



## ***Drynaria* total flavonoids decrease cathepsin K expression in ovariectomized rats**

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**ABSTRACT.** This study investigated the effects of *Drynaria* total flavonoids on cathepsin K serum concentrations and gene expression, biomechanics and bone mineral density (BMD) of the tibial shaft in ovariectomized rat models of osteoporosis, and mechanism in the prevention and cure of osteoporosis. Seventy-two female Sprague-Dawley rats were divided into six groups. The rats in each group were subjected to gastric lavage after the model was established. The tibial shaft of the right hindlimb was obtained to measure the BMD. Serum cathepsin K concentrations were determined. The cathepsin K mRNA expression was also determined using fluorescent quantitative polymerase chain reaction. The three-point bending method was performed to measure the maximum bending load of the tibial shaft. The total flavonoid and normal groups had significant differences in serum cathepsin K concentrations compared with that in the estrogen group ( $P < 0.05$ ). The total flavonoid and sham-operated groups also showed significant differences in cathepsin K mRNA expression compared with that in the normal group ( $P < 0.01$ ). The maximum

bending load of the rats in the total flavonoid group was significantly different from that in the estrogen group ( $P < 0.05$ ) and the sham-operated and normal groups ( $P < 0.01$ ). The high-dose total flavonoid group elicited a better effect on BMD than that by the medium- and low-dose groups ( $P < 0.05$ ). Thus, *Drynaria* total flavonoids inhibited the serum cathepsin K concentration and increased the maximum bending load of the tibial shaft in ovariectomized rats.

**Key words:** *Drynaria* total flavonoids; Ovariectomized rats; Cathepsin K; Biomechanics; Bone mineral density

## INTRODUCTION

Aging is one of the major causes of the rapidly increasing incidence of osteoporosis. In China, approximately 130,000,000 individuals, which accounts for 10% of the total population (aged more than 60 years) are affected, and the incidence rate of osteoporosis reaches approximately 12.4% (8.5% in males and 15.7% in females) (Xu et al., 2008). In 2050, Asians would comprise more than half of the patients with osteoporotic bone fractures worldwide, and most of them would be Chinese (Lane et al., 2000). Therefore, Chinese medical professionals have devoted efforts to decrease the incidence rate of osteoporotic fractures and improve the patients' quality of life to prevent primary osteoporosis.

In this study, *Drynaria* total flavonoids, the main active constituents of Rhizoma Drynariae (a single Chinese drug), were administered to ovariectomized rats for the intervention treatment. To investigate the effect of *Drynaria* total flavonoids on the osteoclasts, the cathepsin K mRNA expression in the proximal metaphysis of the tibia was detected using real-time quantitative polymerase chain reaction (PCR). To investigate the effect of *Drynaria* total flavonoids on bone strength, the maximum load of the tibial shaft was measured using a three-point bending test. The effects of *Drynaria* total flavonoids on serum cathepsin K concentration and bone mineral density (BMD) were also investigated. This study aimed to provide experimental evidence regarding the mechanism of Rhizoma Drynariae for the treatment of osteoporosis.

## MATERIAL AND METHODS

### Subjects and sampling

Seventy-two female Sprague-Dawley rats were divided into six groups: 1) high-dose *Drynaria* group, 2) medium-dose *Drynaria* group, 3) low-dose *Drynaria* total flavonoid group, 4) estrogen group, 5) sham-operated group, and 6) normal group. The concentrations of total flavonoids of Rhizoma Drynariae solution in different groups were 0.216, 0.108, and 0.054  $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  for high-, medium-, and low-dose groups, respectively. For the remaining three groups (estrogen, sham-operated, and normal groups), the total flavonoids of Rhizoma Drynariae were mixed in distilled water P to produce the rat gastric infusion. Four rats in each group were sacrificed immediately, after three months, and after six months. The venous blood was extracted under a sterilized condition and centrifuged.

Seventy-two serum samples were obtained and stored at  $-20^{\circ}\text{C}$ . The proximal metaphysis of the left tibia was obtained. The surrounding flesh and the connective tissues were carefully removed. Afterward, the proximal metaphysis was dehydrated with graded ethanol, and the proximal metaphysis samples ( $N = 72$ ) were stored at  $-80^{\circ}\text{C}$ . This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Zhejiang Chinese Medical University.

### Detection of the serum cathepsin K concentration

The serum cathepsin K level was detected using an enzyme-linked immunosorbent assay (ELISA). The procedures were performed according to manufacturer instructions (Wuhan USCN, China).

### Detection of the cathepsin K mRNA expression

The bone sample that was frozen at  $-80^{\circ}\text{C}$  was ground in liquid nitrogen. Trizol reagent (1 mL) was added and oscillated. Chloroform (0.2 mL) was added and oscillated vigorously. The mixture was allowed to stand at room temperature for 5 min and centrifuged at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 15 min. The upper aqueous phase was transferred into an Eppendorf tube. An equal volume of isopropanol was added, oscillated, and then allowed to stand at room temperature for 10 min. The solution was centrifuged at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. The supernatant was then discarded. Afterward, the precipitates were washed once with 1 mL 75% ethanol [prepared with diethylpyrocarbonate (DEPC) water], centrifuged at  $7500 \times g$ , and dried in air. The sample was diluted with DEPC water. The absorbance ( $A$ ) was determined using an ultraviolet spectrophotometer. The mRNA concentration and the purity of the sample were calculated according to the ratio of  $A_{260}/A_{280}$ . The sample was stored at  $-20^{\circ}\text{C}$ .

### Detection of cathepsin K expression

The total RNA was extracted using the Trizol method and reverse transcribed into cDNA. The PCR reaction system, with a total volume of 50  $\mu\text{L}$ , contained 4.0  $\mu\text{L}$  cDNA, 5.0  $\mu\text{L}$  10X PCR buffer, 3.0  $\mu\text{L}$  25 mM  $\text{MgCl}_2$ , 1.0  $\mu\text{L}$  10 mM dNTPs, 1.0  $\mu\text{L}$  10  $\mu\text{M}$  upstream primers, 1.0  $\mu\text{L}$  10  $\mu\text{M}$  downstream primers, and 5  $\mu\text{L}$   $\beta$ -actin primer mixture (with 28.5  $\mu\text{L}$   $\text{ddH}_2\text{O}$  and 0.5  $\mu\text{L}$  5 U/ $\mu\text{L}$  *Taq*). In the amplification, a pre-denaturation step was performed at  $50^{\circ}\text{C}$  for 2 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 10 min,  $94^{\circ}\text{C}$  for 30 s, and  $61^{\circ}\text{C}$  for 1 min. The primer design and synthesis template had GenBank accession No. NM\_031560.2. Upstream and downstream primer sequences used in this study were 5'-GGGAGACATGACCAGCGAAG-3' and 5'-CTGAAAGCCCAACAGGAACC-3'.  $\beta$ -actin was used as an internal standard. Upstream and downstream primers were 5'-CCGTCTCCCCTCCATCG-3' and 5'-GTCCCAGTTGGTGACGATGC-3'. The PCR product length was 195 bp.

The application amount of each sample was 1  $\mu\text{L}$ . Considering that errors in RNA concentration quantification and RNA reverse transcription efficiency may lead to differences in cDNA contents among samples with the same volume,  $\beta$ -actin was used as the internal

standard. The relative content of the target gene was calculated according to the ratio between the value of the target gene and that of the internal standard.

### Measurement of the maximum bending load

The left tibia (in which the proximal metaphysis was removed) was obtained. The maximum bending load of the left tibia was measured using a WDW-100B electric universal testing machine (Changchun Nonmetallic Testing Machine Factory, China). The maximum bending load of the samples was measured in the same region.

### Measurement of BMD

The tibial shaft of the right hindlimb was obtained to measure the BMD using the human forearm BMD measurement software. All of the samples were measured at the same site. The following laboratory apparatus and reagents were used: rat K (cath-K) ELISA kit, 2010 XH/LAB-YQ08 ELISA analyzer (Zhengzhou Biocell, China), QDR 24000 dual-energy X-ray absorptiometer (HOLOGIC, USA) at a coefficient of variation <0.1%, 2% pentobarbital sodium injection, 75% ethanol, povidone iodine, physiological saline, and surgical instruments.

### Statistical analysis

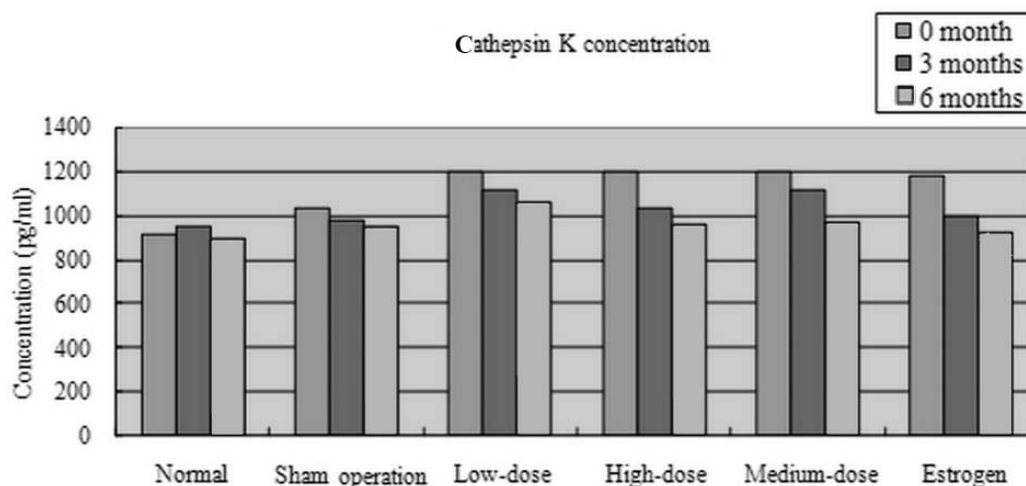
Data are reported as means  $\pm$  standard deviation (means  $\pm$  SD) and analyzed using the GraphPrism 4.0 and SPSS 13.0 software. The Student *t*-test was performed to compare the difference between the means of two samples. One-factor analysis of variance was performed to compare groups.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Cathepsin K concentration

After the model was established, all of the treatment groups showed significant differences in cathepsin K concentration compared with the sham-operated and normal control groups ( $P < 0.05$ ). Ten weeks after ovariectomy, the cathepsin K concentration in the rats increased by varying degrees, indicating that the models were successfully established. After three months, the total flavonoid, estrogen, and normal groups had significant differences compared with the sham-operated group ( $P < 0.05$ ). The total flavonoid and estrogen groups also showed significant differences compared with the other groups ( $P < 0.05$ ). These results indicated that the serum cathepsin K concentration that was expressed in the osteoclasts in the total flavonoid and estrogen groups was inhibited to a certain degree after the three-month drug treatment. Thus, *Drynaria* total flavonoids and estrogen had curative effects on osteoporosis in the ovariectomized rats. In addition, the high-, medium-, and low-dose total flavonoid groups were compared. The results showed that the high-dose group exhibited a significant difference compared with the medium- and low-dose groups ( $P < 0.05$ ), whereas no significant difference was found between the

medium- and low-dose groups ( $P > 0.05$ ). After six months, the cathepsin K concentration of the total flavonoid and estrogen groups showed significant differences compared with the concentration after three months ( $P < 0.05$ ), indicating that the serum cathepsin K concentration did not return to normal levels, although this concentration in the ovariectomized rats was further inhibited after six months. Thus, osteoporosis was partially improved. Furthermore, all of the flavonoid groups showed a significant difference compared with the estrogen group ( $P < 0.05$ ), indicating that *Drynaria* total flavonoids elicited a less significant inhibitory effect on the serum cathepsin K concentration than the estrogens in the treatment of osteoporosis. The results are shown in Figure 1.



**Figure 1.** Cathepsin K concentration in different groups at different time points.

### Cathepsin K mRNA expression

After the model was established, the total flavonoid and estrogen groups showed significant differences in the cathepsin K mRNA expression compared with the sham-operated and normal groups ( $P < 0.01$ ).

After three months, the total flavonoid groups showed significant differences compared with the estrogen group ( $P < 0.05$ ) and the sham-operated and normal groups ( $P < 0.01$ ). Significant differences were also observed at  $P < 0.05$ . These findings indicated that *Drynaria* total flavonoids could reduce cathepsin K mRNA expression. Among the total flavonoid groups, the most significant inhibitory effect was found in the high-dose group, but the effect was observed to a lesser extent in the estrogen group.

After six months, the total flavonoid groups showed significant differences compared with the estrogen group ( $P < 0.05$ ) and the sham-operated and normal groups ( $P < 0.01$ ). Significant differences were also observed at  $P < 0.05$ , in which the high-dose group was significantly different to a moderate extent. The results are shown in Figure 2.

**Maximum bending load**

The maximum bending load of the tibial shaft was measured using a three-point bending test. The results are shown in Figure 3.

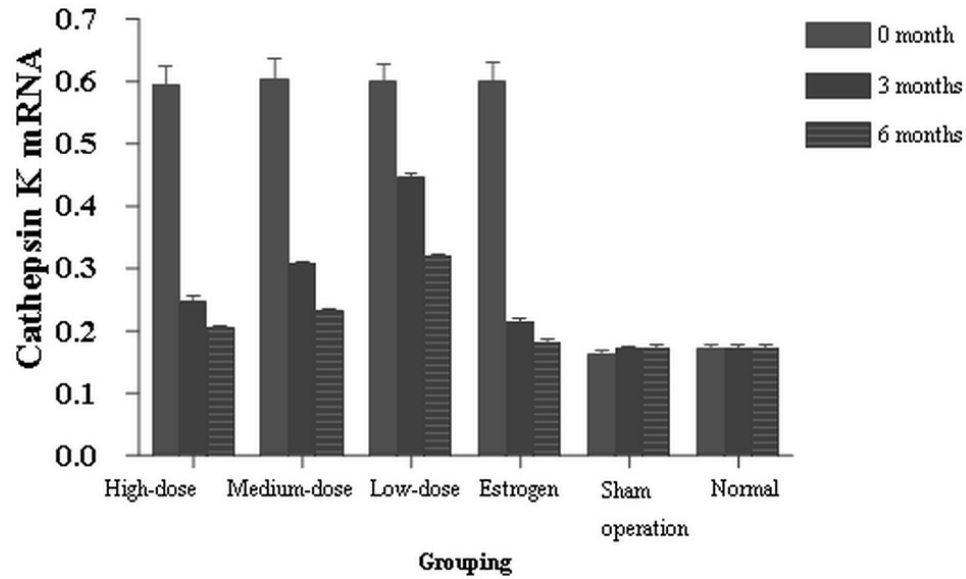


Figure 2. Cathepsin K expression in different groups at different time points (Cathepsin K/ $\beta$ -actin).

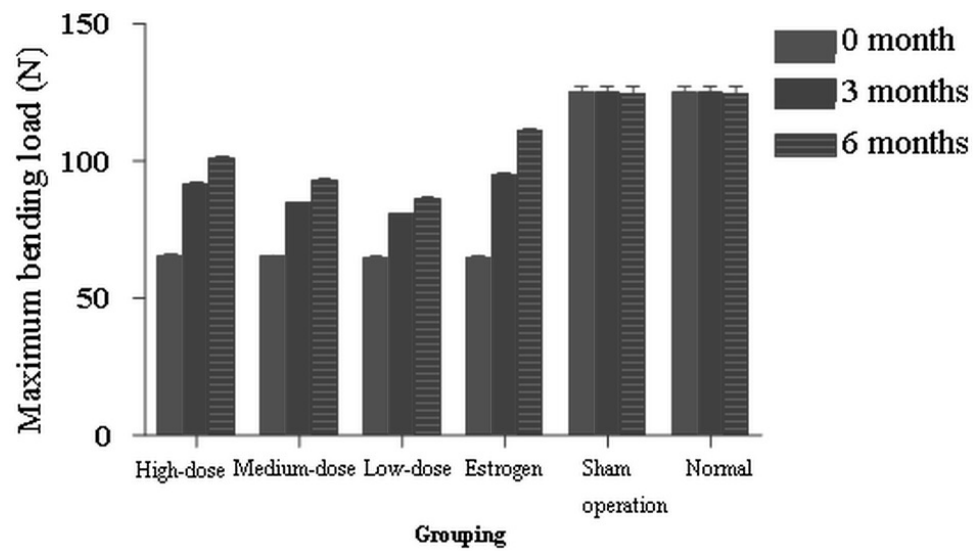


Figure 3. Maximum bending loads in different groups at different time points.

After the model was established, the total flavonoid and estrogen groups showed significant differences in the maximum bending load compared with the sham-operated and normal groups ( $P < 0.01$ ), but no significant difference was found between the flavonoid groups and the estrogen group ( $P > 0.05$ ), indicating that the osteoporotic rat models were successfully established.

After three months, the total flavonoid groups showed significant differences compared with the estrogen group ( $P < 0.05$ ) and the sham-operated and normal groups ( $P < 0.01$ ). These results indicated that *Drynaria* total flavonoids could increase the maximum bending load of the tibial shaft and intensify the BMD. The high-dose group exhibited a significant effect to a lesser extent than the estrogen group ( $P < 0.05$ ).

After six months, the total flavonoid groups showed significant differences compared with the sham-operated and normal groups ( $P < 0.01$ ). The high-dose group showed a significant effect to a lesser extent than the estrogen group ( $P < 0.05$ ). The three total flavonoid groups also showed significant differences ( $P < 0.05$ ), in which the high-dose group was moderately significant.

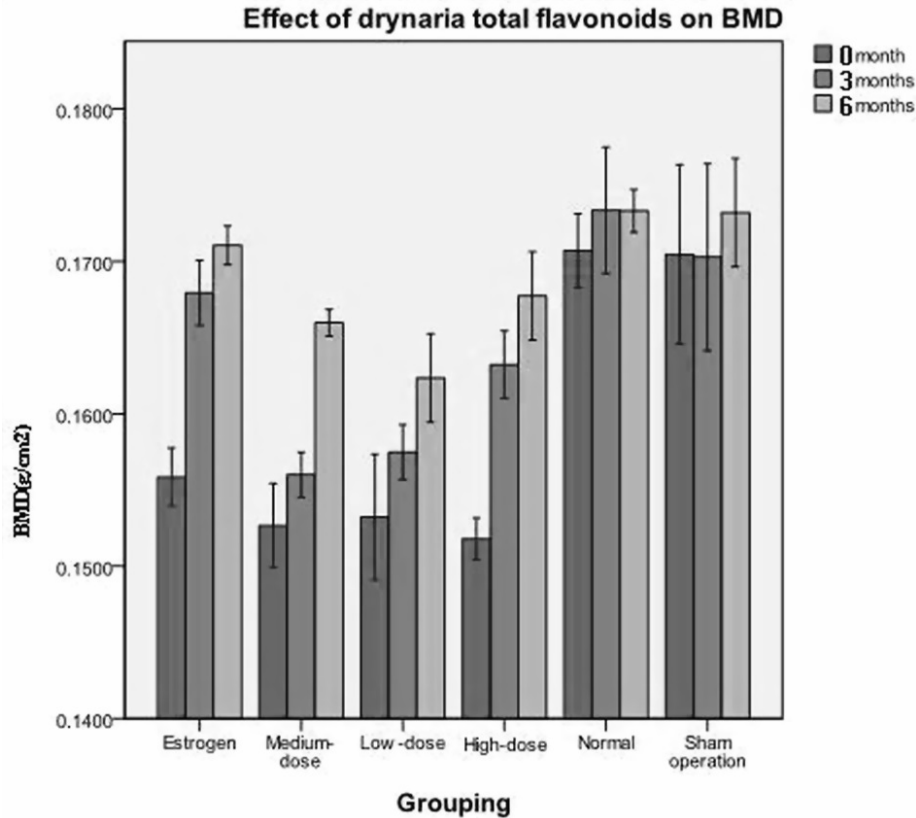
## BMD

After the model was established, the total flavonoid and estrogen groups showed significant differences in terms of BMD compared with the sham-operated and normal group ( $P < 0.05$ ). The BMDs decreased by varying degrees 10 weeks after ovariectomy, indicating that the models were successfully established.

After three months, the total flavonoid and estrogen groups showed significant differences in terms of BMD compared with the sham-operated and normal groups ( $P < 0.05$ ). The BMDs in these groups also showed significant differences compared with those at 0 months ( $P < 0.05$ ). These results indicated that the BMDs in the total flavonoid and estrogen groups were increased by a certain degree after the three-month drug treatment with *Drynaria* total flavonoids and estrogens. These drugs also affected osteoporosis in the ovariectomized rats. The total flavonoid groups showed a less significant effect ( $P < 0.05$ ) than the estrogen group. Among the total flavonoid groups, the high-dose group elicited the highest significant effect, in which the high-dose *Drynaria* total flavonoids had a better effect on BMD than the medium- or low-dose treatment ( $P < 0.05$ ). No significant difference was found between the medium-dose group and the low-dose group ( $P > 0.05$ ).

After six months, the total flavonoid and estrogen groups showed significant differences compared with those after three months ( $P < 0.05$ ), indicating that the BMDs in the ovariectomized rats in these groups were further increased, but these groups still showed significant differences compared with the normal and sham-operated groups ( $P < 0.05$ ). This result indicated that the BMDs in the ovariectomized rats did not return to normal, but osteoporosis was partially improved. Significant differences were observed between the estrogen group and the total flavonoid groups ( $P < 0.05$ ), in which *Drynaria* total flavonoids showed a less significant effect on osteoporosis than estrogens. The results are shown in Figure 4.

The results showed that cathepsin K mRNA expression decreased in the tibial metaphysis and the maximum bending load of the tibial shaft increased after three and six months. Thus, the total flavonoids of *Rhizoma Drynariae* could inhibit the expression of cathepsin K mRNA to prevent bone resorption and increase bone strength.



**Figure 4.** BMDs in different groups at different time points.

## DISCUSSION

Cathepsin K, an endoproteinase that is localized mainly in the lysosomes, is one of the most important enzymes that is expressed highly and selectively in osteoclasts (Lane, 2006). Cathepsin K is located at the ruffled border of the osteoclasts where bone resorption is active, but it is not expressed in the osteoblasts or in the osteocytes; gene silencing can inhibit cathepsin K expression; thus, bone resorption and collagen degradation can be inhibited (Selinger et al., 2005). The overexpression of cathepsin K can increase cancellous bone turnover, suggesting that cathepsin K may be an important target and functions as a highly specific and sensitive biomarker in bone resorption treatment (Holzer et al., 2005).

In humans, variations in cathepsin K expression can cause pycnodysostosis, which may ultimately cause osteoporosis and lead to an increase in osteopsathyrosis (Fratzl-Zelman et al., 2004). Pycnodysostosis is characterized by the same phenotype in rats and humans, in which cathepsin K removal in rats and a lack of cathepsin K in humans cause pycnodysostosis; in both cases, bone resorption is seriously damaged and can lead to osteopetrosis (Duque and Troen, 2008). Therefore, cathepsin K is a potential target in the treatment of pycnodysostosis.



Cathepsin K also elicits different effects on different bones. In particular, the bones with high bone metabolism, such as the long bone and the vertebral column, are more significantly affected than those with low metabolism, such as the cranium and the epiphyses. The cathepsin K mRNA expression is higher when increased amounts of active enzymes are secreted near the resorption lacunae in the osteoclasts. This finding is consistent with that in a previous study, in which cathepsin K mRNA and protein expression were high in human and rat osteoclasts (Troen, 2004).

Drynaria total flavonoids are extracted from the dry rhizomes of *Drynaria fortunei* (kunze), and the major active component is naringin dihydroflavonoid (The People's Republic of China Pharmacopoeia Committee, 2000). These flavonoids have multiple functions, such as increasing bone mass, improving bone quality, maintaining the integrity of bone microstructures, inhibiting articular cartilage lesions, inducing analgesia, reducing blood viscosity, promoting osteoblast proliferation and differentiation, promoting bone formation, intensifying bone mineralization, improving the disorders of bone regulatory hormones, and inhibiting bone resorption. The safety of the use of these flavonoids has been demonstrated according to Rieman et al., 2001; Vääräniemi et al., 2004; Xie et al., 2005; Zhao et al., 2005. These flavonoids also have an important function in preventing post-menopausal osteoporosis and the osteoporosis that is caused by ovarian hypofunction (Jeong et al., 2003; Gu et al., 2006).

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