Archives of Gynecology and Obstetrics (2019) 299:873–882
https://doi.org/10.1007/s00404-018-5033-9

GENERAL GYNECOLOGY

Oviductus Ranae protein hydrolyzate prevents menopausal osteoporosis by regulating TGFβ/BMP2 signaling

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Received: 31 January 2018 / Accepted: 14 December 2018 / Published online: 16 January 2019

Abstract

Purpose It is known that menopausal osteoporosis (MOP) is the most typical form of osteoporosis, which is characterized by low bone mass and microstructure damage of the bone tissue, leading to increased bone fragility and risk of fracture. This study aimed to evaluate the protective effects of Oviductus Ranae protein hydrolyzate (ORPH) on the MOP in vivo.

Methods Osteoporosis model was induced by ovariectomy, treated with ORPH 150 or 75 mg kg^{-1}. Body weight and bone mineral density (BMD) of rats were measured at the beginning and the end of the experiment, and femoral maximum load was determined immediately after killing. The expression levels of alkaline phosphatase (ALP), Smad4, tartrate acid phosphatase (TRAP), BMP2, Runx2, CPB, ColI and osteocalcin were examined by RT-PCR or western-blotting. HE staining was used to observe the pathological changes in the femurs. Immunohistochemistry was used to detect the expression of ALP and BMP2. All data were analyzed by SPSS 13.0.

Results The results revealed that ORPH had no effect on the weight of normal and osteoporotic rats. ORPH could significantly improve the femur BMD and increase the maximum load of the osteoporotic rats. ORPH could significantly upregulate the expression level of bone formation makers, ALP, osteocalcin, ColI, and Runx2, and downregulate the expression level of bone resorption marker, TRAP. In the ORPH group, the expression levels of BMP2, Smad4, and CPB of key proteins in the TGFβ/BMP2 signaling pathway were significantly upregulated. In addition, immunohistochemistry showed that ALP and BMP2 expression in femurs of the ORPH group was stranger. H&E staining showed that ORPH (150 mg kg^{-1}) significantly increased the thickness of trabeculae and decreased fracture risk.

Conclusion Collectively, ORPH plays a role in the prevention and treatment of osteoporosis, which may be a potential anti-osteoporosis drug.

Keywords ORPH · Menopausal osteoporosis · TGFβ/BMP2 · BMD · ALP

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Introduction

Oviductus Ranae (OR), a crude Chinese drug, is sourced from dried tubal products of *Rana temporaria chensinensis* [1]. Pharmacological investigations of OR have mainly focused on anti-aging, anti-oxidation and anti-inflammatory actions, and on improving immunity, nourishing lungs and suppressing cough [2]. Recently, a number of studies have revealed that OR can promote osteoblast proliferation [3, 4]. OR can also increase the synthesis of estradiol (E_2), up-regulate levels of antioxidants, promote follicular development, and improve ovarian function and delay ovarian aging [5]. OR as a nourishing kidneys medicine, can be refined fine marrow; therefore, we speculated that it may be useful in the prevention and treatment of menopausal osteoporosis. However, OR contains a special type of glycoprotein, namely the oviduct glycoprotein. The oviduct glycoprotein is secreted by the oviduct mucosal epithelial cells, and its molecular weight is very large, generally from 3×10^6 to 40×10^6 Da, and the molecular weight is linear. Due to the hydrophilic action of the side-chain glycosyl group, a large amount of water can be bounded around the molecule. The structural feature of the oviduct protein is that the solution of the OR has a very high viscosity [6]. Since the viscosity of the OR is high, and the poor water solubility affects human body absorption and product development. Therefore, this study used protease enzymatic hydrolysis technology to prepare low viscosity and high solubility of ORPH, and the anti-osteoporosis effect of ORPH was studied.

Osteoporosis is a bone metabolism disease that has several features, including low bone mass, bone structure deterioration, and an increased risk of fracture [7]. It causes severe pain in patients and can even lead to death. All countries have a considerable financial burden in treating osteoporosis [8]. Osteoporosis is divided into primary and secondary osteoporosis. Secondary osteoporosis is mainly confined to women undergoing the menopause. There are a variety of drugs currently used for the treatment of osteoporosis, such as parathyroid hormone, calcium, vitamin D, estrogen, estrogen receptor modulators and bisphosphonates [9–11]. Estrogen therapy is one of the most effective treatments; however, it can result in side effects, such as stroke, thrombosis, dementia and other diseases. ORPH is a natural product from OR, and it has potential for the prevention and treatment of menopausal osteoporosis.

There is no direct method for measuring or evaluating bone strength. BMD is often used to diagnose osteoporosis, predict the risk of osteoporotic fracture, and to evaluate the optimal quantitative index of drug intervention [12]. Biochemical markers of bone conversion are classified into bone formation markers and bone resorption markers. Bone formation markers are mainly serum ALP, osteocalcin, Runx2 and ColI, while the main bone resorption marker is TRAP.

The effects of OR on menopausal osteoporosis are not well characterized. Here, we explored the effect of ORPH on MOP by studying its effect on the expression of bone metabolism-related genes, Runx2, ALP, osteocalcin, ColI and TRAP. We also explored whether the therapeutic action of ORPH on menopausal osteoporosis involves the TGFβ/BMP2 pathway by measuring changes in the levels of TGFβ/BMP2 signaling proteins, such as CPB, BMP2 and Smad4.

Materials and methods

Preparation of ORPH

Frozen Oviductus Ranae was thawed and fascia and other contaminating tissues discarded. The Oviductus Ranae was then dried in an oven at 40 °C for 24 h, crushed, and passed through a 100-μm mesh sieve. It was then mixed at a 1:100 ratio with citrate-buffered saline (10 mM, pH 4.5) and extracted at 4 °C for 12 h. Two volumes of pure water was then added, and the solution was boiled for 2 h to reduce the volume to one tenth of the initial volume. The pH was adjusted to 8.0, trypsin added and incubated at 40 °C for 3 h. The hydrolysis was stopped by heating the slurry to 100 °C. The hydrolysates were cooled to room temperature and centrifuged at 10,000*g* for 15 min. The clear supernatant, containing ORPH, was dialyzed overnight, freeze-dried and stored at − 20 °C.

Animal model and drug administration

Fifty female Sprague–Dawley (SD) rats weighing 220 ± 10 g were purchased from Yi Si Experimental Animal Technology (Changchun City, China) and housed in the SPF experimental center of the College of Pharmacy of Changchun University of Chinese Medicine (Agreement Number: SYXK-Ji-2016-0016). The experimental procedures and the animal facility were approved by the Animal Care and Welfare Committee of Changchun University of Chinese Medicine. After a week of acclimatization, 40 rats were ovariectomized (OVX) to induce osteoporosis, while 10 rats were used in the control group. The rats in the OVX group were randomly divided into four groups as follows: model group, estradiol valerate (EV) (0.05 mg kg^{-1}) group, 150 mg kg^{-1} ORPH group and 75 mg kg^{-1} ORPH group. All rats were administered treatments by intragastric administration once per day. Control group and model group rats were administered the same amount of 0.9% normal saline by the same procedure. After treatment every day for 8 weeks, all rats were euthanized.

Table 1 Sequences of primers used for real-time PCR

Genes	Primer sequences		Size (bp)
	Upstream	Downstream	
ALP	CCCACAAGAGCCCACAAT	AACGGCAGAGCCAGGAAT	163
Osteocalcin	ACTTGTGCTGGGTGGTCT	CAATACGCAGTGGCATTAA	181
Runx2	GCACCCAGCCCATAATAGA	TTGGAGCAAGGAGAACCC	229
BMP2	CTCTGACGAGGTCCTGAGCGAGTTC	GGCTGACCTGAGTGCCTGCGATACA	135
ColI	GCCAATGTGGTTCGTGACCGTGAC	GGGCTCCGGATGTTCTCGATCTGCT	88
TRAP	ATGTTAAACAAAGAGGAGACGAAGA	TGTTTTACTGCTGGCCTCACTTATG	108
Smad4	CTCCTGAGTATTGGTGTTCCATTGC	ACTCAAACAAAAGCGATCTCCTCCA	140
CPB	AACCCCTGTGATGAAACTTACTGTG	CAGATATGCCTTGATGGAAGAGAGT	108
β-Actin	ACGTTGACATCCGTAAAGAC	GAAGGTGGACAGTGAGGC	264

BMD measurement

Bone mineral density is the major quantitative indicator for the clinical diagnosis and assessment of osteoporosis. The BMD of the right femur and lumbar vertebrae were measured using Dual-energy X-ray absorptiometry (LUNAR DPX-IQ, USA) immediately before and after treatment. All samples were measured three times and the average values were used.

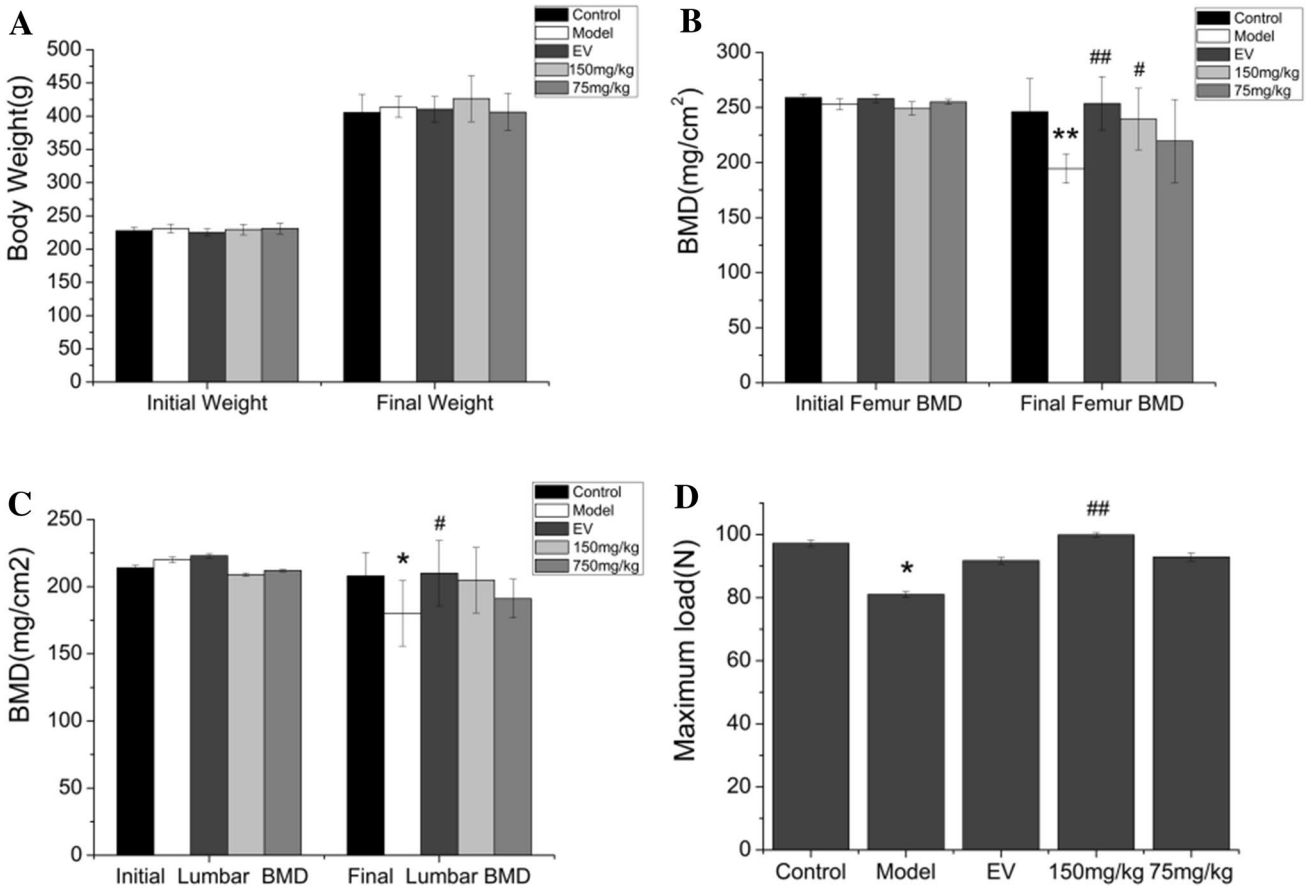
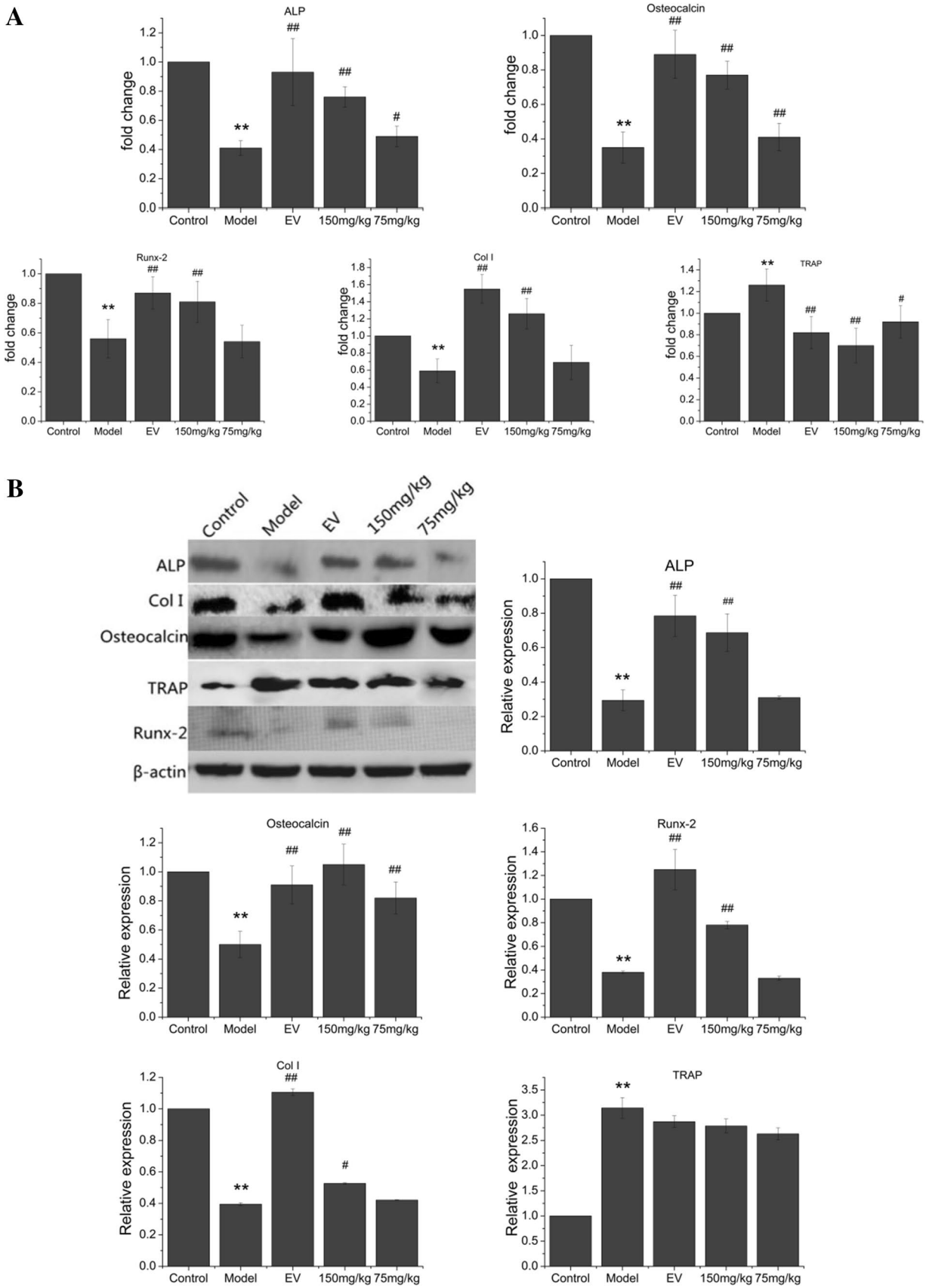


Fig. 1 Effects of ORPH on body weight, femur BMD, lumber vertebrae BMD and maximum femoral load. **a** Initial and final weights of the different groups. **b** Initial and final femur BMD of the different groups. **c** Initial and final lumbar vertebrae BMD of the different groups. **d** Initial and final maximum load of the different groups. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the model group


A
ALP
Osteocalcin
Runx-2
Col I
TRAP
fold change
Control
Model
EV
150mg/kg
75mg/kg
B
Control
Model
EV
150mg/kg
75mg/kg
ALP
Col I
Osteocalcin
TRAP
Runx-2
β-actin
Relative expression

◂**Fig. 2** Effects of ORPH on bone metabolism-related proteins. **a** mRNA levels of ALP, Runx2, ColI, osteocalcin and TRAP in rat femurs of ovariectomy-induced osteoporosis. **b** Protein levels of ALP, Runx2, ColI, osteocalcin and TRAP in rat femurs with ovariectomy-induced osteoporosis

Bone biomechanics

The left femur was fixed on a small animal bone strength tester and pressure was applied to the middle of the femur until the femur broke. The maximum load before fracture was recorded. Bone fractures are common in osteoporosis; therefore, maximum load better reflects the preventive effect of ORPH on postmenopausal osteoporosis.

Histological analysis

Proximal femur samples were fixed in 4% formaldehyde for 24 h, and then dehydrated in 70% ethanol for 2 days. Samples were then decalcified in 10% ethylenediamine tetraacetic acid disodium (EDTA 2Na) buffer at room temperature for 15 days, with the buffer replaced once a day. Samples were then embedded in paraffin and 5-μm coronal sections cut. The sections were stained with hematoxylin and eosin (H&E) and observed under a light microscope (IX53; Olympus, Japan).

Immunohistochemistry

Paraffin sections were deparaffinized in xylene and rehydrated EDTA-Tris. Sections were treated with 3% hydrogen peroxide for 15 min and then incubated with 80 μL BMP2 (1:100, Proteintech, China) or ALP (1:80, Proteintech, China) antibodies at 4 °C overnight. After washing with 5% PBS three times, sections were incubated with the second antibody (1:2000, Proteintech, China) for 1 h at room temperature (RT). Sections were then washed three times in 5% PBS and reactions developed using DAB. Sections were then dehydrated, cleared and coverslips mounted.

Real-time PCR

The expression of ALP, osteocalcin, Runx2, BMP2, ColI, TRAP, Smad4 and CPB in the left femur was detected by RT-PCR. Frozen left femurs were pulverized with a mortar and pestle under liquid nitrogen in RNase-free conditions. Total RNA was extracted from the bone powder using Trizol reagent (Invitrogen, USA), extracted with chloroform and precipitated in isopropyl alcohol. The concentration of RNA was measured by ultraviolet spectrophotometry and purity assessed according the 260 nm:280 nm ratio. The size and integrity of the RNA were determined by 1% agarose gel electrophoresis. Primers were designed and synthesized (General Biological Systems, Anhui, China) (see Table 1 for sequences of primers used for real-time PCR). β-Actin was used as an internal reference gene. RNA was reverse transcribed into cDNA, and then cDNA was used as a template for fluorescence quantitative PCR. All data were quantitatively analyzed by the $2^{-\Delta\Delta C_T}$ method.

Western blotting

Total protein was extracted from femurs using RIPA buffer (Solarbio Science and Technology, Beijing, China) containing 1 mM PMSF. The protein content was quantified using the Bradford method. After quantification, equal amounts of protein (30 μg) were separated by 12% SDS-PAGE and then electro-transferred onto nitrocellulose membranes. After blocking the membranes with 5% skimmed milk for 2 h, the membranes were incubated with the following primary antibodies: anti-β-actin (1:2000, Proteintech, China), anti-BMP2 (1:2000, Proteintech, China), anti-ALP (1:400, Omnimabs, China), anti-Runx2 (1:500, Proteintech, China), anti-TRAP (1:1000, Proteintech, China), anti-CPB (1:1000, Proteintech, China), anti-Smad4 (1:500, Proteintech, China), anti-osteocalcin (1:1000, Proteintech, China), anti-ColI (1:500, Proteintech, China) at 4 °C overnight. After washing, membranes were then incubated with the secondary antibody (1:2000) for 1 h at RT in the dark. After washing with 1 × TBST three times, membranes were visualized using enhanced chemiluminescence (ECL) reagents and an Omega Lum G imaging system (Aplegen, USA). Grayscale images were analyzed using Image J software.

Statistical analysis

All data are expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical tests were performed using SPSS 13.0 software. $P < 0.05$ was considered to be statistically significant.

Results

General condition of rats

The body weights of animals in the different groups were measured immediately before and after treatment. All animal weights before treatment were approximate. After treatment, the weight of rats in the model group was not significantly different compared with the control group ($P > 0.05$); the body weight of rats in the EV and ORPH groups was also not significantly different compared with the model group ($P > 0.05$) (Fig. 1a). ORPH, therefore, had no effect on the weight of normal and osteoporotic rats.

The initial and final femur and lumber vertebrae BMD of all rats was measured. All rats had the same femur and

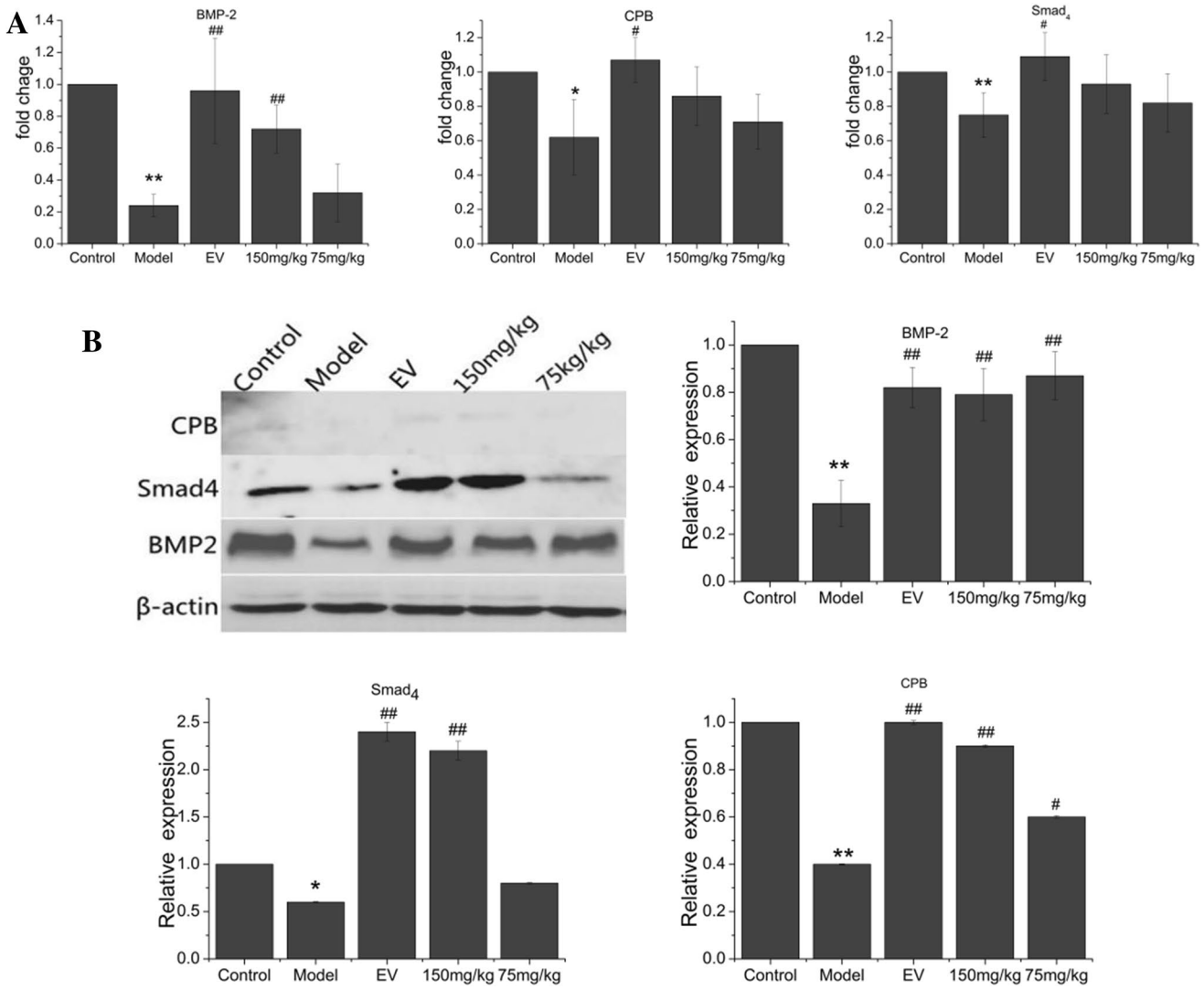


Fig. 3 Effects of ORPH on the TGFβ/BMP2 signaling pathway. **a** mRNA levels of BMP2, Smad4 and CPB in rat femurs of ovariectomy-induced osteoporosis. **b** Protein levels of BMP2, Smad4 and CPB in rat femurs with ovariectomy-induced osteoporosis

lumbar BMD before treatment. After 8 weeks of treatment compared with the control group, the BMD in the femur and lumber vertebrae in the model group was significantly decreased ($P < 0.01$, $P < 0.05$, respectively); compared with the model group, the femur BMD of the EV and ORPH (150 mg kg^{-1})groups was significantly increased ($P < 0.01$); the lumbar BMD of the EV group was also significantly increased compared with the model group ($P < 0.05$). As shown in Fig. 1b, c, ORPH could significantly improve the femur and lumbar BMD of the osteoporotic rats.

Femoral maximum load was determined immediately after killing. Compared with the control group, the maximum load of femurs in the osteoporosis model group was significantly decreased, bone fragility was increased, and calcium loss in femurs was confirmed. The EV group and the ORPH groups were compared with the model group, the maximum femoral loads of the high the ORPH (150 mg kg^{-1}) group was significantly higher than that of the model group ($P < 0.01$). Therefore, ORPH may be able to improve bone strength in osteoporosis patients, and may help to prevent fracture disease in women after the menopause.

Effects of ORPH on bone metabolism-related proteins

The expression levels of bone formation markers, ALP, Runx2, ColI and osteocalcin and of the bone resorption

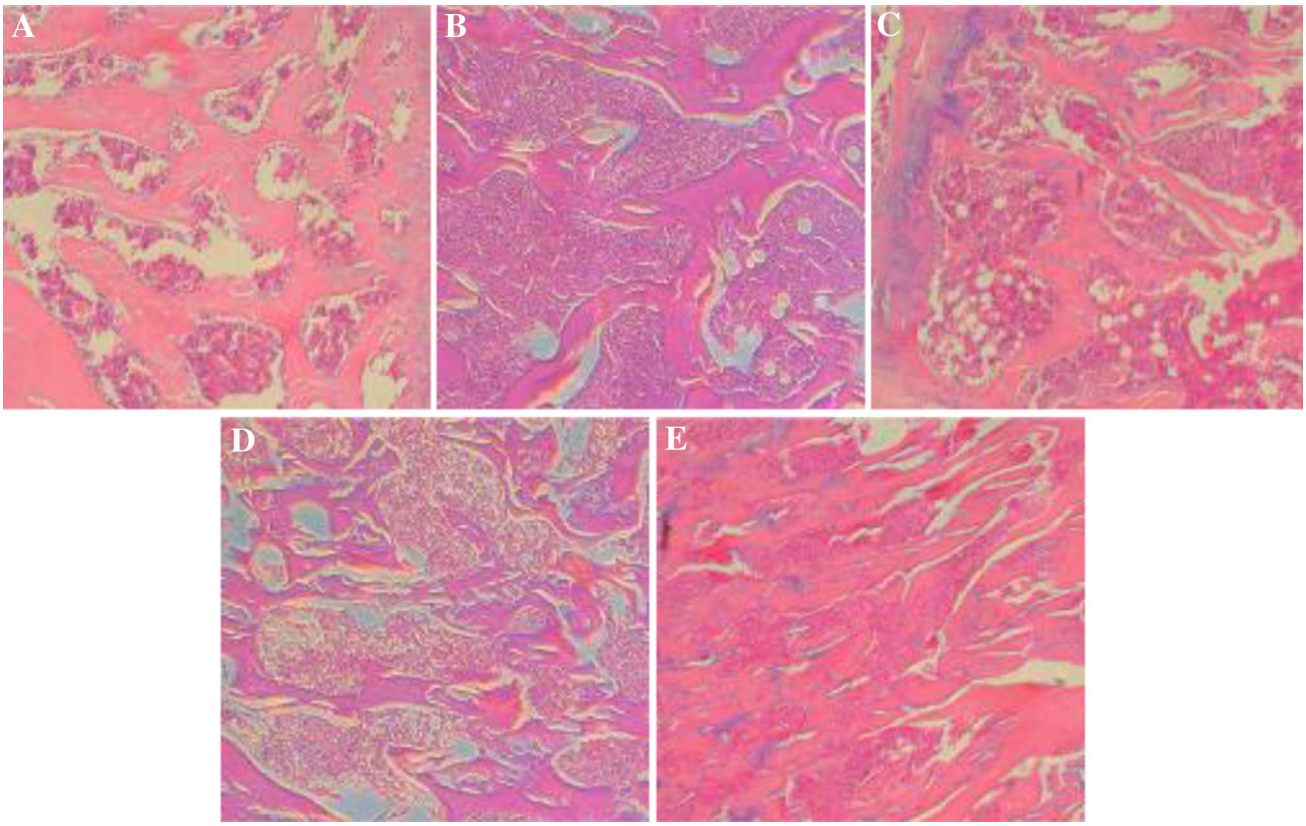


Fig. 4 H&E staining of femur tissue sections from control (**a**), model (**b**), EV (**c**), 150 mg/kg ORPH (**d**), and 75 mg/kg ORPH (**e**) rats. Ovariectomy resulted in obvious damage to femurs and ORPH attenuated the damage

marker, TRAP, were examined. As shown in Fig. 2, the expression levels of ALP, Runx2, ColI and osteocalcin were significantly downregulated and the expression level of TRAP was significantly upregulated in the femurs of rats following ovariectomy. However, treatment with ORPH significantly reversed these changes.

Effects of ORPH on the TGFβ/BMP2 signaling pathway

The expression of key proteins in the TGFβ/BMP2 signaling pathway was measured by western-blotting and RT-PCR. After ovariectomy, mRNA and protein levels of BMP2, Smad4, and CPB were significantly downregulated. However, treatment with ORPH effectively reversed these changes (Fig. 3). These results indicate that ORPH may activate TGFβ/BMP2 signaling to regulate bone metabolism and promote osteogenic differentiation.

Histomorphological changes in rat femurs

H&E staining of control group femurs showed normal trabeculae structure and ordered trabecular arrangement, with few empty bone lacunae and no slight fractures (Fig. 4). In model group femurs, H&E staining showed significantly reduced numbers of disordered and thinned trabeculae, large numbers of empty bone lacunae and slight fractures. However, compared with the model group, 150 mg/kg ORPH significantly increased the thickness of trabeculae and decreased the fracture risk. Trabeculae in femurs of the ORPH group had a more ordered arrangement and more complete structure compared with the model group.

Immunohistochemical detection of ALP and BMP2 in bone

Immunohistochemistry showed a high intensity of ALP and BMP2 in the control group, while ALP in the model group was only weakly positive. Compared with the model group, ALP and BMP2 expression in femurs of the ORPH group was stranger. This result is consistent with results from the RT-PCR and western-blotting experiments. Therefore, ORPH can promote the secretion of ALP and BMP2 in rat femur tissue. This would affect bone metabolism, and promote bone formation (Figs. 5, 6).

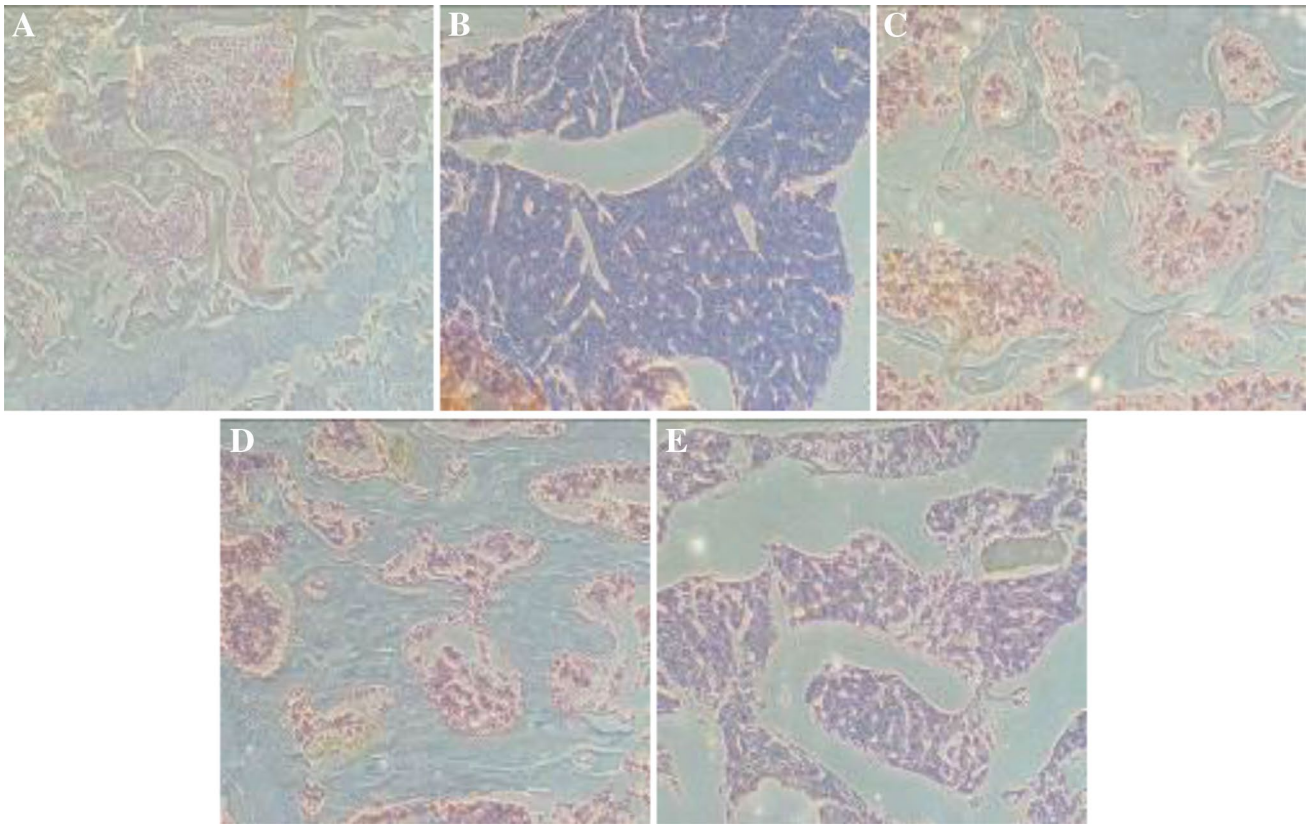


Fig. 5 Immunohistochemistry of ALP in femur sections from control (**a**), model (**b**), EV (**c**), 150 mg/kg ORPH (**d**) and 75 mg/kg ORPH (**e**) rats. ORPH significantly increased the expression of ALP

Discussion

The previous studies on Oviductus Ranae mainly focused on original medicinal material of OR [3]. However, its solubility is poor due to the water absorption and expansion of OR; therefore, it often requires a higher feed fluid ratio and a longer swelling time to get a sample suitable for taking, and this greatly limits its application. This study showed that the ORPH did not affect its activity while reducing viscosity, which provided support for further research on the active ingredients and clinical application of Oviductus Ranae.

In the present study, ORPH affected the regulation of osteoblast differentiation and proliferation in an ovariectomy osteoporosis model. In addition, ORPH modulated the TGFβ/BMP2 signaling pathway. These results indicate that ORPH may protect bone in osteoporosis.

Osteoporosis is a systemic skeletal metabolic disorder that is characterized by low bone mass and microstructure damage of bone tissue, leading to increased bone fragility and risk of fracture. It is common in postmenopausal, elderly women and can endanger the lives of patients. BMD or bone mineral content is a quantitative indicator for the clinical diagnosis and evaluation of the disease [13, 14]. ColI is the most abundant protein in the bone matrix and genes encoding ColI are strong candidates for BMD regulation [15–17]. We found that ORPH enhanced BMD and upregulated the expression of ColI. ORPH had no effect on body weight changes in osteoporotic rats, but it improved the BMD of rats and enhanced the maximum loads of femurs. H&E staining confirmed that ORPH could improve the structure and alignment of trabecular bone. Therefore, ORPH can reduce the occurrence of bone fragility and the risk of fracture by regulating bone metabolism.

Bone ALP and bone calcification are closely related, and ALP is an important marker of bone formation and transformation [18–20]. ALP is known as a catalyst for the process of bone formation, and the activity of ALP can reflect the degree of bone formation and bone turnover [21]. Immunohistochemistry showed that ORPH promoted the expression of ALP, thereby affecting bone formation and turnover. Mutation in human *RUNX2* leads to cleidocranial dysplasia and mutation in mouse *Runx2* results in critical defects in bone formation [22–24]. *Runx2* promotes osteoblast differentiation and chondrocyte maturation in diabetic patients with osteoporosis [25]. In addition, *Runx2* plays a central role in osteogenic differentiation signaling and induces osteoblastic differentiation of stem cells and promotes bone healing [21]. Osteocalcin is the most abundant non-collagenous

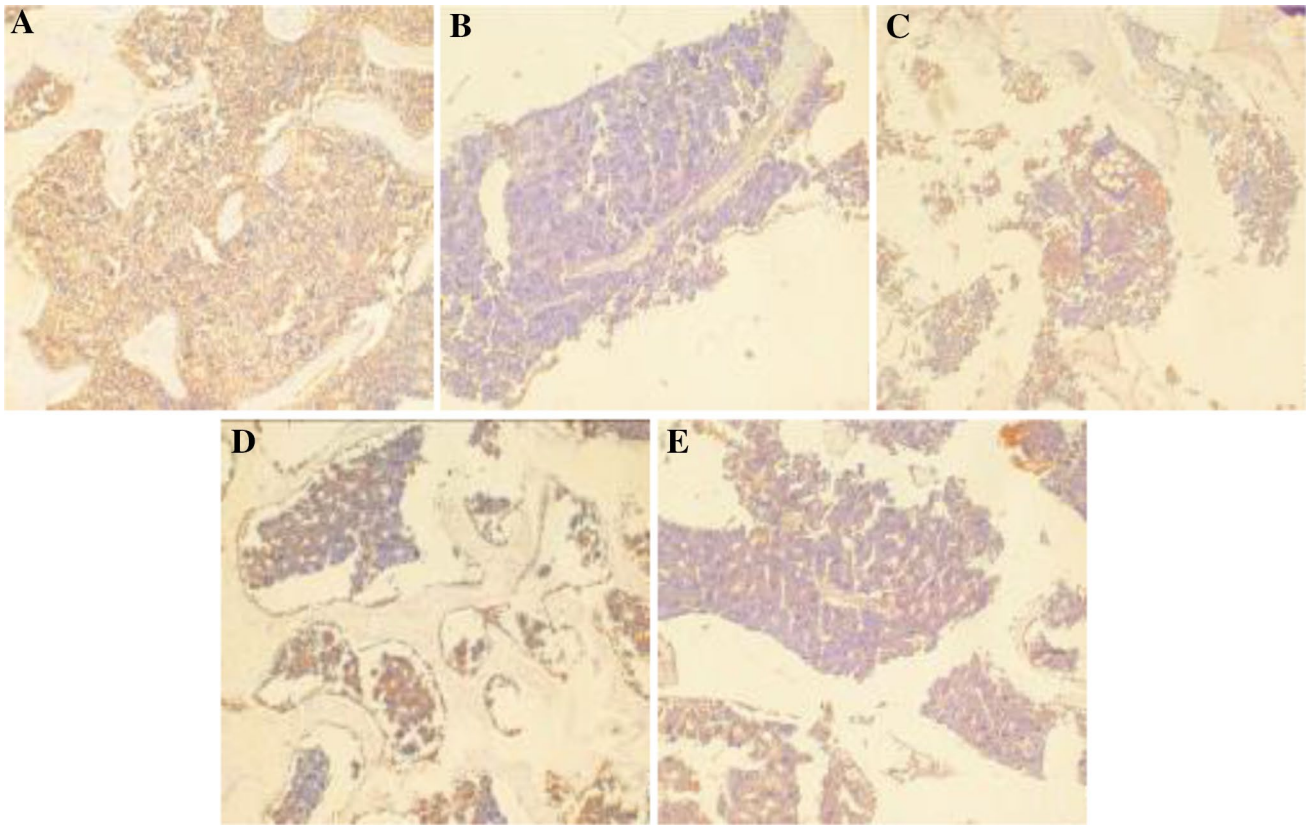


Fig. 6 Immunohistochemistry of BMP2 in femur sections from control (**a**), model (**b**), EV (**c**), 150 mg/kg ORPH (**d**) and 75 mg/kg ORPH (**e**) rats. ORPH significantly increased the expression of BMP2

protein in bone and is released by osteoblasts [26]. It is a vitamin K-dependent calcium-binding protein, and is mainly deposited on the bone matrix. It is a marker of bone regeneration, bone formation, and bone metabolism changes [27, 28]. TRAP was measured in this study as a bone resorption maker; the overexpression of TRAP may lead to osteoporosis. Our study found that ORPH can promote up-regulation of bone formation makers and down-regulation of a bone resorption maker. We, therefore, suggest that ORPH can regulate bone metabolism and can promote bone formation and inhibit bone resorption.

BMP2, located on chromosome 20p12, is a growth factor that belongs to the TGFβ superfamily [29, 30]. BMP2 has a critical role in early embryogenesis, skeletal development, and differentiation of osteoblasts located in the mature skeleton [31–34]. We confirmed that ORPH can increase mRNA and protein levels of BMP2, Smad4, and CPB. ORPH may, therefore, upregulate BMP signaling to promote Smad1/5 phosphorylation. After phosphorylation, Smad1/5 binds to Smad4 and forms a stable complex in the nucleus, where it acts on CPB to promote osteogenic cell differentiation and proliferation. We found that ORPH could promote osteoblast differentiation and proliferation, and inhibit osteoclast differentiation and proliferation though the TGFβ/BMP2 signaling pathway. ORPH can, therefore, play an anti-osteoporosis role and is a candidate drug for the treatment of osteoporosis.

Taken together, our results indicate that ORPH improves BMD and promotes osteogenic differentiation in vivo in the OVX mouse model. Furthermore, our data demonstrate that the promotive effect of ORPH on osteoblasts is because of BMP and TGFβ-induced signaling pathways. Thus, ORPH is a potential therapeutic agent for the treatment of menopausal osteoporosis. In the future, we will use iTRAQ/TMT quantitative proteomics to analyze all proteins in the osteoporosis model and ORPH treatment groups to screen for highly abundant proteins. We predict that this will identify clinical markers and drug targets for osteoporotic diseases.

Acknowledgements This work was funded by Jilin Province Traditional Chinese Medicine Administration and Jilin Province Department of Education.

Author contributions XL, XQ and NL conceived and designed the study. XL, XS, QY, and XS performed the experiments. YL provided the Oviductus Ranae. XL wrote the paper. DH, YL, XH, PY and XQ reviewed and edited the manuscript. All authors read and approved the manuscript.

Funding This study was funded by the Jilin Province Traditional Chinese Medicine Science and Technology Project (2017002, 2018024);

the Jilin Provincial Department of Education Science Research Project (JJKH20181266KJ); the Jilin province science and technology backbone training project for health and hygiene (2018Q043).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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