Dose-dependent effects of genistein on bone homeostasis in rats’ mandibular subchondral bone

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Aim: To investigate the effect of genistein on bone homeostasis in mandibular subchondral bone of rats.

Methods: Female SD rats were administered with genistein (10 and 50 mg/kg) or placebo by oral gavage for 6 weeks. Then the animals were sacrificed, and histomorphology and micro-structure of mandibular condyle were examined using HE staining and micro-CT analysis, respectively. The expression levels of alkaline phosphatase (ALP), osteocalcin (OC), osteoprotegerin (OPG), the receptor activator of nuclear factor κB ligand (RANKL) and estrogen receptors (ERs) in mandibular condyle were detected using real-time PCR. Cultured osteoblasts were prepared from rat mandibular condyle for in vitro study. The cells were treated with genistein (10⁻¹ or 10⁻⁴ mol/L) for 48 h. The expression of the bone homeostasis-associated factors and estrogen receptors (ERs) was detected using real-time PCR, and ER silencing was performed.

Results: At both the low- and high-doses, genistein significantly increased the bone mineral density (BMD) and bone volume, and resulted in thicker subchondral trabecular bone in vivo. In both in vivo and in vitro study, the low-dose genistein significantly increased the expression of ALP, OC and OPG, but decreased the expression of RANKL and the RANKL/OPG ratio. The high-dose genistein decreased the expression of all these bone homeostasis-associated factors. Both the low and high doses of genistein significantly increased the expression of ERβ, while ERα expression was increased by the low dose genistein and decreased by the high dose genistein. ERβ silencing abrogated most of the effects of genistein treatment.

Conclusion: In rat mandibular condylar subchondral bone, low-dose genistein increases bone formation and inhibit bone resorption, while excess genistein inhibits both bone formation and resorption. The effects of genistein were predominantly mediated through ERβ.

Keywords: genistein; estrogen receptor; mandibular subchondral bone; osteoblast; alkaline phosphatase; osteocalcin; osteoprotegerin; the receptor activator of nuclear factor κB ligand (RANKL)

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phatase (ALP) and osteocalcin (OC) are widely used markers for bone formation. Genistein treatment (45 mg/kg by oral gavage) was observed to significantly increase the serum ALP and OC levels in OVX rats\(^{[19]}\). In the process of bone resorption, osteoprotegerin (OPG) and the receptor activator of nuclear factor κB ligand (RANKL) constitute a complex mediator system. The RANKL to OPG ratio is the key regulatory determinant of bone resorption\(^{[19]}\). It is suggested that genistein (10 mg/kg, subcutaneous administration) significantly increased serum OPG level as well as decreased serum RANKL level and the RANKL/OPG ratio in OVX rats\(^{[20]}\). Additionally, the most abundant protein in bone is type I collagen. Type I collagen carboxy terminal telopeptide (CTX) is formed during bone collagen breakdown and is liberated into the circulation. Serum concentration of CTX reflects the degree of bone resorption\(^{[21]}\).

In the literature, the effects of genistein on bone were almost exclusively focused on changes in long bones or lumbar spines in OVX animals or postmenopausal women with estrogen deficiency. It has been suggested that patients with a history of osteoporotic fractures tend to have increased mandibular bone loss and relevant trabecular micro-structural changes\(^{[22]}\). Additionally, a dense trabecular pattern in the mandibular alveolar process has been proven to be a reliable sign of osteopenia\(^{[23]}\). In developed countries, a large proportion of the population visits dentists annually, and dental radiographs are performed routinely. Thus, researchers worldwide have recently tried to develop methods for using the jawbones, especially the mandible, to predict skeletal BMD\(^{[23]}\). The mandibular condyle, one important section of the mandible, is one of the most common sites of OA\(^{[24]}\). Furthermore, the articular surfaces of the mandibular condyle are composed of fibrocancellous cartilage that is distinct from the hyaline cartilage of the appendicular skeleton\(^{[25]}\). Unlike appendicular joints whose cartilage and subchondral bone are separated by tidemark and calcified cartilage, mandibular condylar cartilage and its subchondral bone are not obviously separated by an osteochondral interface\(^{[26]}\). However, in the literature few reports focused on the effect of phytoestrogens on mandibular bone, particularly in intact animals with normal estrogen levels. How genistein affects bone homeostasis in mandibular subchondral bone and whether different doses of genistein play different roles in this process remain unclear. The aim of the present study was to investigate the effect of different doses of genistein on bone homeostasis in the mandibular condylar subchondral bone in intact female rats.

**Materials and methods**

**Genistein treatment in vivo**

All experimental procedures administered to the animals were approved by the Animal Research Committee of the Fourth Military Medical University. Thirty female 7-week-old SD rats, weighing 180–190 g, were provided by the animal center of the Fourth Military Medical University. All animals were housed under conditions of 22°C and 50%–60% relative humidity with a normal day-night rhythm, consisting of a 12 h:12 h light-dark cycle. Animals were randomly assigned into the control, low- or high-dose genistein-treated groups, 10 animals in each group. Rats had free access to tap water and a phytoestrogen-free diet where corn oil replaced soybean oil. Based on a series of safety studies with genistein, it is estimated that the no observed adverse effect level (NOAEL) of genistein is 50 mg kg\(^{-1}\) d\(^{-1}\) for rats, and the no observed effect level (NOEL) is 10 mg kg\(^{-1}\) d\(^{-1}\)\(^{[27]}\). Thus, in the present study, 50 mg kg\(^{-1}\) d\(^{-1}\) genistein was chosen as the high dose, and 10 mg kg\(^{-1}\) d\(^{-1}\) as the low dose. The animals were treated once daily with genistein (10 or 50 mg/kg body weight, 99.5% pure, Winherb Med Sci Co Ltd, China) or placebo by oral gavage. Genistein was dissolved in a placebo solution (0.9% NaCl, 2% Tween 80, and 0.5% methyl cellulose in water). Application volume was 5 mL/kg body weight. Changes in body weight during the experimental period were taken into account in calculating the genistein dosage. All animals were sacrificed following 6 weeks of genistein administration.

**Histological analysis**

Under deep anesthesia, 12 rats (4 in each group) were sacrificed for histological analysis. The right temporomandibular joints (TMJs), including the mandibular ramus, were dissected and fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C and then decalcified for 1 week in Kristensen’s fluid (sodium formate 52.2 g, formic acid 174.2 mL, 1000 mL distilled water). Samples were then dehydrated and embedded in paraffin, followed by cutting into 5 µm middle-sagittal sections. HE staining was carried out for histological analysis. As we previously reported\(^{[26]}\), two square areas (0.5 mm × 0.5 mm) were selected under the interface of the cartilage and subchondral bone. They were located in the center of the middle and posterior third sections of the condylar cartilage that appeared consistent among animals. Using a computer-assisted image analyzing system (Leica Qwin Plus, Leica Microsystems Imaging Solutions Ltd, Cambridge, United Kingdom), the 2D measurements within the selected squares were performed by directed measurement of the trabecular bone area (B.Ar in mm\(^2\)) and perimeter (B.Pm in mm). Then, the histomorphometric parameters of the subchondral trabecular bone were calculated according to Parfitt’s formula as follows: bone volume fraction (BV/TV)=B.Ar/T.Ar; trabecular thickness (Tb.Th)=B.Ar/B.Pm; trabecular number (Tb.N)=B.Pm/T.Ar; trabecular separation (Tb.Sp)=(T.Ar–B.Ar)/B.Pm. The means of the data from two squares were used for statistical analysis.

**Micro-computed tomography (micro-CT) analysis**

As reported previously\(^{[26]}\), the left mandibular condyles from animals for histological analysis were scanned in a micro-CT system (GE eXplore Locus SP, London, UK) at an isotropic spatial resolution of 8 µm and a peak voltage of 80 kV. Twelve condyles, 4 in each group, were inserted in parallel into a home-made, round synthetic foam and mounted in the cylindrical specimen holders. Four projections were performed for each scan angle in each slice. These projections had an
exposure time of 3000 ms each and were averaged in order to improve the signal-to-noise ratio. The time of each scanning was over 270 min. General Electric Health Care MicroView ABA 2.1.2 Software was used to analyze reconstructive images of condyles and to calculate the parameters of architecture and mineralization. Similar to histomorphometric analysis, the interface of the cartilage and subchondral bone was divided into anterior, middle and posterior regions. As shown in Figure 1, two cubic regions of interest (ROI) (0.5 mm×0.5 mm×0.5 mm) were selected in the center of the middle and posterior regions of the condyle for the model-independent, three-dimensional morphometric analysis (Figure 1). The following micro-structural parameters were obtained: 1) BMD; 2) BMC; 3) trabecular bone volume fraction (BV/TV); 4) bone surface-to-volume ratio (BS/BV); 5) mean trabecular thickness (Tb.Th); 6) trabecular number (Tb.N), 7) mean trabecular separation (Tb.Sp), and 8) bone volume (BV). The means of the data from two ROIs were used for statistical analysis.

Quantitative real-time PCR in vivo
Under deep anesthesia, the remaining 18 rats (6 in each group) were sacrificed for real-time PCR. Twelve mandibular condyles in each group were randomly assigned into 3 samples. Mandibular condylar subchondral bone samples were pulverized in liquid nitrogen. The total RNA was isolated using a standard TRIzol® protocol (Invitrogen, Carlsbad, CA, USA), followed by first-strand cDNA synthesis with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed in an ABI 7500 Fast thermal cycler. The protocol comprised 40 cycles of 94°C for 5 s, 62°C for 34 s, and 72°C for 1 min each. The detected cytokines were ALP, OC, OPG, RANKL, ERα, and ERβ. Table 1 shows the sequences of the primers used in this study. Expression levels of all detected cytokines were normalized according to ribosomal protein 18S RNA levels to account for differences arising from reverse transcription efficiency and quality of the total RNA[23].

Table 1. Primer sequences for ALP, OC, OPG, RANKL, ERα, ERβ, and S18.

| Gene       | Sequence                           | Fragment length (bp) | Accession number |
|------------|------------------------------------|----------------------|-----------------|
| ALP        | F: 5′-CGAGCAGGAACAGAAAGTTTG-3′     | 105 bp               | NM_013059       |
|            | R: 5′-TGGCCAAAGCAGATAG-3′          |                      |                 |
| OC         | F: 5′-ATGCCACCTGATGTTTGATA-3′      | 122 bp               | M25490          |
|            | R: 5′-TCCGCTAGCTGTCACAAAT-3′       |                      |                 |
| OPG        | F: 5′-AGCTGACAACTGAGTAA-3′         | 106 bp               | U94330          |
|            | R: 5′-CACATTCCGCACACTGCGTT-3′      |                      |                 |
| RANKL      | F: 5′-GGAGGATTTTCTAGCTCCG-3′       | 103 bp               | NM_057149       |
|            | R: 5′-TGAAAGCCTAAAGTGTCG-3′        |                      |                 |
| ERα        | F: 5′-TGCGCAATGTTAGAAGTTG-3′       | 108 bp               | NM_012689       |
|            | R: 5′-CTCCCTTCCTACATCTG-3′         |                      |                 |
| ERβ        | F: 5′-ATAAATCACTGGCAATGACCTG-3′    | 124 bp               | NM_012754       |
|            | R: 5′-GCTGAATCTCATGCGGTT-3′        |                      |                 |
| S18        | F: 5′-CGGCTACCACTACGAAAG-3′        | 187 bp               | M11188          |
|            | R: 5′-GCTGAAATTCCCGGCCT-3′         |                      |                 |

ALP, alkaline phosphatase; OC, osteocalcin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; ERα, estrogen receptor α; ERβ, estrogen receptor β; S18, ribosomal protein S18, a housekeeping gene.

Serum chemistry
Before animals were sacrificed, blood was taken from the abdominal aorta by puncture exsanguination. Serum was collected by centrifugation and stored at -80°C for further biochemical analysis. Serum bone-ALP, OC, OPG, RANKL, and CTX levels were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (CSB-E11865r, CSB-E05129r, CSB-E07404r, and CSB-E05126r from Cusabio Biotech Co, Ltd, and QRCT-301330013301EIA\UTL from Adlitteram Diagnostic Laboratories) according to the manufacturer’s instructions. The absorbance was read on an Elx800™ microplate spectrometer (Bio-tec).

Culture of primary osteoblasts and genistein treatment in vitro
Primary osteoblasts were obtained from the mandibular subchondral bone of 8-week-old SD rats. In brief, the mandibular condyles were dissected with aseptic technique. The soft tissues and cartilage were removed to obtain mandibular subchondral bone, which was reduced to small fragments and
gently digested at 37 °C with 0.125% trypsin for 8 min. The digestion process was repeated 6 times. Fractionated cells were collected from the final four digestions and combined together as the primary osteoblasts. Cells were plated at a density of 1×10⁴/cm² into appropriate dishes and then cultured under 37 °C and 5% CO₂ conditions. Culture medium was DMEM containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. The cell medium was changed every three days.

For experiments, osteoblasts from the second generation were seeded in 12-well plates at a concentration of 3×10⁴/cm². It has previously been identified that genistein increases OPG levels with a maximum effect at 10⁻⁷ mol/L[29]. Thus, in the present study, 10⁻⁷ mol/L genistein was regarded as physiological or low dose, and 10⁻⁴ mol/L as the high dose in vitro. At confluence, cells were treated with 0.1% DMSO (Sigma D-2650) or genistein (10⁻⁷ and 10⁻⁴ mol/L, Sigma G6649) for an additional 48 h. Cells were collected for total RNA isolation. The mRNA expression levels of ALP, OC, OPG, RANKL, ERα, and ERβ were evaluated by real-time PCR.

**RNAI of ERs and genistein treatment**

Small interfering RNA (siRNA) duplexes targeted against rat ERα and ERβ genes were designed and synthesized by Gene-Pharma (Shanghai, China). The siRNA sequences for ERα (GenBank Accession No NM 012689) were as follows: sense, 5’-GGGACGUGGUCAUAGAUUTT-3’ and anti-sense, 5’-AUCAUAGAACCAGCUCCTT-3’. The siRNA sequences for ERβ (GenBank Accession No NM 012754) were as follows: sense, 5’-GAGCACACCUUACCUGUAATT-3’ and anti-sense, 5’-UUACAGGUAGGUUGUCUATT-3’. The nonsense siRNA sequences were as follows: sense, 5’-UUCUCGGAACGUGCUCUATT-3’ and anti-sense, 5’-ACGUGACCGUUCGAATTT-3’. Briefly, cells were grown in 6-well plates for 36 h and transfected at 70%–80% confluency. 6-well plates for 36 h and transfected at 70%–80% confluency. The mRNA expression levels of ALP, OC, OPG, RANKL, ERα, and ERβ were evaluated by real-time PCR.

**Statistical analysis**

SPSS 13.0 (SPSS Inc, Chicago, IL, USA) was used to analyze and describe the data. Single-factor analysis of variance was adopted. When significant main effects were found, specific comparisons between groups were made by Student’s t test. P-values were considered to be statistically significant when less than 0.05.

**Results**

**Morphology and micro-architectural properties of mandibular condylar subchondral bone**

In the mandibular condyles of control, low-, and high-dose genistein-treated groups, condylar cartilage covering subchondral bone was arranged regularly with good continuity in each layer (Figure 2). As identified by histomorphological analysis, the mandibular subchondral trabecular bone became larger and thicker after both high- and low-dose genistein treatment (Table 2), which was also identified by micro-CT (Figure 1).

As shown in Table 3, genistein induced significant increase in BMD, BMC, and bone volume in both the low-dose (P<0.05, P<0.05, P<0.01, respectively) and high-dose genistein-treated groups (all P<0.01). The BMD in the high-dose genistein-treated group was higher than that in the low-dose group (P<0.05). With respect to the micro-architecture of subchondral bone, the BV/TV and Tb.Th increased in both low-dose (P<0.05, P<0.01) and high-dose genistein-treated groups (both P<0.01), as well as obvious decrease in BS/BV, Tb.N and Tb.Sp in both genistein-treated groups (P<0.05, P<0.01). The BV/TV

**Table 2. Values of the histomorphological parameters after genistein treatment. Mean±SEM.**

| Parameters          | Control group | Genistein (10 mg/kg) | Genistein (50 mg/kg) |
|---------------------|---------------|----------------------|----------------------|
| BV/TV (%)           | 57.76±2.35    | 63.15±2.92           | 72.4±3.37            |
| Tb.Th (µm)          | 58.02±4.66    | 75.45±5.02           | 83.18±3.45           |
| Tb.N (#/mm²)        | 114.73±2.31   | 9.34±0.88            | 9.03±0.81            |
| Tb.Sp (µm)          | 45.64±4.35    | 29.52±2.88           | 26.48±3.35           |

BV/TV, trabecular bone volume fraction; Tb.Th, mean trabecular thickness; Tb.N, mean trabecular number; Tb.Sp, mean trabecular separation.

Figure 2. The histological morphology of mandibular condyle. (A−C) showed the morphology of middle-posterior region of mandibular condyle from control, low dose and high dose genistein-treatment groups. The area of trabecular bone in genistein-treatment group was obviously higher than that in control group. The space between trabecular bones in genistein-treatment group was obviously less than that in control group. C, cartilage; B, subchondral bone; TB, trabecular bone. Scale bar is 200 µm.
in the high-dose genistein-treated group was higher than that in low-dose group ($P<0.01$), while the Tb.Sp was lower in the high-dose group than that in the low-dose group ($P<0.05$).

### Effect of genistein-treatment on the mRNA expression of ALP, OC, OPG, RANKL, ERα, and ERβ in vivo

As shown in Figure 3, gene expression of ALP, OC, OPG, ERα, and ERβ increased in the low-dose genistein-treated group (all $P<0.01$) compared with the control group, but that of RANKL decreased ($P<0.01$). In the high-dose genistein-treated group, however, the gene expression of ALP, OC, OPG, RANKL, and ERα decreased significantly (all $P<0.01$), but that of ERβ still increased compared with the control group ($P<0.01$). Gene expression of ALP, OC, OPG, and ERα in the low-dose genistein-treated group was higher than that in the high-dose group (all $P<0.01$). Additionally, the RANKL/OPG ratio decreased in both genistein-treated groups.

### Effect of genistein-treatment on serum levels of bone-ALP, OC, OPG, RANKL, and CTX

Compared with the control group, the serum b-ALP, OC, and OPG levels increased significantly (all $P<0.01$), but the sRANKL level decreased ($P<0.01$) in the low-dose genistein-treated group. However, compared with the control group, not only the serum sRANKL and CTX (both $P<0.01$), but also b-ALP, OC, and OPG levels decreased ($P<0.05$, $P<0.05$, and $P<0.01$, respectively) in the high-dose genistein-treated group. The serum b-ALP, OC, and CTX levels in the low-dose genistein-treated group were higher than those in the high-dose group ($P<0.01$, $P<0.01$, $P<0.01$, and $P<0.05$, respectively). The RANKL/OPG ratio decreased in both genistein-treated groups (Table 4).

### Table 3. Values of BMD and microstructural parameters after genistein treatment. Means±SEM. $^aP<0.05$, $^bP<0.01$ vs control group. $^cP<0.05$, $^dP<0.01$ vs low dose (10 mg/kg) genistein-treatment groups.

| Parameters | Control group | Genistein (10 mg/kg) | Genistein (50 mg/kg) |
|------------|---------------|----------------------|----------------------|
| BMD (mg/mm$^3$) | 761.273±38.076 | 887.893±31.714$^a$ | 993.276±47.386$^a$ |
| BMC (mg) | 0.097±0.004 | 0.111±0.007$^a$ | 0.124±0.008$^a$ |
| Bone volume (mm$^3$) | 0.079±0.002 | 0.089±0.005$^a$ | 0.093±0.004$^a$ |
| BV/TV (%) | 61.297±1.903 | 67.228±2.578$^b$ | 73.568±1.782$^c$ |
| Tb.Th (μm) | 61.748±1.444 | 72.794±1.999$^c$ | 77.593±7.111$^c$ |
| BS/BV (%) | 31.993±1.184 | 27.806±1.325$^c$ | 25.923±2.112$^d$ |
| Tb.N (#/mm$^3$) | 10.170±0.296 | 9.182±0.611$^b$ | 9.088±0.525$^b$ |
| Tb.Sp (μm) | 0.102±3.654 | 31.204±1.504$^c$ | 28.176±1.850$^e$ |

BMD, bone mineral density; BMC, bone mineral content; BV/TV, trabecular bone volume fraction; BS/BV, bone surface-to-volume ratio; Tb.Th, mean trabecular thickness; Tb.N, trabecular number; Tb.Sp, mean trabecular separation; BV, bone volume.

### Table 4. Values of serum bone markers after genistein treatment. Mean±SEM. $^aP<0.05$, $^bP<0.01$ vs control group. $^cP<0.05$, $^dP<0.01$ vs low dose (10 mg/kg) genistein-treatment groups.

| Parameters     | Control group | Genistein (10 mg/kg) | Genistein (50 mg/kg) |
|----------------|---------------|----------------------|----------------------|
| b-ALP (U/L)   | 118.00±9.90  | 151.33±9.01$^a$     | 98.93±13.02$^b$     |
| OC (ng/mL)    | 9.95±1.27    | 13.93±2.55$^c$      | 7.76±1.73$^b$       |
| OPG (pg/mL)   | 335.24±18.50 | 403.67±19.36$^c$    | 255.64±23.14$^d$    |
| sRANKL (pg/mL)| 22.53±3.18   | 13.15±2.17$^b$      | 11.64±2.56$^b$      |
| CTX (ng/mL)   | 53.93±7.49   | 49.40±8.07          | 38.46±6.62$^e$      |

b-ALP, bone alkaline phosphatase; sRANKL, soluble receptor activator of nuclear factor κB ligand.

### Figure 3. Effect of genistein treatment on the mRNA expression of ALP, OC, OPG, RANKL, ERα, and ERβ. Values were normalized to the expression of S18. Data shown are means±SD (n=3). $^aP<0.01$ indicates significant difference between genistein-treatment and control groups. $^bP<0.01$ indicates significant difference between high and low dose genistein-treatment groups.

### Figure 4. Effect of genistein-treatment on the mRNA expression of ALP, OC, OPG, RANKL, ERα, and ERβ in vitro

As shown in Figure 4, genistein affected osteoblasts from mandibular condylar subchondral bone in a concentration-dependent manner. The presence of $10^{-7}$ mol/L genistein in
the culture medium induced a significant decrease in the gene expression of RANKL (P<0.01), as well as increase in ALP, OC, OPG, ERα, and ERβ (all P<0.01). However, the presence of 10⁻⁴ mol/L genistein reduced the expression of ALP, OC, OPG, RANKL and ERα in the 10⁻⁷ mol/L genistein-treated group were higher than those in the 10⁻⁴ mol/L group (all P<0.01). Alternatively, expression of ERβ was lower in the low-dose group than in the high-dose group (both P<0.01). The RANKL/OPG ratio was decreased by both 10⁻⁴ and 10⁻⁷ mol/L genistein treatment, respectively.

**Effect of ER silencing on the effect of genistein treatment**

In order to explore the function of ERα and ERβ, we performed gene silencing experiments in osteoblasts. The mRNA levels of ERα and ERβ were decreased in cells as detected by real-time PCR (Figure 5). ERα and ERβ siRNA were able to knockdown 72% and 80% of ER expression, respectively. As shown in Figure 6 and Figure 7, ERβ silencing reversed the effect of both 10⁻⁷ and 10⁻⁴ mol/L genistein treatment in the expression of ALP, OC, OPG, and RANKL. ERα silencing only reversed the expression of ALP and OPG (both P<0.01) in 10⁻⁴ mol/L genistein-treatment group, and that of OPG in the 10⁻⁴ mol/L genistein-treated group (P<0.05). Importantly, the effect of ERβ silencing was greater than that of ERα silencing in nearly all of the groups with the exception of OC expression in the 10⁻⁴ mol/L genistein-treated group (all P<0.01).
Discussion

In the literature, the protective effects of genistein in OVX rats and mice preventing OVX-induced bone loss have been reported in appendicular bone or vertebrae for various treatment periods (2–15 weeks), application routes (oral gavage, intraperitoneal administration, subcutaneous administration) and doses of genistein (3–50 mg kg\(^{-1}\) d\(^{-1}\))[18, 20, 30–36]. Similarly, oral administration of genistein (54 mg kg\(^{-1}\) d\(^{-1}\)) increased BMD of the spine and hip in osteopenic postmenopausal Caucasian women with an observation period of 24 months[17]. In the present study on female rats with normal estrogen levels, both low and high doses of genistein treatment were observed to induce significant increase in BMD, BMC and bone volume, as well as inducing thicker and larger trabecular bone in the mandibular subcondral bone. These results were consistent with the above-mentioned studies on appendicular bone or vertebrae[17, 18, 20, 30–36], indicating that the effect of genistein on mandibular subcondral bone is similar to that on appendicular bone or vertebrae.

Similar to the biphasic effects of estrogen depending on dosage[37], genistein was also observed to have a biphasic cell proliferative response, stimulation at low concentrations and inhibition at high concentrations[38–40]. In the present study low dose genistein treatment increased the expression of ALP, OC, and OPG at the serum level and mRNA level both in vivo and in vitro. Low-dose genistein treatment also decreased the expression of RANKL and the RANKL/OPG ratio in agreement with previous studies[20, 41–44]. These data suggest that normal doses of genistein improve bone formation and inhibit bone resorption. However, high-dose genistein treatment decreased not only the expression of RANKL and the RANKL/OPG ratio, but also that of ALP, OC, and OPG. It indicates that excess genistein inhibits both bone resorption and bone formation in rat mandibular subcondral bone. This phenomenon is consistent with the fact that the number of both active osteoclasts and active osteoblasts is increased in postmenopausal osteoporosis induced by estrogen deficiency[45]. Although bone formation was also inhibited by excess genistein, the RANKL/OPG ratio was still lower in the high-dose genistein-treated group than in the control group, which is in line with the increased BMD and bone volume in the high-dose genistein-treated group. Interestingly, though bone formation was inhibited by excess genistein to some extent, high doses of genistein induced more mandibular subcondral trabecular bone compared to low-dose genistein. This discrepancy might be due to the sampling time-point, or the asynchronism between microstructural parameters and the expression of bone homeostasis-associated factors. Allowing for these considerations, further studies with additional time-points are needed.

A mechanism has been proposed in which genistein affects bone homeostasis directly via ER activation in bone[34]. The two subtypes of ER, ER\(_{\alpha}\) and ER\(_{\beta}\), are distinct proteins encoded by separate genes located on different chromosomes. Genistein is almost exclusively ER\(_{\beta}\) selective and binds with a 7- to 30-fold greater affinity to ER\(_{\beta}\) than to ER\(_{\alpha}\)[40]. Further-
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