 (A)). At the end of the treatment with genistein and/or exercise which lasted for 4 weeks, the body weight of OVX-Cont and OVX-GEN groups was approximately 15% higher than that of Sham (p<0.001). The weight gain was somewhat depressed in the two exercising groups, OVX-
Table 1 Effects of ovariectomy (OVX), genistein and exercise on bone properties of femur and fourth lumbar vertebrae (L4) in rats

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<th>Factor</th>
<th>Femur</th>
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<tr>
<td></td>
<td>BMD (mg/cm²)</td>
<td>Ultimate strength (N)</td>
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<tr>
<td>Sham</td>
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<tr>
<td>OVX-Cont</td>
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<td>OVX-GEN</td>
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<td>OVX-GEN-EXE</td>
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The ultimate strength of a femoral shaft (N) was measured with three-point bending methods. Sham; sham-operated, OVX-Cont; ovariectomized, OVX-GEN; OVX genistein dosed with, OVX-EXE; OVX exercised, OVX-GEN-EXE; OVX genistein and exercise treated from the age of 16 to 20 weeks. Values are means ± SE. Statistical significance was evaluated with ANOVA (Fisher’s PLSD). *: p<0.05, **: p<0.01, ***: p<0.001 vs. Sham group. *: p<0.05, **: p<0.01, ***: p<0.001 vs. OVX-Cont group.

Effects on bone status in femurs and fourth lumbar vertebrae

The femoral BMD (mg/cm²; means ± SE) of OVX-Cont significantly declined to 206 ± 5 compared to 226 ± 2 of Sham (p<0.001). OVX resulted in causing the decrease by 9% in the femoral BMD. Genistein dosage and/or habitual resistance exercise induced a slight but significant increase in BMD, such as 217 ± 2 in OVX-GEN (p<0.01), 217 ± 2 in OVX-EXE (p<0.01) and 222 ± 2 in OVX-GEN-EXE (p<0.001) as compared to 206 ± 5 in OVX-Cont. Genistein dosage and resistance exercise equally recovered BMD by 5%, and the combined treatment with genistein and exercise more successfully by 8% (Table 1, Fig. 3 (A)). The representative load-deformation curves to fracture femoral shafts were shown in Fig. 4. Any obvious differences were not observed in the shapes of the curves and presumably in the courses of deformation among the experimental groups. The maximum load at the point of breaking femurs was evaluated as a meaningful measure of ultimate bone strength. Ultimate strength and BMC of femurs in all groups showed a parallel tendency with the femoral BMD (Fig. 3 (B, C)).

The BMD (mg/cm²; means ± SE) of the fourth lumbar vertebrae (L4) in OVX-Cont was significantly reduced to 191 ± 7 (p<0.001), as compared to 225 ± 4 in Sham (Table 1). OVX-Cont caused the decrease of approximately 15% in BMD. The regular exercise effectively served to suppress the OVX induced bone loss and recovered BMD of L4 by 6% each to 205 ± 4 (p<0.01) in OVX-EXE and 203 ± 3 (p<0.01) OVX-GEN-EXE, compared with 191 ± 7 in OVX-Cont. Separate genistein dosage failed to increase BMD of L4 (Fig. 3 (D)). The BMC of L4 in all the groups showed a similar tendency with the BMD (Fig. 3 (E)).

Discussion

The present study was planned to elucidate the suppressive effect of genistein dosage and resistance exercise on ovariectomy (OVX)-induced bone loss. We confirmed that the BMD reduced in twelve-week-old OVX rats to 9% of Sham operated ones was significantly increased by genistein dosage. In the present experiments we used ovariectomized rats with the initial age of 12 weeks as a postmenopausal model. Kalu (1991) designated that rats aged 3 months could successfully be used as a mature rat model, because these rats were not only reproductively mature, but capable of responding appropriately to sex hormone deficiency and its sequela following ovariectomy. Moreover, the characteristics of the bone loss were mostly similar to those of the aged rat model. The results obtained suggest that genistein have suppressive effects on the bone loss caused by OVX, and support the views described in other reports (Fanti et al., 1998; Anderson et al., 1998; Ishimi et al., 1999), although we failed to indicate the preventive effects of genistein on the bone loss of fourth lumbar vertebrae (L4). At present we could not explain the reasons why genistein is effective to femurs and ineffective to L4 for restraining the bone loss in estrogen deficiency. From the constitutional point of view, femurs and vertebrae are comparatively different. Most of the femurs such as other long bones functioning with mechanical strength are

EXE and OVX-GEN-EXE. The former group was lessened by 6% (NS) and the latter by 9% (p<0.05), as compared to OVX-Cont, though the average daily food intake of all the groups was almost similar during 4 weeks of the experimental period.

Uterine weight in OVX-Cont, OVX-GEN, OVX-EXE and OVX-GEN-EXE was markedly reduced to approximately 15% of that in Sham (p<0.001) (Fig. 2 (B)). The spleen was enlarged in two genistein treated groups, OVX-GEN and OVX-GEN-EXE, by 35%, as compared to that in Sham (p<0.001). The spleen was enlarged in two genistein treated groups, OVX-GEN and OVX-GEN-EXE, by 35%, as compared to that in Sham (p<0.001). The spleen was enlarged in two genistein treated groups, OVX-GEN and OVX-GEN-EXE, by 35%, as compared to that in Sham (p<0.001). The spleen was enlarged in two genistein treated groups, OVX-GEN and OVX-GEN-EXE, by 35%, as compared to that in Sham (p<0.001). The spleen was enlarged in two genistein treated groups, OVX-GEN and OVX-GEN-EXE, by 35%, as compared to that in Sham (p<0.001).
composed of cortical bones, while vertebrae, which are metabolically more active than femurs, constituted from 80% or more cancellous bones.

Anderson et al. (1998) and Picherit et al. (2000) demonstrated that the administration of genistein remained ineffective for the suppression of cancellous bone loss in OVX rats. On the other hand, several studies indicated that isoflavone dosage successfully prevented the OVX-induced cancellous bone loss such as L4 (Arjmandi et al., 1996). These results revealed that the
conclusion has not yet reached agreement about the efficacy of genistein on the suppression of L4 in estrogen deficiency and further examination would be needed for drawing conclusions on this unsettled assignment.

In the present study, we tried to develop and evaluate a new type of resistance exercise for increasing bone loss in OVX rats. We loaded a lead plate weighing 100 g on the back, and made weight bearing OVX rats run intermittently at 20 m/min interspersed with short rest on a uphill treadmill, in which the rats could receive more intense imparts on bones than those in previously published studies (Barengolts et al., 1993). We termed this mode of workout as resistance exercise after the general definition that resistance-runs are repeated runs against an added resistance such as a hill, a weight carried, a drag which has to be pulled, or a soft, uneven surface (Kent, 1994).

Our resistance exercise successfully increased the BMD of femurs and L4 of OVX rats by approximately 5% and 7% respectively, compared with sedentary counterparts. Barengolts et al. (1993) reported endurance running exercise (21 m/min, 7% inclination, 40 min/day, 4 days/week for 3 month) increased the ash weight of femurs and tibia. Our resistant running exercise brought almost the same results on the preventive effect on bone loss of femurs as the endurance exercise. As described above, our experimental results presented some evidences that moderate resistance exercise such as intermittent slow running with a heavy weight on the back either separately or combined with phytoestrogen dosage could suppress the bone loss of femurs and a part of vertebrae effectively in OVX rats. However, a lead plate placed on the back of the rats corresponded to the 30% of the body weight and actually too heavy to load on postmenopausal women. Therefore before the preventive and therapeutical application of the same mode of exercise to osteoporosis caused by menopausal, clinical trials are definitely necessary to examine if walking or running exercises bearing a much lighter weight portable without a physical conflict could exert an inhibitory action on the bone loss in postmenopausal women.

When rats walk or run on in usual quadrupedal manners on a flat treadmill, additional gravity load to spine is very small. Iwamoto et al. (1998) observed no significant difference in a maximum breaking force of the 5th lumbar vertebral body among rats loaded running at different speed and frequency of the exercise (12 m/min 1 h/day, 18 m/min 1 h/day and 12 m/min 2 h/day), and concluded that beneficial effects of treadmill running on bone strength came out only in weight-bearing bones. In the present study we loaded a weight of corresponding to about 30% of body weight on the back of exercising rats and drove them to run on a treadmill inclined up by 11%. The exercise trial we loaded exerted small but significant effects on the bone loss of OVX rats, and could be assessed to be a suitable way for transmitting gravity stimulus to L4 in rats.

In our present trial treated together with genistein dosage and the resistance exercise, we obtained the results that the combined treatments cooperatively function for suppressing the bone loss more effectively than each separate treatment in OVX rats. Yeh et al. (1994) evaluated the combined effect of 17 beta-estradiol (E2) replacement (10 micrograms, twice a week) and treadmill exercise (18 m/min, 45 min/day) for 16 weeks on bone status in rats, and found out that the bone-conserving effects of E2 and exercise appeared most significantly on femoral (+3%) and lumbar-5 (+3%) density. In our study, the enhancing effects of combined treatments with genistein and exercise on femoral BMD and max load point of breaking a femoral shaft, which meant the increased bone strength to resist against fractures were confirmed, but not in L4. The reason why our trial failed to affect L4 is unclear. Since the bone metabolic markers ware not estimated in the current study, we are unable to establish whether the increased bone mass induced by the combined treatment of genistein and exercise would be entirely due to suppressed bone loss or be partially due to increased bone formation. Yeh et al. (1993) demonstrated that the high levels in bone mass following the combined intervention with estrogen and exercise in OVX rats were due primarily to decreased bone resorption. In this study similar mechanism seems to operate but further study on the metabolic turnover of bone is needed to determine the details of the mechanism involved.

In conclusion, we evaluated the suppressive effects of genistein dosage, our new type of resistance exercise and the combined treatments of both measures on the bone loss caused by OVX in rats. The following results were obtained:

1) Genistein dosage by itself restrained the bone loss of femurs, but not of L4;
2) Loading the separate resistance running successfully suppressed the bone loss of femur and L4; and
3) Combined treatments of genistein and new type of resistance exercise cooperatively acted for the prevention of bone loss.

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