

Original Article

Pycnogenol® treatment inhibits bone mineral density loss and trabecular deterioration in ovariectomized rats

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Abstract: Context: Pycnogenol® extracted from French maritime pine bark (*Pinus pinaster* Ait. subsp. *atlantica*) is functional for its antioxidant activity. Objective: To investigate the effects of Pycnogenol® on bone mineral density (BMD), trabecular microarchitecture and bone metabolism in ovariectomized (OVX) rats. Materials and methods: Thirty Sprague-Dawley rats were randomized into 3 groups: SHAM group (sham-operated rats), OVX group (OVX rats), and treatment group (OVX rats supplemented with 40 mg/kg Pycnogenol® by oral gavage). Serum levels of procollagen type I N-terminal propeptide (PINP), alkaline phosphatase (ALP) and minerals were detected at the end of 9 weeks of gavage. Deoxypyridinoline/creatinine (DPYD/Cr) and N-telopeptide of type I collagen/creatinine (NTX/Cr) rate in urine were also calculated. Left femora were collected for BMD determination, and the right distal femora were made into undecalcified specimens for histomorphometry analysis. Results: At the end of study, PINP level, DPYD/Cr and NTX/Cr rate were significantly increased, and femoral BMD were dramatically decreased in OVX group compared with SHAM group ($P < 0.01$) while serum minerals and ALP concentrations showed no significant difference. The treatment group had dramatically decreased biomarkers and increased BMD than OVX group ($P < 0.01$). Histomorphometry analysis showed worse bone microarchitecture parameters in the OVX group compared with the SHAM group which were significantly improved in the treatment group compared with the OVX group ($P < 0.01$). Discussion and conclusion: Pycnogenol® (40 mg/kg) can inhibit aggravated bone resorption, prevent BMD loss, and restore the impaired trabecular microarchitecture in OVX rats after 9-week-intervention.

Keywords: Pycnogenol®, ovariectomy, histomorphometry, bone mineral density, bone turnover biomarkers, oxidative stress

Introduction

Osteoporosis, characterized by bone loss and degenerative changes of bone microarchitecture, can lead to an increased risk of bone pain, deformity, or fracture. Patients with osteoporosis in postmenopausal women are twice as many as those in men due to lower peak bone mass, estrogen deficiency and reduction of bone mineral density (BMD) [1]. In Ovariectomized (OVX) rats, bone metabolism biomarkers, e.g. PINP (procollagen type I N-terminal propeptide) for bone formation and deoxypyridinoline (DYPD) for bone resorption, significantly increase to induce bone loss as a result of estrogen deficiency [2, 3]. OVX rats model has been demonstrated to be one of the best experimental models for studying osteopenic changes similar in post-menopausal females and has been widely used to develop new preventive treatments for postmenopausal osteoporosis [4].

Pycnogenol® (PYC), extracted from French maritime pine bark (*Pinus pinaster* Ait. subsp. *atlantica*), is a water-soluble antioxidant composed of procyanidolic oligomers, catechin, epicatechin, taxifoline, and tartaric acid. There are more than 40 functional components in Pycnogenol®, including bioflavonoid, organic acids, and other bioactive ingredients [5, 6]. Antioxidant capacity of Pycnogenol® has been demonstrated in some previous studies, due to its free radical scavenging capacity and antioxidant enzyme activating ability [7-9].

In recent studies, some bioactive compounds such as green tea polyphenols, carotenoid and lycopene could help alleviating bone loss induced by estrogen deficiency in post-menopausal women [10-12]. The common pharmacological activity of these compounds is to scavenge oxygen free radicals [10-12]. It has been demonstrated that oxidative stress is one of the contributing factors to osteoporosis [13].

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Table 1. Comparisons of serum Ca, P, Mg and ALP in OVX group, SHAM group and treatment group (n = 10)

Marker	Model group	Control group	Treatment group
Ca (mmol/L)	2.20 ± 0.16	2.34 ± 0.08	2.22 ± 0.21
P (mmol/L)	1.62 ± 0.24	1.71 ± 0.17	1.82 ± 0.14
Mg (mmol/L)	0.63 ± 0.11	0.52 ± 0.08	0.53 ± 0.12
ALP (IU/L)	3.25 ± 0.19	2.68 ± 0.22	2.92 ± 0.18

Values were expressed as mean ± S.D. Ca, calcium; P, phosphate; Mg, magnesium; ALP, alkaline phosphatase. SHAM, sham-operated; OVX, ovariectomized.

Alleviating oxidative stress has great potential in controlling the incidence and development of osteopenia. Tea polyphenols have been proved to be effective in preventing bone loss because of its antioxidant and anti-inflammatory activities. Pycnogenol®, composed of procyanidolic oligomers (70 ± 5%) and concentrated tea polyphenols (catechin, epicatechin), shows potent antioxidant activity and anti-inflammatory properties [14, 15].

Based on the previous studies, Pycnogenol® can be speculated to have potentially inhibiting effect on osteopenia. In one related research, 30 or 15 mg/kg Pycnogenol® treatment by gavage for 3 months can reduce the bone turnover rate and suppress bone loss in 8-month-old OVX rats [16]. However, different from the previous study, the present study aimed to investigate the preventing effect of 40 mg/kg Pycnogenol® treatment for 9-week on bone loss in 3-month-old OVX rats by detecting the BMD, bone histomorphology and bone turnover biomarkers. The results might provide a theoretical basis for the usage of Pycnogenol® in clinical intervention.

Materials and methods

OVX rats model and Pycnogenol® treatment

Thirty female Sprague-Dawley rats, weighing 220 ± 15 g (aged 3 months), were provided by Department of Laboratory Animal Science, Shanghai Jiaotong University School of Medicine. All the rats were acclimated to the standard clean environmental conditions with 12 h light/dark cycle at temperature of 22 ± 2°C and humidity of 45-65% for 1 week before the experiment. Twenty female rats were subjected to bilateral ovariectomy operation and the other 10 rats underwent a sham operation (SHAM group). One week after surgery, the OVX rats

were randomly assigned into two groups: OVX group and Pycnogenol®-treated OVX group (treatment group). All rats were housed in macrolon cages under the standard clean environmental conditions during 9-week intervention period with *ad libitum* access to water and food. In treatment group, oral administration of Pycnogenol® (40 mg/kg) (GNC, USA) was performed daily through custom-made stomach tubes. This dose was defined based on the GNC recommendation and a previous study [16]. In OVX group, equal amount of distilled water was orally administrated through stomach tubes. Pycnogenol® and distilled water treatment were started from the second week after operation and maintained for 9 weeks. Animals were maintained and processed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Blood and urine collection

At the end of the experiment, 3 mL peripheral blood samples for each rat were collected by cardiac puncture after induction of anesthesia by diethyl ether. Serum was separated by centrifugation (4000 rpm for 20 min at 4°C) and then stored at -20°C for further detecting bone metabolic markers. For urine collection (24 h), rats were placed in metabolic cages at the end of the study with no food but only free deionized water administration. Urine was collected in acid-washed tubes and then frozen at -20°C until required for analysis.

Detection of bone metabolic makers in serum and urine

Plasma calcium (Ca), phosphate (P), magnesium (Mg), alkaline phosphatase (ALP) and urine creatinine (Cr) were measured by colorimetric methods using assay kits (Mabtech, Sweden). The levels of urine DYPD, N-telopeptide of type I collagen (NTX) and serum PINP were determined by corresponding rats ELISA kits (Mabtech, Sweden), respectively. Urine DPYD and NTX excretion rate were expressed as DPYD/Cr and NTX/Cr, respectively. All the measurements were performed using a Cobas-Fara II Clinical Analyzer (Montclair, NJ, USA).

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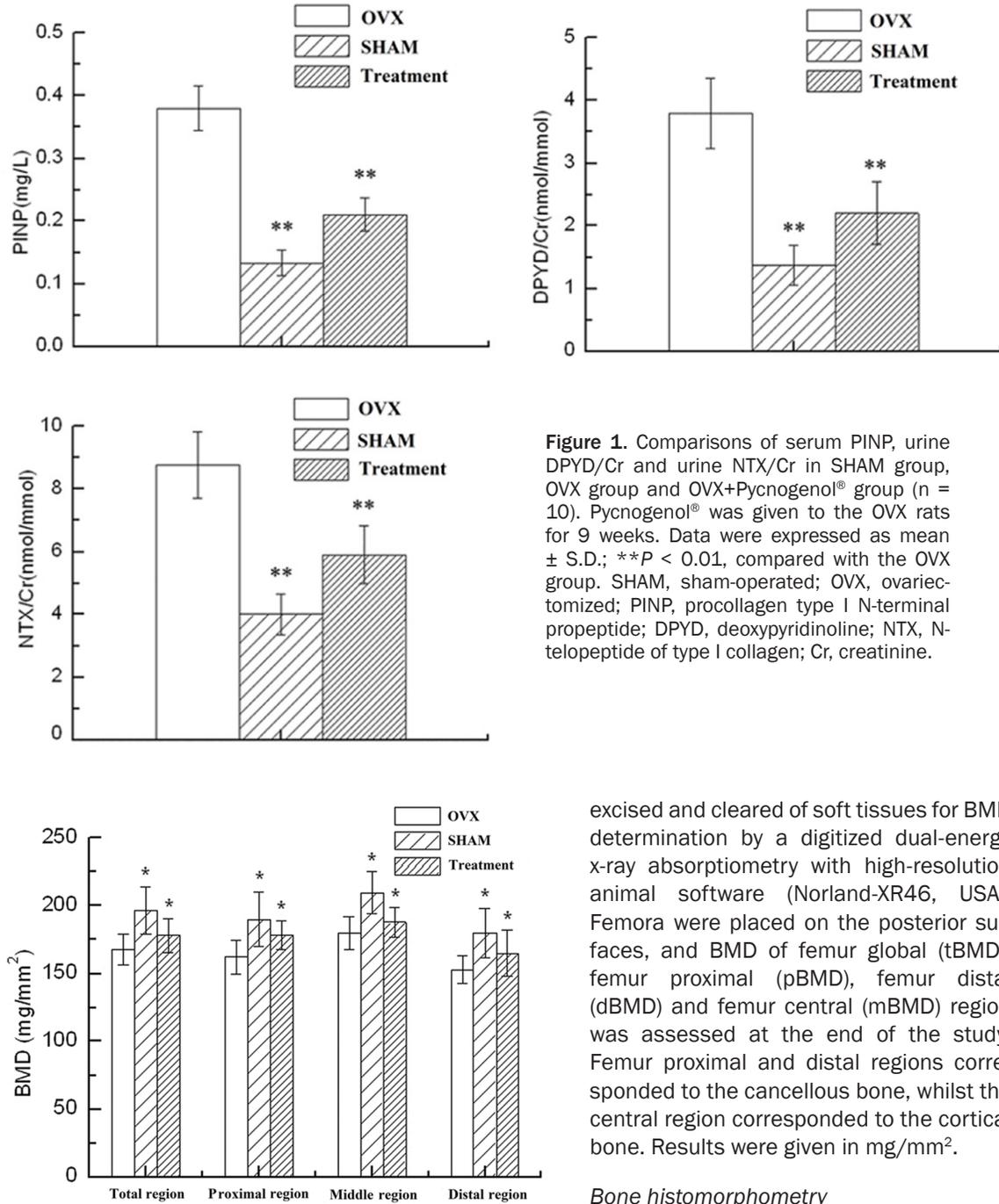


Figure 1. Comparisons of serum PINP, urine DPYD/Cr and urine NTX/Cr in SHAM group, OVX group and OVX+Pycnogenol® group (n = 10). Pycnogenol® was given to the OVX rats for 9 weeks. Data were expressed as mean \pm S.D.; ** $P < 0.01$, compared with the OVX group. SHAM, sham-operated; OVX, ovariectomized; PINP, procollagen type I N-terminal propeptide; DPYD, deoxypyridinoline; NTX, N-telopeptide of type I collagen; Cr, creatinine.

Figure 2. Comparisons of BMD of femur total, femur proximal, femur middle and femur distal region in SHAM group, OVX group and OVX+Pycnogenol® group (n = 10). Pycnogenol® was given to the OVX rats for 9 weeks. BMD of excised left femora was determined. Data were expressed as mean \pm S.D.; * $P < 0.05$, compared with the model group. SHAM, sham-operated; OVX, ovariectomized; BMD, bone mineral density.

excised and cleared of soft tissues for BMD determination by a digitized dual-energy x-ray absorptiometry with high-resolution animal software (Norland-XR46, USA). Femora were placed on the posterior surfaces, and BMD of femur global (tBMD), femur proximal (pBMD), femur distal (dBMD) and femur central (mBMD) region was assessed at the end of the study. Femur proximal and distal regions corresponded to the cancellous bone, whilst the central region corresponded to the cortical bone. Results were given in mg/mm^2 .

Bone histomorphometry

Rats in all groups were injected with 20 mg/kg calcein before sacrifice. The right distal femora of rats in each group were obtained and used as undecalcified specimens for histomorphometry analysis. Samples were fixed in 10% phosphate-buffered formalin (24 h), dehydrated by graded ethanol, and then embedded in methyl methacrylate. Slides with 4 and 8 μm -thicknesses were prepared with a hard tissue microtome (Leica SP 2155, Germany). The 4 μm -thickness

Determination of BMD

At the end of 9 weeks of gavage, all the rats were euthanized. The left femora of rats were

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slides were processed for Von Kossa and Giemsa staining to analyze the femur static parameters: bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, μm), eroded surface to bone surface (ES/BS, %), and osteoclast number (Oc.N, 1/ mm^2). Giemsa staining was employed for bone histological observation, and Von Kossa staining was performed for osteoid observation. The 8 μm -thickness slides were directly processed for dynamic bone histomorphometry analysis including the following parameters: percent osteoid perimeter (O.Pm, %), percent labeled perimeter (L.Pm, %), and bone formation rate per bone volume (BFR/BV, %/yr). Dynamic and static parameters were determined by measuring cancellous bone (secondary spongiosa) 1-4 mm below the distal metaphyseal growth plate of femur using fluorescent or light microscope (Leica DM4000B, Germany). The histomorphometry analysis of images of bone sections was performed using a color pathological image analysis system (Leica, Germany). The nomenclature, symbols and units of bone parameters were described according to the recommendation of American Society for Bone Mineral Research (ASBMR) Nomenclature Committee [17].

Statistical analysis

The statistical analysis was performed by SPSS 13.0. Data were expressed as mean \pm standard deviation (S.D.). Comparisons across groups were performed by one-way analysis of variance with *post-hoc* LSD-*t* test. $P < 0.05$ was considered statistically significant.

Results

Effect of pycnogenol® treatment on serum minerals (Ca, P, Mg) contents and ALP

Comparisons of minerals and ALP content in serum of SHAM group, OVX group and treatment group were shown in **Table 1**. There were no significant differences in serum minerals contents among these three groups. As compared with those in the SHAM and treatment groups, the content of serum ALP was slightly higher in the OVX group ($P > 0.05$).

Pycnogenol® treatment reduced bone turnover markers in OVX rats

Bone metabolic biomarkers in serum and urine were presented in **Figure 1**. The ovariectomy

significantly ($P < 0.01$) increased plasma concentration of PINP as biomarkers of bone formation and urine DPYD/Cr and NTX/Cr as bone resorption biomarkers. However, Pycnogenol® treatment to OVX rats resulted in dramatically reduction in these three bone turnover markers compared with the OVX group. These data suggested that Pycnogenol® treatment might inhibit bone turnover rate that was remarkably elevated under estrogen deficiency.

Pycnogenol® treatment prevented loss of femur BMD due to ovariectomy

Following ovariectomy, OVX rats had significantly ($P < 0.05$) lower tBMD, pBMD, mBMD and dBMD than sham-operated rats in the SHAM group at 10th week after operation (as shown in **Figure 2**). The OVX rats displayed significantly ($P < 0.05$) higher tBMD, pBMD, mBMD, and dBMD in the treatment group as compared with the OVX group, suggesting that Pycnogenol® can prevent the loss of femur BMD caused by ovariectomy.

Pycnogenol® treatment improved trabecular microarchitecture

Trabecular response to Pycnogenol® treatment at the microarchitectural level was quantified. As shown in **Figure 3**, the OVX group had significant microarchitectural deteriorations indicated by remarkably reduced BV/TV, Tb.Th, Tb.N, and BFR/BV values ($P < 0.01$) and increased Tb.Sp, ES/BS and Oc.N values ($P < 0.01$) as compared to the SHAM group. The above indices in treatment group were in the similar range to those in the SHAM group. Pycnogenol® treatment significantly increased the value of L.Pm in treatment group compared with the SHAM group and OVX group, suggesting Pycnogenol® treatment may favor bone formation. Pycnogenol® showed preventive effect on the microarchitectural deteriorations in OVX rats.

Discussion

Estrogen deficiency is a major risk factor for osteoporosis and the OVX rats have been used as the classical animal model for studying postmenopausal osteoporosis [18]. Using this model, this study investigated the effect of Pycnogenol® on osteopenia in OVX rats. Some previous studies have reported the preventative effect of Pycnogenol® on bone loss in OVX animals. In OVX mice, Pycnogenol® treatment prevents BMD loss and deterioration of tra-

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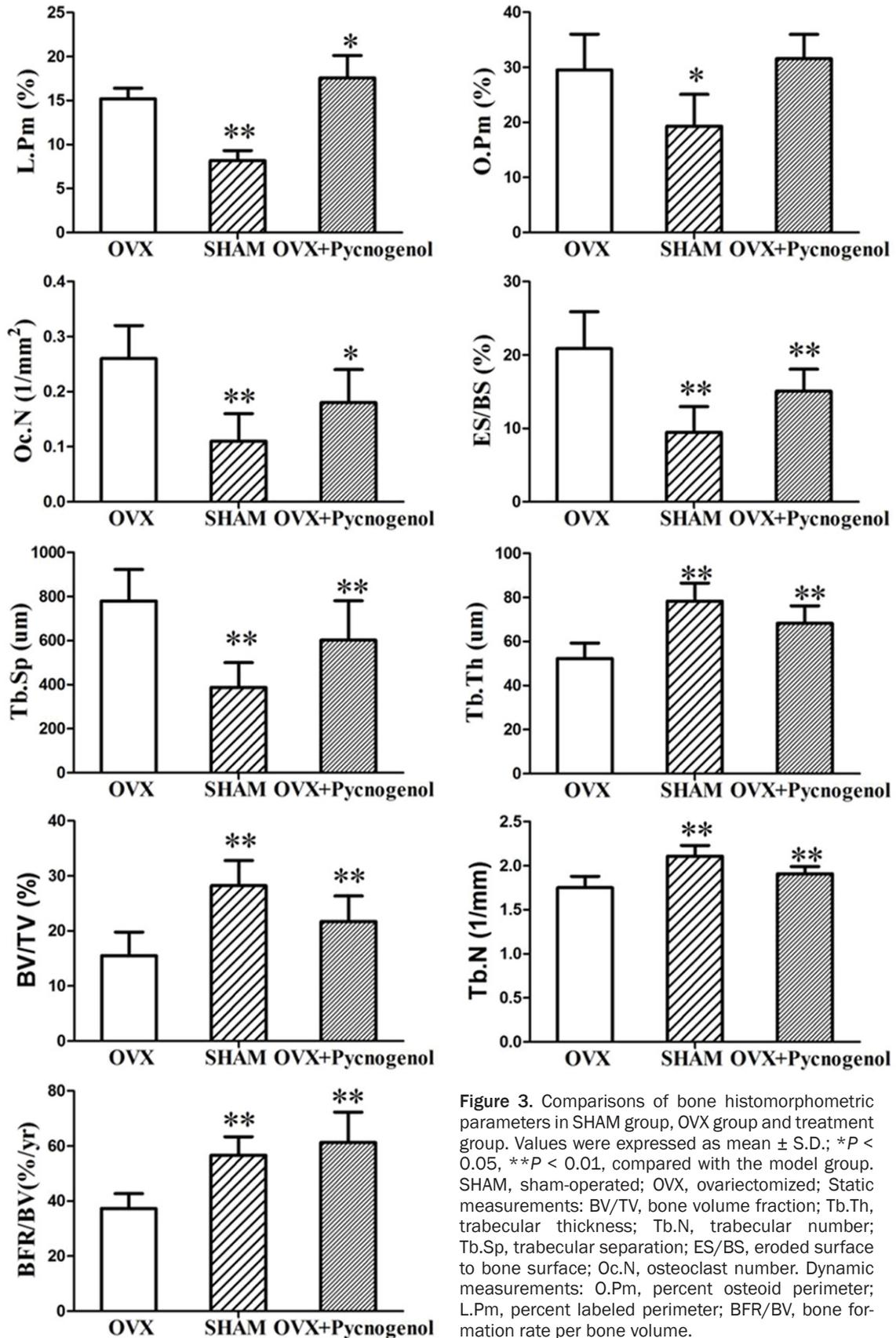


Figure 3. Comparisons of bone histomorphometric parameters in SHAM group, OVX group and treatment group. Values were expressed as mean \pm S.D.; * $P < 0.05$, ** $P < 0.01$, compared with the model group. SHAM, sham-operated; OVX, ovariectomized; Static measurements: BV/TV, bone volume fraction; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; ES/BS, eroded surface to bone surface; Oc.N, osteoclast number. Dynamic measurements: O.Pm, percent osteoid perimeter; L.Pm, percent labeled perimeter; BFR/BV, bone formation rate per bone volume.

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becular architecture by giving water containing 120 mg/L Pycnogenol® for 3 months [19]. In OVX rats, 15 or 30 mg/kg Pycnogenol® administration for 3 months also inhibits BMD loss and protects bone biomechanical strength in 6-month-old rats [16]. However, different from those similar studies, this study used a higher dose of Pycnogenol® (40 mg/kg), younger rats (3-month-old rats) and a shorter intervention time (9-week), which may provide additional information for the clinical usage of Pycnogenol®.

Bone turnover usually increases in OVX rats [20]. The activity of serum ALP shows significant increase in OVX animals [21], but it didn't show significant difference among the three groups in this study. That may be because serum level of bone-specific ALP (BALP) rather than ALP could more effectively reflect bone formation. This study didn't test the serum level of BALP. However, three other bone turnover biomarkers PINP, DPYD/Cr and NTX/Cr were calculated here and showed significant increase in OVX rats. PINP, extracellular catabolite of procollagen type I, is considered as a specific biomarker correlated with collagen I synthesis in new bone formation [22, 23]. DPYD and NTX have been reported as two biomarkers of bone resorption for diagnosis of osteoporosis [24-28], usually expressed as urine DPYD/Cr and NTX/Cr in clinical examination for wiping out the influence of concentration or dilution of urine. Herein, the dramatically elevated levels of serum PINP, urine DPYD/Cr and urine NTX/Cr in OVX group suggested higher bone turnover rate induced by ovariectomy which is similar to postmenopausal osteoporosis [10]. In contrast, oral administration of Pycnogenol® was able to reverse the increase of serum PINP, urine DPYD/Cr and NTX/Cr ratio in the OVX rats, suggesting an inhibition of Pycnogenol® on bone turnover. Besides, results of serum minerals determination showed unchanged levels of serum Ca, P and Mg across groups. This was consistent with a previous study reporting the indiscrimination of serum P and Ca between healthy and osteoporotic postmenopausal women [29]. As the minerals loss is a slow and complex process affected by multiple factors *in vivo*, the levels of serum Ca, P and Mg may be unaffected by ovariectomy.

Besides, in post-menopausal women, the reduction of bone strength and BMD may lead to

an increased risk of fracture due to the osteoporosis [30]. There are several reports demonstrating the occurrence of BMD loss in osteoporosis [31, 32]. BMD is considered as the characteristic parameter in osteoporosis diagnosis. The results of this study showed that the observed decrease in pBMD, mBMD, dBMD and tBMD in OVX rats was partially avoided in the femora of rats treated with Pycnogenol®. Pycnogenol® was supposed to alleviate the symptoms of postmenopausal osteopenia. The decreased BMD of femora in OVX rats may result from the higher bone turnover rate induced by ovariectomy. Thus, it can be speculated that the preventive effect of Pycnogenol® on BMD loss may be due to the inhibition of bone turnover rate.

Moreover, ovariectomy is typically linked with deterioration in trabecular structure [33-35]. Bone mass, microstructure and dynamic metabolism can be detected by bone histomorphometry. Bone trabecular architecture detected by bone histomorphometry can reflect bone metabolism and might contribute to weakened bone strength by its tiny injury. In one related study, the reduced Tb.Th and Tb.N and increased Tb.Sp in trabecular architecture might lead to vulnerability to compression fracture of spine [36]. In this study, changes of characteristic parameters of trabecula in OVX rats were in similar to those of compression fractured trabeculae, suggesting a reduction in biomechanical properties and a higher risk of femora fracture. Pycnogenol® treatment to OVX rats on the other hand resulted in an improvement in the parameters of BV/TV, Tb.Th, Tb.N, Tb.Sp, ES/BS, Oc.N and BFR/BV, suggesting the protective effect of Pycnogenol® treatment on bone microstructure and thus resulting in the decreased risk of fracture. Notably, Pycnogenol® treatment did not reverse the dramatic increases in L.Pm and O.Pm which reflect bone mineralization in OVX rats, suggesting a supporting effect of Pycnogenol® on bone formation. This was inconsistent with the serum PINP presentation whose level was normalized in treatment group. Contradictory emergence of PINP associated with collagen I synthesis and L.Pm and O.Pm related to bone mineralization may imply a complex effect of Pycnogenol® on osteoclast activity. But, undoubtedly, Pycnogenol® treatment had a suppression effect on aggravated bone resorption induced by ovariectomy.

In addition, bone loss in post-menopausal women is usually considered to be caused by an increasing activity of osteoclast [37]. It is now recognized that bone metabolism is in a dynamic balance co-regulated by osteoclast responsible for bone resorption and osteoblast in charge of bone formation in the procedure of bone development. Osteoclasts can express matrix metalloproteinase (MMP) to modulate the remodeling of bone matrix (including collagen type I) [38]. However, MMP activity can be inhibited by Pycnogenol®, thereby probably preventing bone matrix from being abnormal decomposition [39]. Thus, it can be speculated that the protective effect of Pycnogenol® on osteoporosis mainly related to its regulation on the expression of bone-specific genes such as MMP, which needs further study to illuminate the deeper mechanism by molecular biology methods.

Notably, bone loss associated with estrogen deficiency involves several mechanisms in which oxidative stress plays a critical role [18, 40]. The *in vivo* and *in vitro* studies indicated that excessive free radicals generated in the bone cells enhanced bone resorption and reduced bone mineralization [41, 42]. Rutin, quercetin and resveratrol, three widely used antioxidants, are reported to inhibit ovariectomy-induced bone loss in rat model [43-45]. Pycnogenol® is an excellent source of free radical scavengers as described in the introduction. Thus, the protective effect of Pycnogenol® may be due to its components which function as antioxidants. However, further studies are needed to elucidate the mechanisms by which Pycnogenol® prevents bone loss induced by ovariectomy.

In summary, we provided an evidence that 9 weeks of 40 mg/kg Pycnogenol® treatment to osteopenic rats resulted in a remarkable decrease in bone turnover biomarkers including serum PINP, urine DPYD/Cr and NTX/Cr ratio, and an improvement of BMD of femora as well as the trabecular microstructure. These data indicated that 40 mg/kg Pycnogenol® treatment prevented bone loss and repaired bone microarchitecture probably through alleviating the enhanced bone resorption in OVX rats after 9-week-intervention. This study may establish basis of initiating clinical trials with Pycnogenol® in menopausal women.

Disclosure of conflict of interest

None.

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