Genistein Prevents Bone Resorption Diseases by Inhibiting Bone Resorption and Stimulating Bone Formation

Binbin Li* and Shifeng Yu

Department of Oral Pathology, Peking University School of Stomatology; 22 South Zhongguancun Avenue, Haidian District, Beijing 100081, P.R. China. Received January 14, 2003; accepted March 24, 2003

Genistein, a soybean-derived isoflavone, has been shown to suppress osteoclastic bone resorption. To clarify the mechanisms underlying this action, we investigated the effects of genistein on the differentiation, cytoskeleton and function in mice osteoclasts in vitro and bone metabolism in ovariectomized rats. Study design: Primary OCs were isolated from 3 week-old mice and induced by 1,25(OH)2D3. Then OCs were exposed to genistein at various concentration of 0 M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M, and 10⁻⁵ M. The number of TRAP⁺ cells were counted as well as the surface area of bone resorption on bone slice. F-actin change was observed by Confocal. In vivo, forty 12 week-old female SD rats were randomly assigned to four groups: (1) sham operated (Sham); (2) (OVX); (3) ovariectomized and treated with estradiol (OVX-E); (4) ovariectomized and received genistein (OVX-G). After 12 weeks, BMD, body weight, serum level of alkaline phosphatase (ALP), acid phosphatase (ACP), osteocalcin (OC) , IL-1β, TNFα, IL-6 and calcitonin (CT) were evaluated. Femur were sectioned. In addition, the serum estradiol, the weight of uteri and histological behavior were also examined to indicate the side effect of genistein to the uterus. Results: In vitro, the number of TRAP⁺ cells decreased depending on the concentration of genistein as well as the area of bone resorption. F-actin became disorder under Confocal. In vivo, after treated with genistein, BMD and the serum level of ALP , ACP, osteocalcin increased significantly, while the serum level of IL-1β and TNFα decreased. Especially, the increase of ALP and osteocalcin of OVX-G was higher than that of OVX-E. Histologically, the pachy-trabecula were observed as well as the more mineral deposition lines. Additionally, the uterus weight index and the estrus estradiol in OVX-G rats were lower significantly than those of OVX-E. The epithelia of uterine gland in OVX-G appeared cubic while those of OVX-E became squamous. Conclusions: Genistein can prevent bone resorption diseases by the promotion of bone formation and the prevention of bone resorption with slight side effect.

Key words genistein; bone resorption; bone formation

Bone resorption is a very common disease especially for postmenopausal women. Estrogen treatment, or hormone replacement therapy (HRT) is accepted by many to be the best method to prevents bone loss.1) However, many women do not tolerate the numerous side effects, or are concerned about the possibility of increased rate of uterine and/or breast cancer.2)⁵—10) So there remains a need for highly efficacious antiresorptive agents with an excellent safety and tolerability profile. Fortunately, the natural alternatives, phytoestrogens such as soy isoflavones, compounds that exert estrogen activity on several tissues and are thus being investigated as possible alternatives to HRT.11)

Genistein, known as phytoestrogen, is a kind of soybean-derived 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,12) and has the suppression effect on osteoclastic bone resorption.13)⁵—10) Osteoclast is the main functional cell which leads to bone resorption. So it’s important to study how genistein acts on the osteoclast and finally influence the bone metabolism in the whole. My work focus on the exact cellular and biological mechanisms of genistein’s inhibition effect on osteoclastic bone resorption.

MATERIAL AND METHODS

Chemicals αModified Eagle medium (αMEM) was obtained from GIBCO laboratories (Grand Island, NY, U.S.A.). Genistein was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). It was dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentration in the standard extracellular solution just before each experiment. The final DMSO concentration in the solution was less than 0.1%.

Osteoclast Preparation Osteoclasts were obtained from the marrow cells of the femur and tibia of adult ICR mouse (3—5 weeks old). The mice were anesthetized with diethyl ether and killed by decapitation. The femora and tibiae were removed and isolated from adhering soft tissues. Both femur and tibia will be flushed with αMEM media (Gibco, Gaithersburg, MD, U.S.A.) in a sterile syringe with a 25 gauge needle. The resulting suspension will be washed twice, resuspended and incubated in a 75 cm² flask at a density of 10⁶ cells/ml αMEM for 1 h. After 1 h, nonadherent cells will be harvested, washed and resuspended in αMEM containing 10⁻⁸ M, 1,25(OH)₂D₃ at a concentration of 10⁶ cells/ml. This suspension will be added to the wells of 24-well plates containing sterilized glass coverslips or devitalized bovine bone slices. The cells in the experiment group were grown in 10% (volume fraction) fetal calf serum (FCS)+ αMEM+ various concentrations of genistein (10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M)+10⁻⁸ M, 1,25(OH)₂D₃. The control groups were grown in 10% FCS+ αMEM+0.1% DMSO+10⁻⁸ M, 1,25(OH)₂D₃. Cultures will be fed every 3 d by replacing half the medium with fresh medium and reagents.

Enzyme Histochemistry At the point of 3rd day, 5th day and 8th day, cells adherent to the 24-well plates were stained for TRAP, a marker enzyme of osteoclasts,11) and counted the positive cells having one, two or above two nuclei.

Pit Formation Assay At the 12th day of growth, selected bone slices were sonicated to strip off cells and evalu-
ated by scanning electron microscopy for resorption pits. The surface area of bone resorption was assayed by the software of Image-Pro Plus.

**Laser Scanning Confocal Analysis of the Effect of Genistein on Actin Arrangement of the Osteoclasts in Mice**

The osteoclasts suspension of 0 M and 10^-5 M group were incubated in glass slices and be stained by 20 nm phalloidin-actin at the 8th day. F-actin change was observed by Confocal by step 0.3—0.6 μm.

**Animals and Administration Procedure**

Forty adult female SD rats (weighing 230±20 g) from the Animal Center of National Birth Control Board were kept at constant temperature and humidity during the experiment period. (temperature, 22±3 °C; humidity, 50±20%; 12 h light: 12 h dark cycles, light on 07:15 a.m.) This animal experiment was performed under the guidelines and permission of Chinese Animal Care Committee. They were randomly divided into the four groups with ten rats each: (1) Sham: sham surgery; (2) OVX: bilaterally ovariectomized under isoflurane anesthesia; (3) OVX-E: treated with 10 μg/kg estradiol once every two days after ovariectomized; (4) OVX-G: treated with genistein after ovariectomized. Genistein was dissolved in 0.5% CMC–Na and was given daily by oral gavage at 45 mg/kg body weight. Sham and OVX group were treated with vehicle. The general conditions were observed and the body weight of rats were weighed weekly.

One side femur were removed and fixed in 10% buffered formalin for subsequent measurement of BMD by NORDLAND XR-36 dual-energy X-ray absorptiometry (DEXA). The detected sites were 1 cm below the proximal end and the 1 cm above the distal end of the femur.

The another side femur were also removed, fixed in 10% buffered formalin decalcified by 15% EDTA, and paraffin embedded. Sections of 4—5 μm were cut and stained with hematoxylin-eosin. Histopathologic examination of tissue slides was performed by light microscopy.

Blood samples were collected by exsanguinations from the femoral artery and centrifuged for 10 min at 3000 rpm and the serum was removed, transferred to new tubes and frozen at −20 °C until assay.

The cytokines associated with bone formation or bone resorption, such as osteocalcin, IL-1β, IL-6, TNFα, CT were examined by immunoradiometric assay.

The enzymes associated with bone metabolism, such as ALP and ACP, were detected by the test kit produced by RANDOX (U.K.).

The uteri were also removed, stripped of remaining fat, squeezed out gently the liquid in the cavity, weighed by the electronic scale. The ration between the weight of uterus and the body weight is the uterus weight index. The serum of estradiol were tested by radio-immunity too. The uteri were fixed in 10% buffered formalin and paraffin embedded. Sections of 4—5 μm were cut and stained with hematoxylin-eosin. Histopathologic examination of tissue slides was performed by light microscopy.

**Statistical Analysis**

Statistical analyses were performed using the SPSS Statistical Analysis System, Version 10.0. One way analysis of variance (ANOVA) tests were used for comparisons between every two groups. All results are expressed as means±S.D. Significance was considered at p<0.05.

**RESULTS**

**Role of Genistein in the Osteoclasts in Vitro**

The effect of genistein on the osteoclasts formation in the mouse marrow culture system was examined. There began to appear various number of TRAP-positive cells at the third day after cells suspension were added to the sterilized glass coverslips (Fig. 1). At the 8th, the number of TRAP-positive cells with three nuclei were most (Fig. 2). TRAP-positive cells with three or more nuclei will be counted as osteoclasts. Resorption lacunae could be found when the cells cocultured with devitalized bone slices. Both morphological and functional studies showed that the isolated cells shared some of the characteristics typical of osteoclasts.

Extracellularly applied genistein inhibited the differentiation of osteoclasts in a concentration-dependent manner. As for the TRAP+ cells with single nucleus, the presence of genistein (10^-7 to 10^-5 M) in the culture medium caused a significant decrease at the third day (p<0.05, p<0.01, p<0.01 respectively). At the 5th day, there were great significances in the presence of 0 to 10^-3 M (p<0.05). At the 8th day, the presence of genistein (10^-8 to 10^-5 M) in the culture medium caused a significant decrease in the number of TRAP+ cells with single nucleus between that of 0 M. As for the TRAP+ cells with two and above two nuclei, genistein in
The patterns of osteoclastic F-actin became aggregated and shortened by 10^{-5} M genistein. Figure 4 is a panorama about the F-actin arrangement of one osteoclast and forcefully shows the changes of F-actin eliminating the limitation of the plane (Figs. 3, 4).

We also examined the effect of genistein on the surface area of resorption pits formed over 12 d too. It showed that genistein (10^{-8} to 10^{-5}M) significantly inhibited the formation of pits on the bone slice in a dosage-dependent manner. The percentage decreased 52.38%, 63.21%, 66.39%, and 82.01%, respectively. Additionally, there also had significances between 10^{-5} M and 10^{-3} to 10^{-5} M, between 10^{-4} to 10^{-3} M and 10^{-5} M (Fig. 5).

Effect of Genistein Administration on Bone Metabolism in Ovariectomized Rats. Changes of Body Weight of the Four Groups There were no infection in the wounds of all animals, and the general conditions were normal. During the test period, all the animals gained weight than the beginning (p<0.01). The OVX rats had the significant weight increase (58.27%) compared with Sham animals (p<0.05). The weight decreased significantly compared with OVX group after treated with estradiol and genistein (p<0.01, p<0.05 respectively) (Table 4).

Effect of Genistein on BMD OVX resulted in causing the marked decrease femoral BMD (p<0.01). After treated with estradiol, the BMD raised to near Sham’s. After treated with genistein, the BMD of femur were all increased significantly compared with OVX group (p<0.01, p<0.05 respectively). There was no difference among the OVX-E and the OVX-G groups (Table 5).

Histological Behavior of Femur after Treated with Genistein Histologically, there were more fat tissues in the femur marrow cavity of OVX. As a contrast, the pachy-trabecula and more mineral deposition appeared in the femur of OVX-E and OVX-G, with active bone formation in the metaphysis (Figs. 6—9).

Effect of Genistein on Serum Enzyme The serum levels of ALP in OVX rats were higher significantly than Sham animals (p<0.05). The serum level of ALP of OVX-G group was also higher significantly than the other three groups (p<0.01). The ACP of OVX, OVX-E and OVX-G groups were all higher significantly than Sham animals (p<0.01) (Table 6).

Effect of Genistein on Biochemical Marker of Bone Remodelling The serum levels of IL-1β and osteocalcin of OVX rats were higher significantly than Sham animals’s
(p<0.05), while no difference in IL-6 and CT compared with Sham group. After treated with estradiol, TNFα, IL-1β and osteocalcin had significant decrease (p<0.05, p<0.01 respectively). After treated with genistein, osteocalcin increased significantly (p<0.01), especially compared with OVX-E rats (p<0.01). However, their serum level of IL-1β was lower than OVX animals (p<0.01), while TNFα was lower significantly than Sham and OVX group (p<0.05, p<0.01). No difference was detected in the serum of IL-6 and CT among all the four groups (Table 7).

Effect of Genistein on Uterus Weight, Serum Level of Estradiol and Histological Behavior of the Uteri Uterine weight in OVX, OVX-E, and OVX-G were markedly reduced compared with Sham’s (p<0.05). The oviduct of OVX-E rats appeared thick and rich in blood vessels, whose weight of uteri were higher than OVX rats (p<0.05), and the significance of the uter weight index was more higher (p<0.01). The uteri weight of OVX-G rats was significantly lower than OVX-E (p<0.05), and the significance of the uterus weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial weight (g)</th>
<th>End weight (g)</th>
<th>Increase rate of body weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>217.10±11.29</td>
<td>300.20±33.99</td>
<td>38.28</td>
</tr>
<tr>
<td>OVX</td>
<td>214.70±14.77</td>
<td>339.80±24.95</td>
<td>58.27*</td>
</tr>
<tr>
<td>OVX-E</td>
<td>227.50±30.67</td>
<td>325.40±53.40</td>
<td>43.03ΔΔ</td>
</tr>
<tr>
<td>OVX-G</td>
<td>219.20±12.65</td>
<td>327.30±38.62</td>
<td>49.32ΔΔ</td>
</tr>
</tbody>
</table>

*: p<0.05, compared with Sham; #: p<0.01, compared with OVX; ΔΔ: p<0.01 compare with OVX.

<table>
<thead>
<tr>
<th>Group</th>
<th>BMD of distal part of femur</th>
<th>BMD of proximal part of femur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.1760±0.0155</td>
<td>0.2014±0.0177</td>
</tr>
<tr>
<td>OVX</td>
<td>0.1530±0.0177**</td>
<td>0.1680±0.0166**</td>
</tr>
<tr>
<td>OVX-E</td>
<td>0.1733±0.0160ΔΔ</td>
<td>0.1906±0.0220ΔΔ</td>
</tr>
<tr>
<td>OVX-G</td>
<td>0.1698±0.0186ΔΔ</td>
<td>0.1823±0.0134ΔΔ</td>
</tr>
</tbody>
</table>

*: p<0.05, compared with Sham; #: p<0.01, compared with Sham; #: p<0.01 compare with OVX.
index was more higher \((p<0.01)\). The serum level of estradiol of OVX was significantly lower than that of Sham \((p<0.01)\). However, no difference in the serum estradiol was found among Sham, OVX-E and OVX-G groups (Table 8).

Histologically, the epithelia of uterus gland in Sham animals are cubic or column-like (Fig. 10). The epithelia of uterus gland became thin and flat after ovariectomy (Fig. 11). After treated with estradiol, the epithelia became squamous (Fig. 12), while the epithelia of OVX-G group just appeared cubic (Fig. 13).

**DISCUSSION**

**The Effect of Genistein on the Osteoclasts in Vitro**

Genistein has structural similarity to \(17\beta\) estradiol so that it

**Table 7. The Effect of Genistein on Biochemical Marker of Bone Remodelling**

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1(\beta) (ng/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>TNF(\alpha) (ng/ml)</th>
<th>CT (pg/ml)</th>
<th>OC (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.225±0.084</td>
<td>112.85±32.17</td>
<td>0.161±0.032</td>
<td>32.07±9.35</td>
<td>3.054±0.624</td>
</tr>
<tr>
<td>OVX</td>
<td>0.475±0.204*</td>
<td>127.96±24.75</td>
<td>0.169±0.047</td>
<td>34.44±9.95</td>
<td>3.943±1.088*</td>
</tr>
<tr>
<td>OVX-E</td>
<td>0.153±0.096(\Delta)</td>
<td>119.16±31.98</td>
<td>0.135±0.032(\Delta)</td>
<td>37.11±14.66</td>
<td>2.776±0.609(\Delta)</td>
</tr>
<tr>
<td>OVX-G</td>
<td>0.172±0.096(\Delta,\\Delta)</td>
<td>127.22±17.81</td>
<td>0.122±0.021(\Delta,\\Delta)</td>
<td>33.17±5.01</td>
<td>4.588±0.666(\Delta,\\Delta)</td>
</tr>
</tbody>
</table>

\* \(p<0.05\), compared with Sham; ** \(p<0.01\), compared with Sham; \(\Delta\) \(p<0.05\), compare with OVX; \(\Delta,\\Delta\) \(p<0.01\), compare with OVX; \(\=\) \(p<0.01\), compared with OVX-E.
can bind to estrogen receptors, and is believed to exhibit beneficial effect on bone tissue by mimicking the biological responses of estrogens. Estrogen receptors lie both on the osteoblasts and the osteoclasts. So it is believed that genistein could bind to the estrogen receptors on osteoclasts to regulate osteoclast activity, and could bind to the receptors on osteoblasts, stimulated osteoblasts to secret collagenase and cytokines, then accelerated the bone formation.

It is known that 1,25(OH)2D3 can stimulate the formation of the osteoclast-like cells and therefore promote the osteoclastic bone resorption in vitro. Our data showed that the effect of 1,25(OH)2D3 in increasing osteoclast-like cells formation was inhibited markedly in the presence of genistein, especially from 10^{-7} to 10^{-5} M. From the view of osteoclasts function, genistein is effective in inhibiting osteoclast resorption in vitro. The pits excavated by osteoclasts decreased in genistein above 10^{-8} M and the inhibitory effect was depended on the dosage. Actin filament organization is essential for endocytosis in the osteoclasts. Actin rings in osteoclasts have recently increased much attention in bone biologists because they might change their structures depending on the osteoclast activity. We investigated that actin rings expressed in mouse bone marrow osteoclasts was changed by immunofluorescence techniques using a laser confocal microscope. This indicates that the cellular mechanism by the effect of genistein is partly involved with the change of F-actin.

Our result is consent with Gao’s. They think genistein inhibited the increase of osteoclast-like multinucleated cells stimulated by PTH, PGE2, VD3 and LPS. The inhibitory effect of 10^{-7} M genistein was equal to that of 10^{-8} M 17 beta-estradiol, 10^{-9} M calcitonin, or 10^{-5} M zinc sulfate. They suggested that the inhibitory action of genistein may involve in cyclic AMP signaling.

**The Effect of Genistein on Bone Metabolism in Vivo**

We found that genistein increase the BMD in the femur of...
and TNFα. Spontaneous increases in the expression and secretion of the proinflammatory cytokines IL-1, IL-6, and TNFα with estrogen deficiency were first noted several years ago in ex vivo cultures of circulating monocytes,\textsuperscript{16,17} bone marrow macrophages,\textsuperscript{18,19} and osteoblasts.\textsuperscript{20} In our experiment, after treated with genistein, the serum level of IL-1, IL-6, and TNFα increased of IL-6 in the rats were too little to detect. However, interestingly, different from several studies,\textsuperscript{21–23} the serum level of IL-6 in our experiment had no changes, suggesting that there were some differences in the mechanism between genistein and estradiol. Some researchers have reported that IL-6 exerts its effect in the initial stage of osteoclasts differentiation, while IL-1 plays its role in the later phase.\textsuperscript{24,25} So we raise the hypothesis that genistein may produce its marked effect in the late stage. Therefore, there may have several distinct pathways by which genistein may affect cytokine gene expression. On the other side, because of the different experiment circumstance, the increase of IL-6 in the rats were too little to detect.

Calcitonin is a peptide hormone produced by the parafollicular cells of thyroid gland that binds to seven transmembrane calcitonin receptor on osteoclasts and inhibits osteoclast activity. In our experiment, there were no significant changes among the four groups, which suggest that CT were in the balance of compensation through some unknown ways. Brandi\textsuperscript{26} believed that isoflavone didn’t influence the level of CT or the bone mass, but it could elevate CT’s sensitivity to calcium.

Osteocalcin is a protein that is secreted by mature osteoblasts, and it has been shown to correlate with the bone turnover rate. Serum osteocalcin concentration has correlated with both active bone formation and resorption. Taken the other finding together, the obvious elevation of osteocalcin is directly related with the active bone formation. ALP is an enzyme that is expressed by osteoblasts and is another marker of osteoblast activity. The significant increase in ALP of OVX-G rats also indicated the stimulation to the bone formation of genistein.

Additionally, Ishimi\textsuperscript{27} also examined the possible role of genistein in hemopoiesis and bone metabolism. Their results indicated that genistein exhibits estrogenic action in bone and bone marrow, to regulate B-lymphopoiesis and prevent bone loss, without exhibiting estrogenic action in the uterus. Taking our results and Ishimi’s together, we can deduce that phytoestrogens genistein are useful for preventing bone loss caused by estrogen deficiency in females.

**Summary of Genistein Effect**

1. Genistein can improve the bone metabolism through the promotion of bone formation and the prevention of bone resorption.
2. Genistein has slight side effect on uterus and estradiol level compared with the hormone replacement therapy.
3. Genistein provides an additional viable way to therapies for bone resorption diseases, for example osteoporosis.

**Acknowledgements**

We thank Professor Kui-Hua Zhang for helpful reviewing of the manuscript. This work was supported by the National Natural Sciences Foundation of China (39830430) and the “985 Promotion Plan” of Peking University.

**REFERENCES**