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Original Article

Relationship between osteogenesis and angiogenesis in ovariectomized osteoporotic rats after exercise training

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Abstract: To reveal the role of exercise training in regulating osteogenesis and angiogenesis in rats after ovariectomy (OVX), then further examine the relationship between osteoporosis (OP) and local blood supply. Ninety-six rats were randomly assigned to OVX, OVX + exercise (OVX + Exe), sham operation (CON), and sham + exercise (CON + Exe) groups. Bone mineral density (BMD) was determined by dual-energy X-ray absorptiometer (DXA). Microarchitecture and angiogenesis of the femoral condyles were measured by micro-cumputed tomography (micro-CT). The femoral artery was separated for evaluating the vasodilation and endothelial nitric oxide synthase (eNOS) gene expression. BMD was markedly lower in the OVX than CON and CON + Exe groups. The trabecular bone integrity was better in the OVX + Exe than OVX group, but BMD showed no significant difference. The bone parameters indicated OP characteristics in rats after OVX, parts of them were relatively improved after exercise training. Acetylcholine-induced vessel vasodilation was enhanced by exercise training, but blocked by a NOS inhibitor. eNOS gene expression in femoral vessels showed a downward trend after OVX, and a upward trend in CON group, but seemed not to be affected by exercise training. Angiogenesis in the femur was lower in the OVX group than the CON group, but substantially better in the OVX + Exe than OVX group. Exercise training enhancing acetylcholine-induced femoral artery vasodilation and bone angiogenesis improve the blood supply of bone to facilitate osteogenesis in the OP site and therefore delay the progression of OP after menopause.

Keywords: Osteoporosis, ovariectomy, exercise training, osteogenesis, angiogenesis

Introduction

Osteoporosis (OP) is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of the skeleton leading to enhanced bone fragility and an increased risk of fracture. It is generally thought to be caused by an imbalance between osteogenesis and bone resorption [1]. A reduction in BMD with an increase in the likelihood of falls results in a high incidence of OP fractures in the older population [2]. OP-related fractures are a major cause of morbidity and disability in this population [3].

Research indicates that exercise can reduce falls, fall-related fractures, and risk factors for low BMD [4]. Additionally, exercise can reduce bone resorption, facilitate osteogenesis, offset aging-related BMD decline, and improve BMD [5], thereby increasing the BMD of the lumbar spine and femoral neck in older individuals [6]. Therefore, exercise training may reduce OP-related morbidity and mortality [7].

Exercise also plays a major role in angiogenesis. Physical exercise can increase the number of endothelial progenitor cells, suppress neointimal formation, and enhance angiogenesis [8]. Moreover, angiogenesis is considered to be an adaptive response to repeated exercise through gene expression of vascular endothelial growth factor (VEGF) and release of endothelial progenitor cells [9]. Exercise plays a positive role in bone metabolism, including stimulation of cell proliferation and differentiation, increases in BMD and bone strength, inhibition of bone resorption, and promotion of VEGF-mediated angiogenesis in bone remodeling [10]. Holstein et al. [11] found that exercise promoted healing of bone defects and stimulated angiogenesis during bone repair in mice with bone defects.

Therefore, exercise can promote bone angiogenesis.

The development of OP in postmenopausal women due to estrogen withdrawal is a global public health problem [12]. It has been found that postmenopausal women with exercise have less BMD loss in the lumbar spine and hips than do postmenopausal women without exercise. Therefore, exercise may become a safe and effective way to improve BMD in postmenopausal women [13]. We speculate that exercise may stimulate osteogenesis, inhibit bone resorption, and promote angiogenesis to preserve BMD, thereby influencing the progression of OP. However, few studies have investigated the relationship of exercise with osteogenesis and the local blood supply in postmenopausal OP, or the role of exercise in regulating the interaction between the latter two factors. In the present study, we simulated the menopausal state of women using rats after ovariectomy (OVX). The effect of exercise training on various bone parameters and vascular function of rats with OP was assessed. Further, the relationship between OP and the local blood supply was examined.

Materials and methods

Experimental design and grouping

Ninety-six 7-month-old female non-pregnant Sprague-Dawley rats weighing approximately 250 g were randomly assigned to four groups: OVX, OVX + exercise (OVX + Exe), sham-operated (CON), and sham + exercise (CON + Exe). All animals were raised in the SPF Animal Breeding Center of Soochow University with free access to sterilized pellet feed (calcium, 0.95%; phosphorus, 0.67%) and water. The room temperature was controlled at approximately 22°C, and the relative humidity was approximately 56%. Conventional ultraviolet disinfection and regular ventilation were performed.

Rats in the OVX group were anesthetized by ether inhalation. After rigorous disinfection, an approximately 1-cm-long longitudinal incision was made 2 cm above the posterior iliac crest and 1 cm from the spine. The skin, subcutaneous tissue, and muscle were incised to expose the abdominal cavity. Both ovaries were excised; the stump was ligated using silk thread and the incision closed with layer-by-layer suturing. After complete removal of both ovaries, the skin was disinfected with penicillin powder and then sutured. In the CON group, the same surgical procedure was followed to remove a small amount of adipose tissue surrounding the ovaries, without excision of the ovaries. After surgery, the animals were placed into cages and allowed free activity and access to food. Penicillin was administered for 3 days by intramuscular injection. Significant atrophy of the uterus was used as evidence of the success of OVX.

One week after OVX, rats in the CON + Exe and OVX + Exe groups started physical exercise. In the first week of exercise (the training period), the training parameters were as follows: 5 days of treadmill at 15 m/min for 15 min/day. On day 8, the speed of the treadmill was maintained at 15 m/min and the duration of exercise was increased to 25 min. The duration of exercise then increased by 5 min every day and reached 45 on day 12. Thereafter, the treadmill exercise continued at 15 m/min for 45 min/day, 5 days per week, for a total of 6 weeks.

After completion of the exercise, six randomly selected rats from each group were sacrificed, and the ipsilateral femur was removed for hematoxylin and eosin (H&E) staining. Next, six randomly selected rats from each group were anesthetized and the ipsilateral femoral artery was immediately separated for the following in vitro experiments. 1) Acetylcholine was added to evaluate vasodilation. 2) N'-nitro-arginine methyl ester HCl solution (L-NAME, a nitric oxide synthase [NOS] inhibitor) was added to assess vascular endothelial signaling pathways. 3) Polymerase chain reaction (PCR) was performed to measure endothelial NOS (eNOS) expression.

Next, six randomly selected rats from each group were anesthetized before femoral artery perfusion, and angiogenesis in the femur was evaluated by micro-computed tomography (micro-CT). Finally, the last six rats in each group were anesthetized, and the femur was taken to analyze the BMD and microarchitecture of the femoral condyles.

H&E staining of bone tissues

The distal femur was fixed in 4% paraformaldehyde in a refrigerator at 4°C for 48 hours and

then decalcified with a fast decalcification solution for 24 hours. The endpoint of decalcification was determined as softening of the bone that can be pierced with a pin. The decalcification solution was then removed and the sample was washed with normal saline, dehydrated in an ethanol gradient, embedded in paraffin, and sliced into 4-μm-thick sections. Sectioned specimens were deparaffinized with xylene and washed with an ethanol gradient to distilled water. After H&E staining, the specimens were dehydrated, cleared, and mounted following conventional procedures. Tissue sections of the distal femur of rats were examined under light microscopy.

BMD measurement

The femoral specimens were thawed and rewarmed at room temperature. BMD was measured on a dual-energy X-ray absorptiometer (QDR Discovery A; Hologic Inc., Bedford, MA, USA). The machine's software dedicated to small animals was used (Regional High Resolution version 4.76; Hologic QDR Discovery A), and the same scanning measurement was made at the same fracture site in the contralateral femur. Each specimen was repeatedly measured three times. The coefficients of variation between and within groups were <2% in our laboratory.

Micro-CT and three-dimensional reconstruction and morphometric analysis of the femur

The microarchitecture and morphology of the femoral condyle specimens were examined by micro-CT (GE eXplore Locus SP MicroCT; GE Healthcare Technologies, Waukesha, WI, USA). The isolated femoral specimens were properly fixed on a micro-CT scan bed. The scanning method was as follows: 45 μm, 24 R, 18 min. The parameters were set as follows: tube voltage, 80 kV; tube current, 450 μA; scan mode, 360°C rotation; scan time, 18 min; average frame number, 1; angle gain, 0.5; exposure time, 400 ms; detector assembly mode, 2 × 2; and voxel resolution, 45.0 × 45.0 × 45.0 μm. The Fluo mode was used to scan the distal portion of the femur. Meanwhile, a standard phantom was scanned to calibrate the CT values.

After the scan, the CT values were manually calibrated and the overall structural reconstruction was completed in the scanning area using the isotropic voxel resolution of 45.0 × 45.0 × 45.0 μm. We chose 1.2-mm-thick bone tissue of the distal femur at a distance of 0.8 mm from the distal end of the growth plate as the region of interest. Cancellous bone in the region of interest was chosen for three-dimensional (3D) visualization. Quantitative analysis was performed using MicroView 2.0 + ABA software (GE eXplore Locus SP MicroCT; GE Healthcare Technologies). Parameters of 3D structural analysis included the bone volume fraction (BV/TV, %), trabecular bone pattern factor (Tb.PF), mean trabecular thickness (Tb.Th), mean trabecular separation (Tb.Sp), mean trabecular number (Tb.N), and structural model index (SMI).

Microfilangiography

Microfil contrast agent (Flow Tech, Carver, MA, USA) was mixed proportionally according to the specification of the product. The contrast agent comprised 42% Microfil MV-122, 53% diluent, and 5% fixative. After the selected rats were anesthetized, the thoracic cavity was opened and perfusion-fixation of the ascending aorta was applied using 10% formalin solution through the left ventricle for 30 min. Next, the abdominal cavity was opened and contrast agent was infused through the abdominal aorta (above the femoral artery branches), allowing the contrast agent to be distributed in the main nutrient vessels of the femur and their branches. Filling of the hepatic or intestinal mesangial capillaries with the yellow contrast agent indicated successful perfusion. After completion of the contrast agent perfusion, the animals were stored in a refrigerator at 4°C overnight. The femur specimens were then further fixed in 10% formalin solution for approximately 1 week and decalcified before micro-CT scanning.

Determination of acetylcholine-induced vasodilation rate in secondary branches of femoral artery

After the rats had been anesthetized, the femur was taken and the abdominal cavity was simultaneously opened to fully expose the abdominal aorta and its both sides. The branches of the abdominal aorta on both sides and the surrounding tissue were taken together (without dragging) and placed in chilled oxygenated Krebs-Henseleit solution. Tissue surrounding the arteries was removed under microscopy.

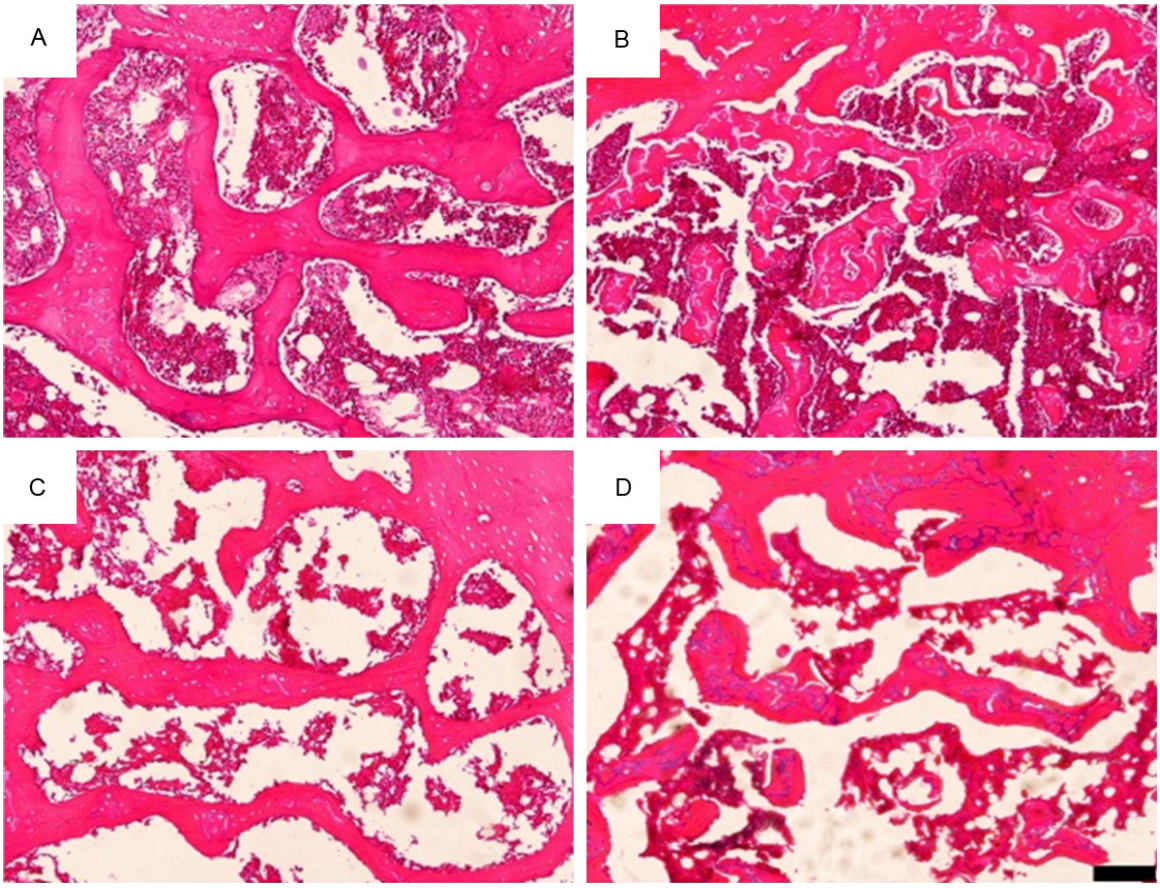

Figure 1. The results of H&E staining of femoral tissues of four groups.

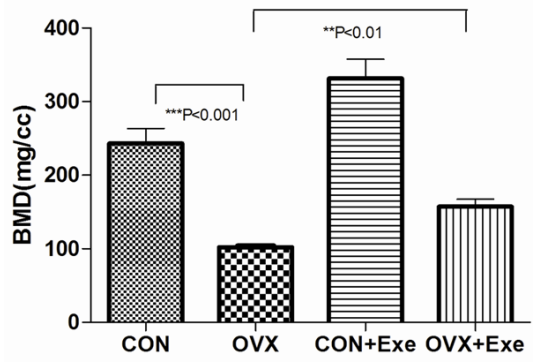

Figure 2. The results of BMD of the four groups.Values are means ± SD of 24 mice in each group. Values are means ± SD of 24 mice in each group. ***, P<0.001 versus CON group, **, P<0.05 versus OVX + Exe group.

The separated and clean secondary femoral arteries were cut into 3- to 5-mm vascular rings. A hook was passed through the blood vessels and connected to a fixing device of the tension transducer. The blood vessels were placed in a bath containing the Krebs-Henseleit solution at a constant temperature of 37°C and continuously aerated with a mixed gas of 95% oxygen + 5% carbon dioxide. The preload of the specimens was adjusted from 0.6 to 0.7 g for 60 min of equilibrium, and the nutrient solution was replaced once every 15 min. After stabilization, a phenylephrine HCl solution was slowly added along the bath wall. At peak contraction of the vascular rings, the NOS inhibitor L-NAME and the placebonormal saline (NS) were added for 3 to 5 min of stabilization. An acetylcholine solution was then added sequentially from a low to high concentration, and every minimum tension was recorded. The vasodilation rate was calculated as follows: vasodilation rate (%) = (maximal contractility of normal blood vessel - minimal contractility after drug administration)/maximal contractility of normal blood vessel × 100.0%. We recorded the preload, the maximal vascular tension after administration of phenylephrine HCl, and the minimal vascular tension after administration of varying concentrations of acetylcholine solution (final concentrations: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}

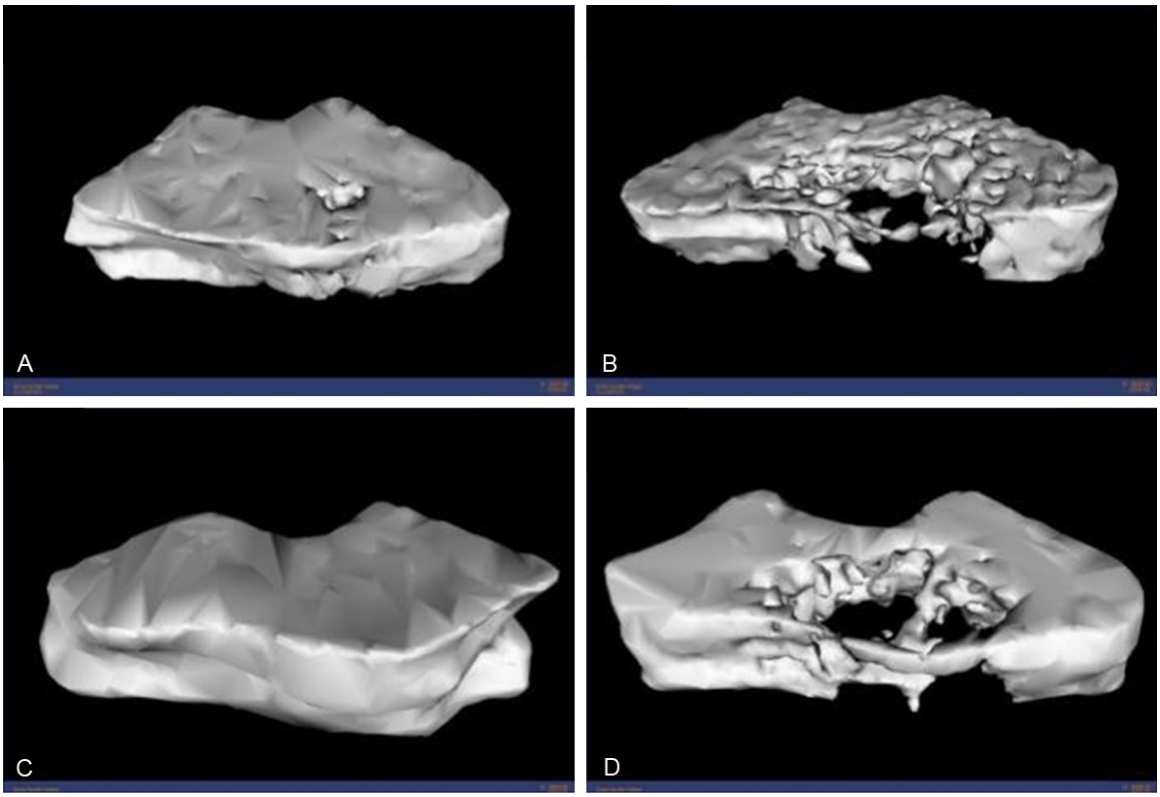

Figure 3. Micro-CT images of three-dimensional reconstruction of the rat femurs.

Table 1. Bone parameters of the rat femur

Group	Bone volume fraction (%)	Trabecular thickness (um)	Trabecular number (N/mm^3)	Trabecular separation (um)	Trabecular bone pattern factor (mm^2/mm^3)	Structure model index (SMI)
sham	35.2±5.4	127.9±16.5	4.41±0.56	246.3±30.9	14.2±2.4	1.15±0.44
Model	6.9±3.2**	86.7±18.6	1.57±0.08**	644.2±32.5**	15.5±2.7	1.39±0.51
sham + Exe	55.4±7.4§§	255.2±39.37§§	8.27±1.12§§	131.0±18.8§§	9.30±1.47	1.12±0.44
Model + Exe	9.2±1.6$^{\#\#}$	121.0±22.9$^{\#}$	1.59±0.07$^{\#\#}$	634.8±28.0$^{\#\#}$	14.38±2.08$^{\#}$	1.56±0.25

Values are means ± SD of 24 mice in each group. n=6. **P<0.01 vs sham group; $^{\#}$P<0.05 vs sham + Exe group. $^{\#\#}$P<0.01 vs sham + Exe group. §§P<0.01 vs sham group.

mol/L). The percentage rate of drug-induced vasodilation was calculated and comparatively analyzed.

Real-time PCR

A portion of the femoral vessels obtained from the above experiments were crushed immediately ex vivo and placed in 1.5-mL RNase-free centrifuge tubes. One milliliter of Trizol agent (Tiangen, Beijing, China) was added to each tube, and all tubes were kept at room temperature for 5 min before adding 0.2 mL of chloroform. The mixture was gently oscillated and then centrifuged at 4°C (12,000 rpm × 10 min). The upper aqueous phase of the solution was transferred into a new 1.5-mL microfuge tube. An equal volume of isopropanol (0.5 mL) was added, and the tube was inverted to mix the contents and then allowed to stand at room temperature for 15 min. The mixture was then centrifuged and the supernatant was discarded. The reminder was washed with 1 mL of 75% ethanol and then dried. The extracted RNA was dissolved with diethylpyrocarbonate water.

Total RNA was reverse-transcribed using a reverse transcription kit (Takara, Dalian, China).

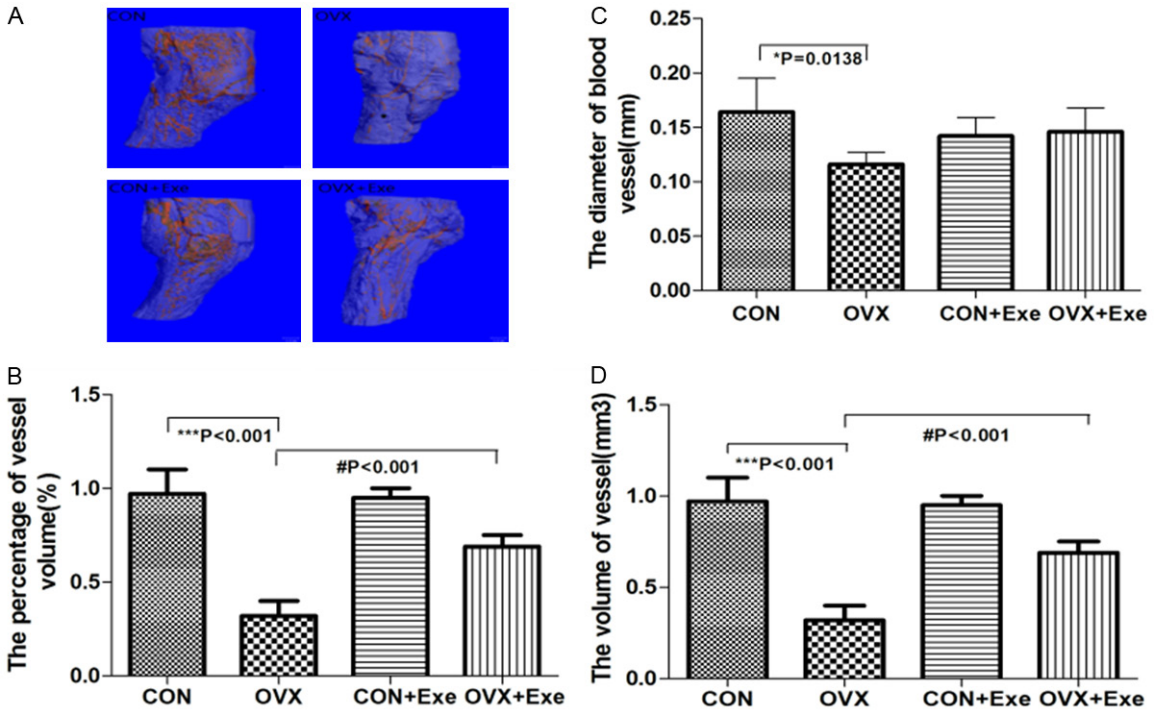

Figure 4. Micro-CT reconstruction and Parameters of femoral artery. A. Micro-CT reconstruction of femoral artery of four groups. B. The percentage of vascular volume in the four groups. C. The diameter of blood vessel in the four groups. D. The volume of vessel in the four groups. Values are means ± SD of 24 mice in each group. ***, P<0.001 versus CON group, #, P<0.001 versus OVX + Exe group.

A 20-μL reaction contained 4 μg total RNA sample, 4 μL 5 × RT buffer, 1 μL PrimerScript RT enzyme mix, and 1 μL RT primer mix. The reaction was first incubated at 37°C for 15 min and then heated at 85°C for 5 s to deactivate the reverse transcriptase. RT-PCR was performed to measure NOS and mRNA expression in the secondary branches of the femoral artery. A 20-μL reaction contained 5 μL template, 0.5 μL each of the upstream and downstream primers, 13 μL SyberGreen mix, and 1 μL deionized water. The PCR program was run as follows: pre-denaturation, 95°C × 30 s; degeneration, 95°C × 5 s; annealing, 60°C × 20 s; and extension, 72°C × 30 s (40 cycles in total).

The primers used were as follows: 5'AGGAAGTAGCCAATGCAGTGAA3' and 5'AGCCATACAGGATAGTCGCCTT3'.

Statistical analysis

Datas are presented as mean ± standard deviations. One-way analysis of variance (one-way ANOVA) was performed using SPSS 19.0 software, with P<0.05 indicating statistical significance.

Results

Numbers of experimental animals

Ninety-six rats were assigned to 4 groups. No animals died within the observation period. No animals had significant complications, and all were included in the analysis.

H&E staining of bone tissues

The results of H&E staining of femoral tissues are shown in **Figure 1**. In the CON and CON + Exe groups, a large amount of trabecular bone showed a well-arranged structure and good continuity. In the OVX group, the trabecular bone showed poor continuity and alignment. The OVX + Exe group showed better trabecular bone integrity than the OVX group, but the bone integrity was not better than that in the CON and CON + Exe groups.

Bone mineral density

The results of BMD are shown in **Figure 2**. The OVX and OVX + Exe groups had markedly lower BMD than the CON and CON + Exe groups. The

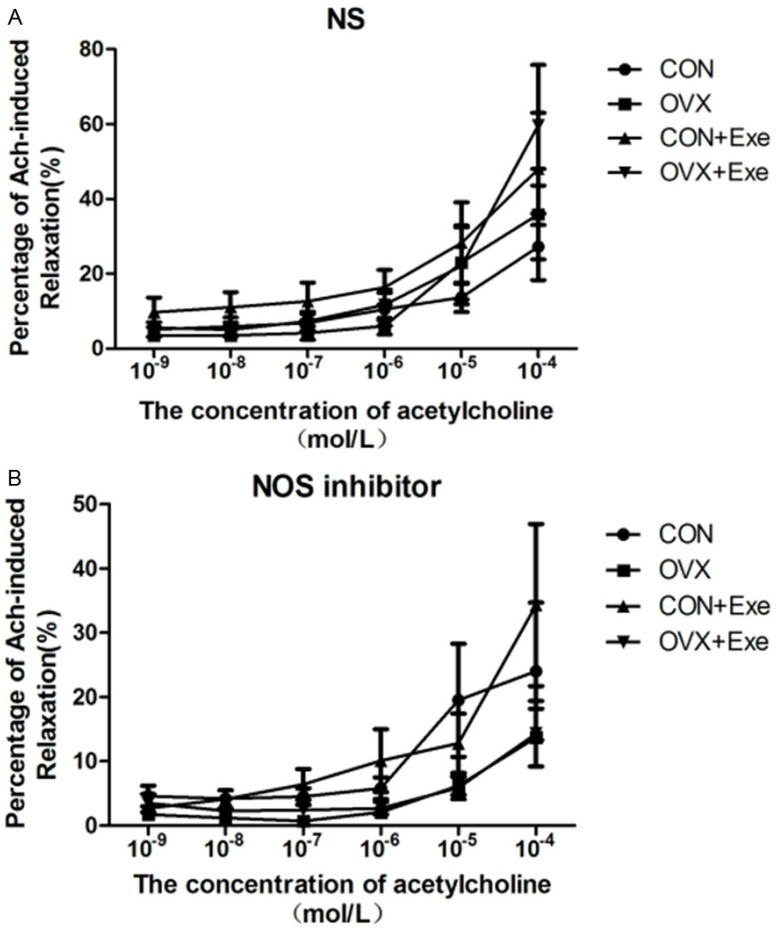

Figure 5. The results of acetylcholine-induced femoral artery vasodilation. A. The femoral artery vasodilation in different concentration (10^{-9}-10^{-4} mol/L) of acetylcholine solution after treatment of normal saline in the four groups. B. The femoral artery vasodilation in different concentration (10^{-9}-10^{-4} mol/L) of acetylcholine solution after treatment of N'-nitro-arginine methyl ester HCl solution in the four groups.

BMD in the OVX group was lower than that in the OVX + Exe group, and the BMD in the CON group was lower than that in the CON + Exe group.

Femoral micro-CT and bone parameters

Micro-CT images and bone parameters of the rat femur are shown in **Figure 3** and **Table 1**. The bone tissue in the OVX group showed significant OP characteristics. The level of OP in the OVX + Exe group was alleviated but still significant compared with the CON and CON + Exe groups. In the CON + Exe group, the parameters of the rat femur were improved over those in the CON group; the improvements included an increase in BV/TV, Tb.Th, and Tb.N and a decrease in Tb.Sp. In the OVX group, bone parameters including BV/TV, Tb.Th, Tb.N, and Tb.Sp showed varying levels of OP-like characteristics. Among all parameters tested, BV/TV and Tb.Th were both improved in the OVX + Exe group.

Micro-CT reconstruction of femoral artery and relevant parameters

The results of micro-CT reconstruction of the femoral artery are illustrated in **Figure 4**. The OVX group showed significantly decreased femoral angiogenesis compared with the CON group. The OVX + Exe group showed markedly improved femoral angiogenesis compared with the OVX group. However, the CON + Exe group did not show significantly improved angiogenesis compared with the CON group. The femoral vascular volume and volume percentage of the tissue were substantially lower in the OVX than CON and OVX + Exe groups. The OVX + Exe group had a lower vascular volume percentage of the tissue than did the CON + Exe group.

Acetylcholine-induced vasodilation rates in secondary branches of femoral artery

The results of acetylcholine-induced femoral artery vasodilation are shown in **Figure 5**. Irrespective of assignment to the CON or OVX group, exercise training enhanced acetylcholine-induced femoral artery vasodilation to a certain level. This enhancement effect was blocked by the NOS inhibitor.

eNOS gene expression

The results of RT-PCR analysis are illustrated in **Figure 6**. Gene expression of eNOS in femoral vessels showed a downward trend in the OVX group, and exercise seems to have no affect on the gene expression. The CON group showed

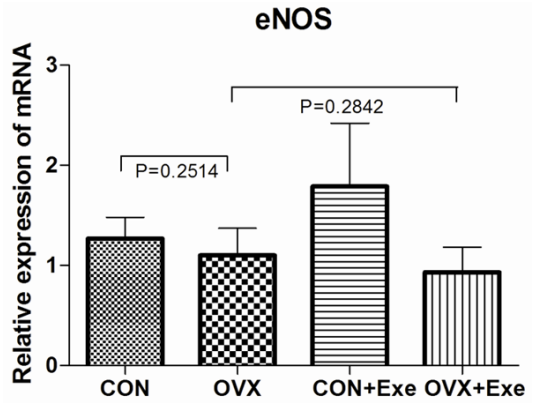

Figure 6. The results of RT-PCR analysis of eNOS gene expression.

an upward trend in eNOS gene expression after exercise.

Discussion

In this study, we found that exercise promoted osteogenesis in postmenopausal rates with OP. This may be associated with the effect of exercise on improving NOS signaling pathways, increasing NO bioavailability, and facilitating angiogenesis in bone tissues, which improves the local blood supply in the OP site. We believe that exercise can delay the progression of OP.

Postmenopausal OP is mainly caused by estrogen withdrawal [14, 15]. Estrogen withdrawal leads to greater bone resorption than osteogenesis, thus causing bone mass loss and ultimately OP. Estrogen plays a role in promoting osteogenesis [16], inhibiting bone resorption [17], and suppressing bone marrow adipogenesis [18], thereby inhibiting the development of OP. Estrogen replacement therapy can inhibit bone mass loss in postmenopausal women [19-21], thus suppressing and delaying the development of postmenopausal OP. However, there is a risk that estrogen replacement therapy can increase the incidence of arteriosclerotic disease, venous thromboembolism, and breast cancer, among other diseases [22, 33]. It has been proposed that exercise may become a safe and effective way to prevent bone loss in postmenopausal women [13]. Kemmler et al. [24] found that exercise significantly improves BMD and reduces the risk of falls in old women, as revealed by a 18-month exercise training project in women aged 65 years. Li et al. [25] studied a rat model of postmenopausal OP and found that exercise inhibits IL-1, IL-6, and Cox-2 expression to negatively regulate osteoclast formation and bone resorption; increases osteogenesis by affecting the expression of hormones (e.g., calcitonin, osteocalcin, and parathyroid hormone) that play a major role in bone remodeling; and can elevate serum estrogen levels and thus significantly increase osteogenesis. Menuki et al. [10] found that mountaineering promotes osteoblast differentiation and suppresses adipocyte differentiation. Chen et al. [26] reported that running inhibits the expression of PPARc (an adipocyte differentiation factor) and promotes the expression of Runx2 (an osteogenesis promoting factor) to prevent bone mass loss caused by menopause. David et al. [27] observed an increase in Runx2 protein expression levels and a decrease in PPAR2 protein expression levels; the authors believed that a mechanical load down-regulates PPAR2 protein expression levels and thereby leads to an increase in osteoblasts at the expense of reducing adipogenesis. Taken together, the above studies indicate that exercise can increase osteoblast differentiation to promote osteogenesis; it can also inhibit adipocyte differentiation to suppress adipogenesis and inhibit osteoclast formation to suppress bone resorption. Furthermore, exercise can prevent the adverse consequences of drug use. In the present study, H&E staining, BMD measurement, and femoral micro-CT all revealed OP-like changes in the femur of rats after OVX. After exercise, rats that had undergone OVX showed marked improvements in femoral BMD, bone parameters, and femoral micro-CT; however, there were still differences from the CON and CON + Exe groups. Therefore, we believe that the rats that had undergone OVX developed OP-like changes because estrogen withdrawal led to decreased osteogenesis. Taking into account the increased osteogenesis in rats after exercise, we consider that exercise can promote osteogenesis in patients with postmenopausal OP.

Although postmenopausal OP is caused by estrogen withdrawal, numerous studies [28-32] have indicated a close relationship between the decrease in the local blood supply and the development of postmenopausal OP. Exercise plays a positive role in promoting angiogenesis and improving vascular function. Thus, exercise can affect the development and progression of

postmenopausal OP by altering the local blood supply. Therefore, we believe that a causal relationship may exist between osteogenesis and the blood supply. Yao [33] found that the expression of VEGF and its receptor VEGF-R1 is up-regulated after exercise; this change results in an increase in angiogenesis in the cancellous bone and periosteum, thereby increasing osteogenesis and decreasing bone resorption and ultimately reducing bone mass loss. Our study also showed that the rats that had undergone OVX had markedly lower angiogenesis in the femur than did the rats in the CON group. However, angiogenesis in the femur was significantly better in the OVX + Exe than OVX group, and an improvement in angiogenesis was also observed in the CON + Exe group compared with the CON group. These results further prove that the development of postmenopausal OP is associated with a reduction in angiogenesis, whereas exercise can promote angiogenesis and thus increase osteogenesis; the promoting effect of exercise is more evident after menopause.

The blood supply to bone tissue is associated with both the formation and function of blood vessels, particularly vasodilation. Exercise has a positive impact on the endothelial vasodilation of blood vessels. Braga et al. [34] found that aerobic exercise training increases NO bioavailability and reduces reactive oxygen species (ROS) levels to improve endothelium-dependent vasodilation in rats that have undergone OVX. Tanaka et al. [35] also believed that exercise improves the endothelial function of blood vessels by increasing NO bioavailability; acute exercise can significantly improve acetylcholine-induced endothelium-dependent maximum vasodilation and the sensitivity of the endothelium to acetylcholine. Moreover, it can markedly enhance the capacity of eNOS phosphorylation, but simultaneously increase the production of superoxide dismutase and catalase, so that the increase in partial arterial vasodilation is almost completely prevented by catalase [35]. Therefore, exercise plays an essential role in maintaining the balance between eNOS and ROS, which is closely associated with exercise intensity. Murias et al. [36] found that in rats with type 1 diabetes, high-intensity resistance training recovered the vasodilation lost due to diabetes, accompanied by increased eNOS protein in the vascular wall. This indicates that exercise can not only increase NO bioavailability and eNOS activity, but also facilitate eNOS expression and thereby increase NO synthesis. Maeda et al. [37] found that chronic aerobic exercise training promotes gene expression of eNOS and elevates its mRNA levels, thereby increasing eNOS protein levels. Boa et al. [38] also observed that chronic aerobic exercise improves endothelial function and promotes eNOS expression. In our study, eNOS gene expression in femoral vessels showed a downward trend in rats after OVX, while gene expression did not seem to be affected by exercise. This indicates that estrogen plays an essential role in eNOS expression. Therefore, the rats in the CON group showed an upward trend in eNOS gene expression after exercise. Nevzati et al. [39] treated human umbilical and brain endothelial cells with estrogen and found that estrogen elevated eNOS protein expression and phosphorylation levels with a significantly increased NO level. These effects were blocked by estrogen and eNOS antagonists. The authors therefore concluded that estrogen induces the production of NO by activating eNOS [39]. Two similar studies also found that estrogen increases eNOS expression, indicating that estrogen has an essential role in maintaining normal vascular function [40, 41]. Our results showed that eNOS gene expression decreased in rats after OVX, namely estrogen withdrawal; this effect was not reversed by exercise. In the rats in the CON group, however, eNOS gene expression relatively increased after exercise. Again, this proves that estrogen plays an indispensable role in the promotion of eNOS gene expression by exercise. Moreover, we found that irrespective of assignment to the CON or OVX group, exercise training enhanced acetylcholine-induced femoral artery vasodilation, and this enhancement was blocked by a NOS inhibitor. In summary, exercise can increase NO bioavailability, enhance eNOS activity, and promote eNOS expression, thereby increasing the synthesis of NO; meanwhile, exercise can reduce the expression levels of ROS and thereby improve endothelium-dependent vasodilation. Therefore, the rats in both the OVX and CON groups exhibited enhanced vasodilation after exercise, whereas those in the OVX + Exe group did not show improved eNOS expression after exercise. The enhancement of vasodilation may be associated with an increase in eNOS activity or NO bio-

availability, or a reduction in the expression of ROS, by exercise. Furthermore, the enhancement of vasodilation may be attributed to other vasodilators, such as prostaglandins. Prostacyclin (PGI2) is a strong vasodilator and antiplatelet aggregation agent produced from free arachidonic acid via the catalytic activity of two enzymes (COX-1 and COX-2). Estrogen can promote vascular endothelial production of PGI2, possibly because the estrogen-dependent pathway leads to an increase in the activity and expression of COX-1 and COX-2. Therefore, estrogen can reduce vascular tension and improve endothelium-dependent vasodilation [42]. The aim of the present study was to assess the effect of exercise on the changes in the NOS signaling pathways after OVX and further examine the role of exercise in vasodilation, thereby revealing the relationship between OP and the blood supply.

In summary, estrogen withdrawal after menopause leads to lower eNOS gene expression. Although exercise cannot reverse the alteration in eNOS gene expression, it exerts a compensatory effect by enhancing eNOS activity and increasing NO bioavailability. With normal estrogen levels, exercise can increase eNOS gene expression. Therefore, irrespective of assignment to the CON or OVX group, exercise enhanced acetylcholine-induced femoral artery vasodilation, and this enhancement was blocked by a NOS inhibitor. Therefore, the weak endothelium-dependent vasodilation in postmenopausal rats with OP is associated with decreased NO bioavailability due to an alteration in the NOS signaling pathways. Exercise can enhance acetylcholine-induced femoral artery vasodilation in postmenopausal rats with OP by improving the NOS signaling pathways and increasing NO bioavailability. Moreover, micro-CT reconstruction of the femoral vessels showed that femoral angiogenesis decreased after menopause, while angiogenesis increased after exercise. Therefore, we believe that exercise promotes osteogenesis in rats with OP, possibly because it improves NOS signaling pathways, increases NO bioavailability, and facilitates bone tissue angiogenesis to improve the local blood supply in the OP site, thereby preventing the progression of postmenopausal OP. Although treatment of OP by simple exercise is obviously imperfect, exercise is an adjunct treatment that has great implications for preventing the progression of postmenopausal OP. Further exploration of the relationship between OP and the blood supply, as well as elucidation of the regulatory mechanism, will help to identify new therapeutic targets for the treatment of OP.

The present study had a few limitations. First, we did not detect the downstream products of the NO and PGI2 signaling pathways. Because of the lack of strong experimental evidence, we could only analyze the factors influencing vascular endothelial function at the NOS level. Second, we did not detect ROS and thus could not assess their relationship with estrogen in the experiments or the role of NOS antagonism in regulating the vascular endothelial function. These limitations need to be improved in the next study. Finally, we did not examine how exercise changes the COX signaling pathways and how the pathway change affects blood supply in the OP site. We will further improve our research by taking the above limitations into account.

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Disclosure of conflict of interest

None.

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