Effect of esculetin on bone metabolism in ovariectomized rats


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Abstract
OBJECTIVE: To determine the effect of an esculetin formulation (at 97.4% purity) on osteoporosis, and to investigate the potential underlying molecular mechanism(s).

METHODS: Sixty specific pathogen free-grade female Wistar rats were randomly assigned to three groups: blank control (n = 12), sham (n = 12), and model (n = 36). The model group were bilaterally ovariectomized. The sham group had the tissue surrounding the ovaries removed, while the ovaries were retained. After 3 months, the model group was randomly divided into three subgroups: OVX (n = 12), positive control (n = 12), and esculetin (n = 12). The positive control group and the esculetin group were intragastrically administered diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹) or esculetin (384 mg·kg⁻¹·d⁻¹), respectively, once per day for 6 consecutive days; medication administration was then stopped for 1 d, before being administered for another 6 consecutive days. All rats were treated for 3 months. Samples were collected at the end of the treatment period. An Osteocore3 Digital 2D bone densitometer was used to test the bone mineral density, and histomorphometric analysis was performed to measure bone mass, bone formation, and bone resorption. Enzyme-linked immunosorbent assay analysis was used to measure the serum concentrations of interleukin-6 (IL-6), osteoprotegerin (OPG), and receptor activator of nuclear factor-kappa B ligand (RANKL). Immunohistochemistry and in situ hybridization were performed to detect the protein and mRNA expressions of OPG and RANKL in osteoblasts and bone marrow stromal cells.

RESULTS: Compared with the OVX group, the esculetin group had significantly greater femoral bone mineral density and tibial trabecular bone volume, and significantly smaller trabecular resorption surface. The percentage of trabecular formation surface, average osteoid width, trabecular bone mineralization rate, and cortical bone mineralization rate did not significantly differ between groups. Compared with the sham group, the esculetin group had significantly decreased serum levels of IL-6 and RANKL, and significant downregulation of RANKL protein and mRNA expression levels in osteoblasts and bone marrow stromal cells; however, there was no significant difference between groups in OPG.
CONCLUSION: Esculetin can increase bone mass by upregulating RANKL expression in osteoblasts and bone marrow stromal cells, and decreasing serum IL-6 concentration. This indicates that the therapeutic effect of esculetin on osteoporosis occurs via decreased bone resorption.

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Keywords: Esculetin; Osteoporosis; Osteoprotegerin; RANK ligand; Bone resorption

INTRODUCTION

Osteoporosis is a systemic metabolic bone disease that involves low bone mass and bone microstructure destruction, which leads to increased bone fragility and fracture. Recent research has revealed that several signaling pathways are involved in the pathogenesis of osteoporosis, including transforming growth factor-β/Smad, Wnt/β-catenin, janus kinase/signal transducer and activator of transcription, P38/mitogen-activated protein kinases, bone morphogenetic protein 2/ Runx-related transcription factor 2/mitogen-activated protein kinase 1, and osteoprotegerin (OPG)/receptor activator of nuclear factor-kappa B ligand (RANKL). Of these signaling pathways, the OPG/RANKL pathway provides a very important osteoclast differentiation regulatory signal.1 OPG in combination with RANKL inhibits osteoclast differentiation and maturation, thus reducing bone resorption and decreasing loss of bone mass. RANKL is the only cytokine that can directly stimulate osteoclast development and activation, and it binds to receptor activator of nuclear factor-kappa B on the surface of osteoclasts, vigorously promoting the formation, differentiation, and maturation of osteoclasts, enhancing the mobility of osteoclasts, inhibiting osteoclast apoptosis, and prolonging the survival time of osteoclasts.2 3 Therefore, the OPG/RANKL pathway is the essential link between the many factors that regulate bone metabolism [interleukin-6 (IL-6), transforming growth factor-β, and tumor necrosis factor-α], and plays a vital role in osteoclast generation, development, maturation, and activation processes.

Cortex Fraxini (CF) is a commonly used Traditional Chinese Medicine, the major components of which are esculin, esculetin, fraxetin, and fraxoside.4 Of these components, esculetin has been most investigated for its biologic activities. Previous research shows that CF and its main component, fraxetin, produce beneficial preventive and therapeutic effects on osteoporosis. Liu et al.5 reported that CF significantly decreased prostaglandin E2 (PGE2) levels in synovial fluid. PGE2 plays an important role in the balance between osteoblasts and osteoclasts. PGE2 regulates the expression of RANKL by pre-osteoclasts, and the induction of differentiation and inhibition of apoptosis in osteoclasts. Hence, PGE2 activates osteoclasts to destroy bone and cartilage;6 7 this leads to greater bone resorption than bone formation, thereby causing osteoporosis. Hence, it is proposed that CF may manifest therapeutic and preventive effects in osteoporosis via the inhibition of PGE2. Kuo et al.8 discovered that esculetin mediated the maturation and differentiation of osteoblasts by inhibiting pro-inflammatory cytokines that induced osteoblast apoptosis, and augmenting the production of bone morphogenetic protein-2 and bone morphogenetic protein-4, indicating that fraxetin plays an important role in preventing and treating osteoporosis. However, the effects of esculetin on osteoporosis have not yet been reported. Esculetin and fraxetin are two major components of CF, and possess the same coumarin nucleus and other similar chemical structures (Figure 1); they are also both aglycones and have “ortho bisphenol structures” (related to the antioxidant activity of coumarin). Thus, it is anticipated that esculetin may show potential therapeutic effects regarding osteoporosis.

Figure 1 The structures of the major active components of Cortex Fraxini.
A: Esculetin; B: Fraxtin; C: Fraxoside; D: Esculin.

In the present study, we evaluated the effects of esculetin on osteoporosis in rats, and investigated the underlying mechanisms involved by using bone histomorphometry and focusing on the OPG/RANKL signaling system.

MATERIALS AND METHODS

Experimental animals

Sixty specific pathogen free-grade female Wistar rats, weighing 200-220 g, were provided by the National Institute for Food and Drug Control [license #SCXK (Jing) 2009-0017]. Rats were kept in the barrier facility of the Clinical Institute of China-Japan Friendship Hospital [Laboratory Animal Facility permit No. SYXK (Jing) 2010-0011].

Reagents and drugs

The esculetin standard was purchased from the National Institute for Food and Drug Control (Beijing, China, cat #741-9404); esculetin was purchased from Wuhan Chifei Chemical Co., Ltd. (Wuhan, China, content 97.4%); pentobarbital sodium was purchased from Merck & Co. (Kenilworth, New Jersey, USA, cat #20121220); tetracycline hydrochloride was purchased from Amresco (Solon, Ohio USA, cat #20121116);
methyl methacrylate was purchased from Beijing Yili Fine Chemicals Co., Ltd. (Beijing, China, cat # 20090301); beryllium methacrylate was purchased from Beijing Yili Fine Chemicals Co., Ltd. (Beijing, China, cat #980716); polyethylene glycol 400 was purchased from Beijing Chemicals Company (Beijing, China, cat #050425); benzoate was purchased from North China Specialty Chemicals Development Center (Tianjin, China, cat #980923); benzyl peroxide was purchased from Beijing Jinlong Chemical Co., Ltd. (Beijing, China, cat #20000420); N,N-dimethyl-p-toluidine was purchased from Aldrich (St. Louis, MO, USA lot # 04327MB-235); rat OPG enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&B company (Santa, CA, USA, cat #07081102); rat RANKL ELISA kit was purchased from R&B company (Santa, CA, USA, cat #07081103); rat IL-6 ELISA kit was purchased from e Bioscience (Vienna, Austria, cat # 65033023); rat OPG antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA, cat #K1309); rat RANKL antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA, cat #E0911); rat OPG and RANKL in situ hybridization kits were all purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China). For the OPG in situ hybridization kits, the oligonucleotide probes targeting OPG were labeled with digoxin, and the mRNA sequences targeting the rat OPG gene were (a) 5'-TGGAC AACCAG AGGAA ACCTT TCCTC CAAA-3', (b) 5'-TTTCG CTGGG ACCAA AGTGA ATGCA GAGAG-3', (c) 5'-AGAAA TGATA 3'-TGGAC AACCC AGGAA ACCTT TCCTC CAAA-3', (b) 5'-GCCAG CGGAG ACTAC GGCAA GTACCC TGCGC-3', (b) 5'-GGCCGA GGTTG TCTGC AGCAT GCCTC TGTC-3', (c) 5'-TTAT AGAAT CCTGA GACTC CATGA AAACG-3'.

**Instruments**

The instruments used were a microtome cryostat (Thermo, Waltham, MA, USA ), Leica M60 microscope (Leica Corp, Bensheim, Germany), Osteocore3 Digital 2D bone densitometer (Mediplink, Perols, France), Leica CTR6000 microscope (Leica Corp, Bensheim, Germany), Leica QWin Image Analyzer System (Leica Corp, Bensheim, Germany), Multiskan MK3 microplate reader (Thermo, Waltham, MA, USA), and a Wellwash 4 Mk2 microplate washer (Thermo, Waltham, MA, USA).

**Esculetin content measurement**

Chromatographic conditions were as follows: columns, Agilent Extend C18 (4.6 mm × 250 mm, 5 µm); mobile phase, methanol:0.4% acetic acid (25: 75); detection wavelength, 340 nm; column temperature, 40 °C; flow rate, 1.0 mL/min; injection volume, 10 µL.

The control solution was prepared by accurate measurement of appropriate amounts of the esculetin control, and dissolution in methanol to create a 0.1100 g/L control solution.

The experimental solution was prepared by accurate measurement of an appropriate amount of esculetin sample, dissolution in methanol to create a 0.1082 g/L sample solution, thorough mixing, and filtration through a 0.45 µm syringe filter.

**Grouping and modeling**

The 60 rats were randomly assigned to three groups: blank control (n = 12), sham (n = 12, and model (n = 36). All rats were acclimated for 1 week. The model group was anesthetized by intraperitoneal injection of 45 mg/kg sodium pentobarbital, and fixed in the prone position. Hair was removed to expose the surgical field under the last ribs at the intersection of the axillary line and approximately 1 cm lateral to the midline of the spine. Alcohol (75%) and iodine were applied for disinfection before the skin, back muscles, and fascia were incised. Tweezers were used to gently pull out the white cellulite from the incision. The ovary could then be found once the cellulite was separated. The oviduct was ligated with a surgical suture, and the ovary was then removed. The contralateral ovary was removed in the same way. After the incision was sutured, powdered anti-inflammatory drugs were applied. Surgery was also performed on the sham group, but only a small amount of fatty tissue was removed, while the ovaries were left intact.

Three months postoperatively, the model group was randomly divided into three subgroups: OVX (n = 12), positive control (n = 12), and esculentin (n = 12). The positive control group and the esculetin group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹) or esculentin (384 mg·kg⁻¹·d⁻¹) by oral gavage, respectively, once per day for 4 consecutive days; medication administration was then stopped for 1 d. Before being administered for another 4 consecutive days. The rats were weighed weekly, and the doses were adjusted accordingly. All rats were treated for 3 months. The blank control, sham, and model groups were administered distilled water in the same fashion.

**Sample collection**

For bone fluorescent labeling, the rats were given 30 mg/kg tetracycline hydrochloride via intraperitoneal injection at 16 d and 3 d before euthanasia. After treatment, the rats were anesthetized and whole blood was collected via the femoral artery. The whole blood was left to stand for 1 h, and then centrifuged at 2000 rpm for 10 min before serum was collected. Serum cytokine levels were subsequently measured with ELISA. At the end of the treatment period, the rats were euthanized. The left femurs were removed for bone mineral density (BMD) testing; the proximal third of the right tibia was collected, and undecalcified slides were prepared for histomorphometric analysis; the proximal third of the left tibia was collected, and decalcified frozen sections were prepared. Immunohistochemistry and in si-
tu hybridization were performed on these decalcified frozen sections to detect the protein and mRNA expressions of OPG and RANKL in osteoblasts and bone marrow stromal cells (BMC) in the bone marrow cavity.

Measurement of BMD
The femoral BMD in each group was detected with an Osteocore3 Digital 2D bone density instrument.

Analysis of bone mass, bone absorption, and bone formation
The tibiae were fixed in neutral paraformaldehyde, and then embedded in methyl methacrylate. Tungsten carbide knives were used on a Leica M60 microtome (Leica Corp, Bensheim, Germany) to obtain two longitudinal 5 μm, undecalcified bone sections; one section was stained with toluidine blue, while the other was observed directly for fluorescence. Bone histomorphometry was performed, and a Leica QWin image analysis system was used for quantitative analysis. Bone mass was measured as the percentage of trabecular bone volume (TBV%), which represents the percentage of the TBV relative to the total volume of the measured bone marrow cavity. TBV% is considered to be a main indicator of bone mass level.

Bone resorption was measured as the percentage of trabecular resorption surface (TRS%), which represents the percentage of the irregular, uneven surface of the trabecular bone relative to the total trabecular bone surface. TRS% can also be used to determine osteoclast activity.

Bone formation was measured as: (a) the percentage of trabecular formation surface (TFS%), which represents the percentage of osteoblast-covered osteoid surface relative to trabecular surface, and thus can be used to determine osteoblast activity; (b) the trabecular bone mineralization rate (MAR), which was calculated by dividing the mean distance between the two fluorescent bands by the interval (in days) between the two labeling procedures; (c) the average osteoid width (OSW) in the cortical bones covered by osteoblasts; (d) the cortical bone mineralization rate (mAR), which was calculated by dividing the mean distance between the two fluorescent bands inside the cortical bone by the interval (in days) between the two labeling procedures.

Measurement of the Serum Concentrations of IL-6, OPG, and RANKL
The frozen serum samples were brought to room temperature before analysis. Serum concentrations of IL-6, OPG, and RANKL were measured using ELISA, in accordance with the manufacturer’s instructions.

Measurement of the protein and mRNA expression levels of OPG and RANKL
Immunohistochemistry and in situ hybridization were performed in accordance with the instruction manuals to measure the protein and mRNA expression levels of OPG and RANKL in osteoblasts and BMC in the rat tibiae.

The cytoplasm of positive cells in osteoblasts and BMC was stained brown. Histomorphometric analysis was done using a Leica QWin image analysis system. Five or more random fields below the epiphyseal plate were analyzed at ×400 magnification, the positive area was calculated as a percentage of each field, and the mean value was computed. The positive area percentage was calculated as the percentage of the area of the positive cells with brown-stained cytoplasm relative to the area of the whole field, and was defined as the positive cell density (positive density).

Statistical analyses
All experimental results were presented as the mean ± standard deviation (x̄ ± s). SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. One-way analysis of variance was used for data that followed a normal distribution and showed equal variance, while Kruskal-Wallis one-way analysis of variance non-parametric testing was performed for non-normally distributed data. P < 0.05 was considered statistically significant.

RESULTS
Esculetin content measurement
Standard and sample solutions were accurately aspirated in 10 μL aliquots and measured using previously described chromatographic conditions, and the esculetin content was determined to be 97.4% by the external standard calculation method. The chromatography is shown in Figures 2 and 3.

BMD
Compared with the sham group, the femoral BMD in the OVX group was significantly decreased. Compared with the OVX group, the esculetin group had significantly greater femoral BMD. However, the esculetin
group still had a lower femoral BMD than the sham group (Table 1).

**Table 1** Effect of esculetin (384 mg·kg⁻¹·d⁻¹) on BMD in rats treated for 3 months (± s.d.)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0.33±0.029</td>
</tr>
<tr>
<td>Sham</td>
<td>12</td>
<td>0.32±0.024</td>
</tr>
<tr>
<td>OVX</td>
<td>12</td>
<td>0.19±0.027</td>
</tr>
<tr>
<td>E₂</td>
<td>12</td>
<td>0.26±0.026</td>
</tr>
<tr>
<td>Esculetin</td>
<td>12</td>
<td>0.22±0.025</td>
</tr>
</tbody>
</table>

Notes: sham group: a small amount of fatty tissue around the ovaries was removed, while the ovaries were left intact. OVX group were ovariectomized but were not administered any medication. E₂ group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹), 6 d per week, for 3 months. Esculetin group: treated with esculetin (384 mg·kg⁻¹·d⁻¹) by oral gavage, 6 d per week, for 3 months. BMD: bone mineral density; OVX: ovariectomized; E₂: diethylstilbestrol. *P < 0.01 compared with the sham group.* 

**Bone histomorphometry**

Compared with the sham group, the tibiae in the OVX group had significantly lower TBV% and significantly greater TRS%, TFS%, OSW, MAR, and mAR. Compared with the OVX group, the tibiae in the esculetin group had significantly greater TBV% and lower TRS%, while there was no significant difference between the OVX and esculetin groups. However, compared with the sham group, the esculetin group still had greater serum concentrations of IL-6 and RANKL, and a significantly lower serum concentration of OPG (Table 2, Figure 4).

**Table 2** Effect of esculetin on bone histomorphometry in rats treated for 3 months (± s.d.)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TBV (%)</th>
<th>TRS%</th>
<th>TFS (%)</th>
<th>OSW (µm)</th>
<th>MAR (µm/d)</th>
<th>mAR (µm/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>36.8±5.20</td>
<td>3.37±1.31</td>
<td>5.93±1.36</td>
<td>9.00±2.03</td>
<td>0.94±0.23</td>
<td>1.48±0.30</td>
</tr>
<tr>
<td>Sham</td>
<td>12</td>
<td>37.05±6.24</td>
<td>3.16±1.12</td>
<td>5.68±1.59</td>
<td>9.13±2.14</td>
<td>0.99±0.28</td>
<td>1.43±0.31</td>
</tr>
<tr>
<td>OVX</td>
<td>12</td>
<td>14.04±4.30</td>
<td>8.93±2.45</td>
<td>11.52±2.03</td>
<td>15.70±3.33</td>
<td>1.89±0.37</td>
<td>2.29±0.43</td>
</tr>
<tr>
<td>E₂</td>
<td>12</td>
<td>29.08±5.87</td>
<td>3.75±1.45</td>
<td>6.23±1.79</td>
<td>9.57±2.43</td>
<td>1.15±0.19</td>
<td>1.63±0.28</td>
</tr>
<tr>
<td>Esculetin</td>
<td>12</td>
<td>19.05±3.50</td>
<td>4.43±1.24</td>
<td>10.08±2.08</td>
<td>14.69±3.07</td>
<td>1.81±0.38</td>
<td>2.25±0.33</td>
</tr>
</tbody>
</table>

Notes: sham group: a small amount of fatty tissue around the ovaries was removed, while the ovaries were left intact. OVX group were ovariectomized but were not administered any medication. E₂ group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹), 6 d per week, for 3 months. Esculetin group: treated with esculetin (384 mg·kg⁻¹·d⁻¹) by oral gavage, 6 d per week, for 3 months. BMD: bone mineral density; OVX: ovariectomized; E₂: diethylstilbestrol; TBV%: percentage of trabecular bone volume; TRS%: percentage of trabecular resorption surface; TFS%: percentage of trabecular formation surface; OSW: average osteoid width; MAR: trabecular bone mineralization rate; mAR: cortical bone mineralization rate. *P < 0.01, †P < 0.05, compared with the sham group.* 

**Serum concentrations of OPG, RANKL, and IL-6**

Compared with the sham group, the OVX group had significantly greater serum concentrations of IL-6 and RANKL, and a lower serum concentration of OPG. Compared with the OVX group, the esculetin group had significantly decreased serum concentrations of IL-6 and RANKL, while the serum OPG concentration did not significantly differ between the OVX and esculetin groups. However, compared with the sham group, the esculetin group still had greater serum concentrations of IL-6 and RANKL, and a significantly lower serum concentration of OPG (Figure 5).

**Protein and mRNA Expressions of OPG and RANKL in Tibial Osteoblasts and BMC**

Compared with the sham group, tibiae in the OVX group had significantly lower positive densities of OPG protein and mRNA in osteoblasts and BMC, as well as significantly greater positive densities of RANKL protein and mRNA. In the esculetin group, the positive densities of RANKL protein and mRNA were significantly lower than those in the OVX group, but were still greater than those in the sham group. However, the OPG protein and mRNA expressions in the esculetin group were not significantly different from those in the OVX group, but were still lower than those in the sham group (Figures 6, 7).

Figure 4 Effect of esculetin on bone formation

A: control group; B: sham group; C: OVX group; D: OVX + E₂; E: OVX+esculetin group. Sham group: a small amount of fatty tissue around the ovaries was removed, while the ovaries were left intact. OVX group were ovariectomized but were not administered any medication. OVX + E₂ group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹), 6 d per week, for 3 months. OVX + Esculetin group: treated with esculetin (384 mg·kg⁻¹·d⁻¹) by oral gavage, 6 d per week, for 3 months. The orange arrow shows the trabecular bone. Trabecular bone formation was visualized using methylene blue staining. The details can be seen in the Methods section of the text.
DISCUSSION

Esculetin has a beneficial inhibitory effect on bone resorption

Our results show that 3 months after ovariectomy, the femoral BMD and the bone mass indicator TBV% in the model group were significantly decreased compared with the sham group, while the bone resorption indicator TRS% and the bone formation indices TFS%, MAR, mAR and OSW were significantly increased;

![Graph showing the effects of esculetin on bone resorption](image1)

**Figure 5** Effect of esculetin on the serum concentrations of IL-6, OPG, and RANKL

A: IL-6; B: OPG; C: RANKL. Sham group: a small amount of fatty tissue around the ovaries was removed, while the ovaries were left intact. OVX group were ovariectomized but were not administered any medication. E2 group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹), 6 d per week, for 3 months. Esculetin group: treated with esculetin (384 mg·kg⁻¹·d⁻¹) by oral gavage, 6 d per week, for 3 months. IL-6: interleukin-6; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-kappa B ligand; OVX: ovariectomized; E2: diethylstilbestrol.

![Graph showing the protein expression of OPG and RANKL](image2)

**Figure 6** Effect of esculetin on the protein expressions of OPG and RANKL

A1-A5: immunohistochemical stain of OPG; B1-B5: immunohistochemical stain of RANKL; A1, B1: control; A2, B2: sham; A3, B3: OVX; A4, B4: OVX+E2; A5, B5: OVX+Esculetin. C: positive densities of OPG and RANKL protein expressions. OPG and RANKL protein expression data were obtained from panels A and B of this figure, see details in the Methods section of the text. Sham group: a small amount of fatty tissue around the ovaries was removed, while the ovaries were left intact. OVX group were ovariectomized but were not administered any medication. OVX+E2 group were treated with diethylstilbestrol (0.046 mg·kg⁻¹·d⁻¹), 6 d per week, for 3 months. OVX+Esculetin group: treated with esculetin (384 mg·kg⁻¹·d⁻¹) by oral gavage, 6 d per week, for 3 months. OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-kappa B ligand; OVX: ovariectomized; E2: diethylstilbestrol.
this indicates that ovariectomy caused high bone turnover osteoporosis in our animal model, exhibiting pathologic characteristics similar to those of postmenopausal osteoporosis in human females. After 3 months of treatment, BMD and TBV% were significantly increased in the esculetin and positive control groups compared with the OVX group; and the esculetin and positive control groups had significantly lower TRS% compared with the OVX group. The OVX group and the esculetin group had similar TFS%, MAR, mAR, and OSW. Collectively, our data indicate that esculetin (384 mg·kg⁻¹·d⁻¹) manifests a therapeutic effect on ovariectomy-induced osteoporosis in rats, and that this may occur through its inhibitory effect on bone resorption.

Mechanism of the therapeutic effect of esculetin in osteoporosis
Esculetin reportedly shows estrogenic effects in vivo at a dosage of 50-100 mg/kg.₁¹ Estrogen can effectively control the bone resorption effect of osteoclasts, and its attenuation can directly increase the expression levels of matrix metalloproteinase-1 and matrix metalloproteinase-2, and increase the bone resorption activity of osteoclasts, leading to the occurrence of high-turnover postmenopausal osteoporosis.₁² The OPG/RANKL system is an important pathway for estrogenic regulation of osteoclastogenesis and anti-bone resorption.₁³ Females with postmenopausal osteoporosis reportedly have a lower serum OPG concentration than postmenopausal females without osteoporosis, and those with low OPG are at greater risk of fracture.₁⁴ In contrast to OPG, RANKL expression levels are reportedly significantly increased in the bone marrow of mesenchymal cells in postmenopausal females.₁⁵ The present results show that rats treated with esculetin for 3 months had significantly decreased serum IL-6 concentration. Compared with the sham group, the esculetin group showed no significant differences in serum OPG concentration or in densitometric mea-
measurements of OPG protein and mRNA expression levels, while there were significant decreases in RANKL protein and mRNA expression levels, as well as serum RANKL levels. Our results demonstrated that although esculetin had no significant effect on OPG expression, it reduced the serum concentration of IL-6 (a bone resorption stimulator), and it also downregulated the expression levels of RANKL, an osteoclast differentiation regulatory factor. Decreasing the levels of IL-6 and RANKL could, in turn, substantially inhibit osteoclast activity and reduce bone resorption. The present results also revealed that esculetin had minimal effects on bone formation, and that its therapeutic effect on osteoporosis in rats was mainly exerted through decreased bone resorption.

In conclusion, esculetin increases bone mass by upregulating RANKL expression in osteoblasts and BMC, and by decreasing serum concentration of IL-6. This indicates that esculetin exerts its therapeutic effect in osteoporosis by decreasing bone resorption.

REFERENCES