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TRADITIONAL CHINESE MEDICINE IN ORTHOPAEDICS

Bionic Tiger-Bone Powder Improves Bone Microstructure and Bone Biomechanical Strength of Ovariectomized Rats

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Objective: To study the curative effect of bionic tiger-bone powder on osteoporosis in ovariectomized rats and investigate its mechanism.

Methods: Overall, a 120 female Wistar rats were randomly divided into Sham (sham-operated group), ovariectomy (OVX, ovariectomized group), TB (bionic tiger-bone powder treatment group after ovariectomy) and TB + VD groups (bionic tiger-bone powder + vitamin D treatment group after ovariectomy). The osteoporotic rat model was established 3 months after ovariectomy, and rats were intragastrically administrated with the corresponding drugs. Serum and bone tissue samples were collected from 10 rats in each group at weeks 4, 12 and 24 after intragastric administration. The bone microstructure of L_6 vertebrae was analyzed by MicroCT, the biomechanical strength of left femurs was measured by the three-point bending test, and serum bone metabolism markers (P1NP and CTX) were detected by ELISA. Changes in bone collagen were analyzed by Masson's trichrome staining and hydroxyproline detection, and members of the BMP2/SMAD/RUNX2 and OPG/RANKL/RANK signal pathways were detected by immunoblotting.

Results: Compared with the OVX group, the serum level of P1NP in the TB and TB + VD groups was higher ($P < 0.05$), while the CTX level was lower ($P < 0.05$). Bone collagen fiber structures in the TB and TB + VD groups were repaired, and the collagen content was significantly higher than that in the OVX group ($P < 0.05$). In the TB group, BMP-2, P-SMAD1/5, RUNX2 and OPG levels were increased in bone tissue ($P < 0.01$), RANKL levels were decreased ($P < 0.01$), and the bone microstructure and biomechanical strength were improved.

Conclusion: Bionic tiger-bone powder promotes osteogenesis by activating the BMP2/SMAD/RUNX2 signaling pathway, suppresses osteoclasts by downregulating the OPG/RANK/RANKL signaling pathway, increases bone collagen content, and improves bone microstructure and bone biomechanical strength.

Key words: Bionic tiger-bone powder; Osteoclasts; Osteogenesis; Osteoporosis; Ovariectomized rats

Introduction

Osteoporosis is a metabolic disease caused by an imbalance in bone turnover where bone resorption exceeds bone formation, and is characterized by loss of bone mass, deterioration of bone microstructure and increase in bone brittleness[1, 2]. Decreased bone strength and bone quality increases the risk of fracture[3], disability, and death. There are multiple causes of osteoporosis, including aging, endocrine disorders, limb disuse, malnutrition, and heredity[4]. Postmenopausal osteoporosis induced by ovarian hormone deficiency, also known as type I osteoporosis, is the most common type of osteoporosis[5]. Currently, many synthetic drugs including

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diphosphonate, calcitonin, and hormone replacement therapy, have been used in the treatment of postmenopausal osteoporosis. However, long-term usage of drugs can lead to side effects such as increased risk of endometrial cancer, breast cancer and deep venous thrombosis[6, 7]. Therefore, safer and more effective drugs are urgently needed.

Traditional Chinese medicine (TCM) has unique advantages for the treatment of osteoporosis, and it has the characteristics of syndrome differentiation and comprehensive treatment[8]. Tiger bone is a traditional Chinese medicine material. Because tigers are protected animals, bionic tiger-bone powder has been developed as a substitute for tiger bones, and its composition and curative effect are similar to that of natural tiger bones[9]. Studies have confirmed that bionic tiger-bone powder promotes the proliferation of osteoblasts and expression of type I collagen *in vitro*, reduces bone turnover markers[10], and increases bone mineral density in postmenopausal patients with osteoporosis[11]. However, the *in vivo* effect of bionic tiger-bone powder on collagen content and structure in bone has not been reported.

BMP-2 is an extracellular signaling molecule that promotes bone formation, induces osteoblast differentiation and the formation of bone, cartilage and bone-related connective tissue as an autocrine/paracrine factor *in vivo*[12]. BMP-2 is activated by binding to the cell surface receptors BMPR-I and BMPR-II, and has an osteogenic role by activating downstream Smad signals to regulate osteogenic gene transcription. Runx2, a core-binding factor, is an important osteoblast differentiation transcription factor. Runx2 is involved in the regulation of extracellular matrix protein gene expression during osteoblast differentiation, and controls bone formation by regulating the differentiation of cartilage and osteoblasts, which is important for the maturation and stability of osteoblasts. The expression of Runx2 is regulated by several growth factors and hormones involved in osteoblast differentiation. BMP-2 is the most effective inducer of osteoblast differentiation and bone formation, and upregulates the expression of Runx2 resulting in activation of the BMP-2/Smad/Runx2 signal pathway[13].

Osteoprotegerin (OPG)/receptor activator for nuclear factor κB and its ligand (OPG/RANKL/RANK) pathway has an important role in regulating the differentiation and activation of osteoclasts[14]. Inhibition of the OPG/RANKL/RANK pathway effectively inhibits the differentiation of osteoclasts. In this study, the expressions of key proteins in the BMP-2/P-Smad1/5/Runx2 and OPG/RANKL/RANK signaling pathway were detected by western blot to explore the potential mechanism of bionic tiger-bone powder for the treatment of postmenopausal osteoporosis.

Materials and Methods

Animals and Grouping

One hundred and twenty 100-day-old female Wistar rats (general cleanliness, 244–300 g each) were provided by the Shandong University Laboratory Animal Center (Jinan, China). After 3 months in cages kept at 26°C under a 12 h/12 h light–dark cycle, the animals were randomly divided into four groups (n = 30 each group): Sham group (sham-operated + placebo), OVX group (ovariectomy + placebo), TB group (ovariectomy + bionic tiger-bone powder) and TB + VD group (ovariectomy + bionic tiger-bone powder + vitamin D). After intraabdominal injection of 10% chloral hydrate (Qilu Hospital of Shandong University, Shandong, China), rats in the OVX, TB and TB + VD groups underwent bilateral ovariectomy, while rats in the Sham group had only part of the fat surrounding the ovary resected. After surgery, all rats had free access to a casein-based diet (C1000 0.9% Ca, 0.7% P, and choline chloride 1012 mg/kg; Altromin, Lage, Germany) and water. The rat model of osteoporosis was established 3 months after ovariectomy (OVX). After establishment of the model, rats were treated with the corresponding drugs by intragastric administration three times per day. Drug sources: bionic tiger-bone powder (Ginwa Enterprise (Group) Inc., Xi'an, China), and Vitamin D (Liqing tablet, 0.5 μg, Chongqing Yaoyou Pharmaceutical Co., Ltd., Chongqing, China). Interventions: (i) Sham group (sham-operated group): n = 30, deionized water perfusion; (ii) OVX group (ovariectomy): n = 30, deionized water perfusion; (iii) TB group (bionic tiger-bone powder perfusion after ovariectomy): n = 30; bionic tiger-bone powder 0.306 g/kg/d; and (iv) TB + VD group (artificial tiger bone + vitamin D after ovariectomy): n = 30; (bionic tiger-bone powder 0.306 g + vitamin D 0.0525 μg)/kg/d. The animals were weighed each week during the procedure and the drugs were gastrically administrated according to their weight. The daily dose for animals = the daily dose for humans × 6.3/60. At weeks 4, 12 and 24, 10 rats per group were randomly euthanized for sampling. The animal operating procedure was approved by the Qilu Hospital of Shandong University.

Collection of Blood and Tissue Samples

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Blood samples were collected from the right ventricle under anesthesia, placed at 4°C for 3 h, then centrifuged (3000 × g/min, 4°C, 20 min). Serum was collected into EP tubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80°C until further analysis. After collecting blood samples, the rats were euthanized with 10% chloral hydrate. The bilateral femurs and L_6 vertebrae were quickly isolated and the tissues on the bone surface including the muscles and ligaments were removed. The left femurs were stored at −20°C for the three-point bending test and determination of hydroxyproline content. The right femurs were preserved in formalin and stored at 4°C for paraffin embedded sections and Masson's trichrome staining, to observe the changes in bone collagen. L_6 vertebrae were treated with formalin for 24–48 h, rinsed with 75% ethanol, stored in 75% ethanol at 4°C, and used for the analysis of bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular space (Tb.Sp) and

bone mineral density (BMD) by MicroCT. The complete gastrocnemius muscles were isolated, weighed and recorded.

Determination of Hydroxyproline in Bone

The frozen left femur sample was crushed with a standard bone mill. The hydroxyproline concentration was determined according to previously reported procedures[14, 15]. The sample was alkaline-hydrolyzed with a hydroxyproline assay kit (KeyGEN BioTECH, Jiangsu, China), and the absorbance (OD value) at 450 nm was measured. The hydroxyproline concentration was calculated accordingly.

Bone Collagen Staining

After carefully removing the surrounding soft tissues, fresh left femurs were fixed with 10% formalin (pH7.4) for 24 h, decalcified in a solution containing 10% EDTA for 3 weeks, embedded in paraffin, and sliced at 3 μm. After Masson's trichrome staining, the stained slides were observed using a slide scanner (3D HISTECH, Hungary).

Bone Biomechanical Test

The three-point bending test of the right femur was carried out at room temperature on a general testing machine. After preadjusting the sample before the experiment, a bending moment was applied to the sample at 5 mm/min until the sample was destroyed. After the experiment, data from each sample were automatically printed. The parameters reflecting the structural characteristics of the bone were then calculated, including fracture load (the force causing fractures) and fracture displacement (distance of the discharge head movement before fracture).

MicroCT Analysis of Bone Microstructure

The sixth lumbar vertebra was analyzed using three-dimensional MicroCT. The lumbar vertebra was scanned by MicroCT (SkyScan1174, Skyscan, Belgium) to evaluate the trabecular structure of cancellous bone. The scan was carried out at intervals of 10 mm, voxel sizes of 14.47 μm, 50 kV, 800 μA, and exposure time of 1000 ms. The trabecular area was separated from the cortical area of each 2D image by manual profile analysis. The trabecular volume of interest (VOI) of cancellous bone was reconstructed and defined by 3D measurements. The following parameters were obtained by analyzing the VOI: bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp). Bone mineral density (BMD) was obtained by using the same phosphate HA synthetic bone as a reference.

Detection of Bone Metabolism Markers in Serum

All serum samples were thawed at room temperature, mixed well, and centrifuged (3000 × g/min, 4°C, 20 min). Serum PINP (CSB-E12776r, Wuhan, China) and CTX (CSB-E12774r, Wuhan, China) were detected by ELISA according to the manufacturer's instructions. The reaction plates were read by a Microplate Reader at 450 nm, and the sample concentration was calculated by the measured optical density according to the standard curve.

Gel Electrophoresis

Total proteins in femurs of the Sham, OVX and TB groups at week 24 were extracted for gel electrophoresis. The bone tissue was removed from −80°C storage, ground into a powder using a mortar supplemented with liquid nitrogen, and then transferred to a pre-chilled centrifugal tube for total protein extraction. Standard western blot analysis was carried out as described previously[16]. The obtained PVDF membrane was incubated with primary antibodies: BMP-2 (1:1000), RUNX2 (1:1000), p-smad1/5 (1:1000), OPG (1:1000) and RANKL (1:1000) at 4°C overnight (Cell signaling Technology, Beverly, MA, USA). Then, goat anti-rabbit IgG (H + L) HRP secondary antibody (1:5000, ZSB-BIO, Beijing, China) was incubated with the membrane at room temperature for 1 h. The membrane was scanned and the results were normalized to GAPDH (1:1000, Cell Signaling Technology, USA) as a control. CHIDIOC Touch imaging system and Image Lab Touch Software (BioRad, CA, USA) were used to record and quantify the signals. The experiment was conducted in triplicate.

Detection Indicators

Hydroxyproline (HYP) is an imino acid that is unique to collagen, accounting for about 13% of all collagen amino acids. The hydroxyproline content in bone tissue represents the bone collagen content. Masson's trichrome staining uses a mixture of two or three anion dyes to stain collagen fibers, mucus and cartilage blue, and the cytoplasm, muscle, cellulose and glia red, which is suitable to identify collagen fibers in tissues. The three-point bending test is a common mechanical experiment. Fracture load and fracture displacement represent the force and deformation degree when the material is broken and was used to indicate the strength and toughness of the femur in this study. MicroCT was used to detect the structure of lumbar cancellous bone trabeculae. BV/TV indicates the proportion of bone tissue in the whole tissue, Tb.N indicates the number of bone trabeculae, Tb.Th represents the trabecular thickness, and Tb.Sp indicates the space between bone trabeculae. The serum turnover markers PINP and CTX represent the synthesis and decomposition of bone, respectively, and indicate the formation and absorption of bone *in vivo*. The wet weight of gastrocnemius muscle indicates the growth and development of skeletal muscle. In gel electrophoresis, BMP-2, SMAD1/5 and RUNX2 antibodies were used to detect BMP-2/SMAD1/5/RUNX2 signaling pathways, OPG and RANKL antibodies were used to detect OPG/RANKL signaling pathways, and GADPH was used as an internal reference.

Statistical Analyses

All statistical analyses were performed using Graph Prism Program, Version 7. 0 (GraphPad Software, Inc., La Jolla, CA, USA). All the data are presented as the mean ± SD.

Differences between two or more groups was compared by one-way ANOVA and unpaired Student's *t*-test, respectively. Significant differences were set as $P < 0.05$.

Results

Bionic Tiger-Bone Powder Enhanced the Content of Hydroxyproline and Ossein in Bone Tissue

The hydroxyproline content in bone tissue was determined (Fig. 1A). The femoral hydroxyproline content of the Sham group decreased gradually. Compared with the Sham group, the hydroxyproline content in the OVX group was significantly lower at weeks 4, 12 and 24 ($P < 0.001$). Compared with the OVX group, content of hydroxyproline in the TB group was higher at all time points ($P < 0.05$), but there was no significant difference with the TB + VD and TB groups.

At week 24, Masson's trichrome staining showed that cancellous bone in the Sham group was arranged neatly, bone trabeculae were intact, and bone collagen was continuous and connected into a network. In the OVX group, the arrangement of bone trabeculae was sparse and disordered, showing significant absorption, thinness, or complete loss. Distortion and fracture of bone trabeculae were common with enlarged spacing. The content of bone collagen was decreased, and the continuity of collagen was destroyed. Compared with the OVX group, the arrangement of bone trabeculae in the TB group was improved; the number of trabeculae was increased, the staining area of bone collagen was increased significantly, and the structure of bone collagen had recovered. However, there were still signs of bone trabeculae fracture (black arrows) and incomplete connection of bone collagen (green arrows). The bone cerebellum of the TB + VD group was arranged in a more orderly manner than that of the TB group, although there was still incomplete bone collagen connection (red arrows), with thicker bone trabeculae and tighter collagen structures (Fig. 1B).

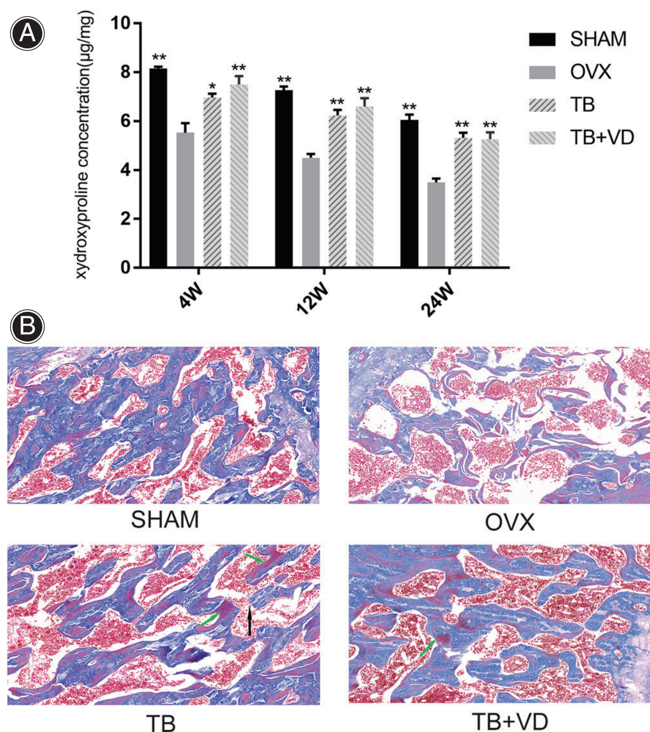


Fig. 1 Determination of hydroxyproline in bone tissues and bone collagen staining. (A) Femoral hydroxyproline contents of each group at weeks 4, 12 and 24 (compared with the OVX group, *: $P < 0.05$, **: $P < 0.01$; compared with the TB group: #: $P < 0.05$). (B) Collagen Masson's trichrome staining of cancellous bone in the proximal femur at week 24 (magnification × 20).

Bionic Tiger-Bone Powder Improved the Fracture Load and Fracture Displacement of Femur

Figure 2 shows the fracture load and fracture displacement of the shaft of the femur. The results of a three-point bending test showed that compared with the Sham group, ovariectomy resulted in a significant decrease of fracture load and fracture displacement in all stages ($P < 0.05$). Compared with the OVX group, the fracture load and fracture displacement of the TB group were significantly increased ($P < 0.05$). However, the fracture load of the femur in the TB + VD group was significantly higher than that in the TB group only at week 24 ($P < 0.05$).

Detection of the Parameters of Lumbar Vertebrae Trabeculae by MicroCT

MicroCT analysis was performed on the lumbar vertebrae L_6 of rats in each group at week 24. The quantitative indexes of bone trabecular changes of lumbar vertebrae are shown in Fig. 3. Compared with the Sham group, BV/TV, Tb.Th, Tb.N, and BMD were significantly decreased and Tb.Sp was significantly increased in the OVX group. Compared with the OVX group, the values of BV/TV, Tb.Sp, Tb.Th, Tb.N, and BMD in both drug treatment groups were significantly improved ($P < 0.05$). Compared with the TB group, BV/TV, Tb.Th, and BMD were increased in the TB + VD group, but the combined treatment showed no significant effect on Tb.N and Tb.Sp.

Serum Bone Metabolism Indexes

In this experiment, compared with the Sham group, the serum PINP level in rats in the OVX group decreased, and decreased further with time. Compared with the Sham group, the concentration of CTX-I was increased, and the difference was most obvious at week 4. Compared with the OVX group, the serum PINP level of the TB group was increased at all time points ($P < 0.05$), while the CTX-I level was decreased. Compared with the OVX group, serum PINP in the TB + VD group was higher at week 12 ($P < 0.05$), while there was no significant difference at other time points. Compared with the TB group, the CTX-I level was further decreased in the TB + VD group at all treatment periods ($P < 0.05$) (Fig. 4).

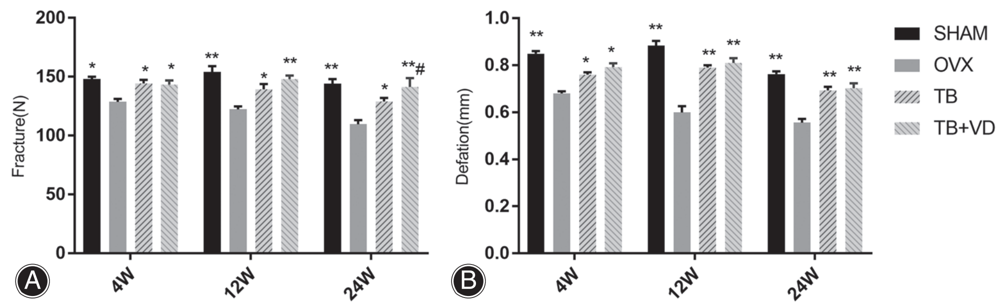


Fig. 2 The fracture load (A) and fracture displacement (B) of each group at different time points (compared with the OVX group: * $P < 0.05$, ** $P < 0.01$. Compared with the TB group, # $P < 0.05$).

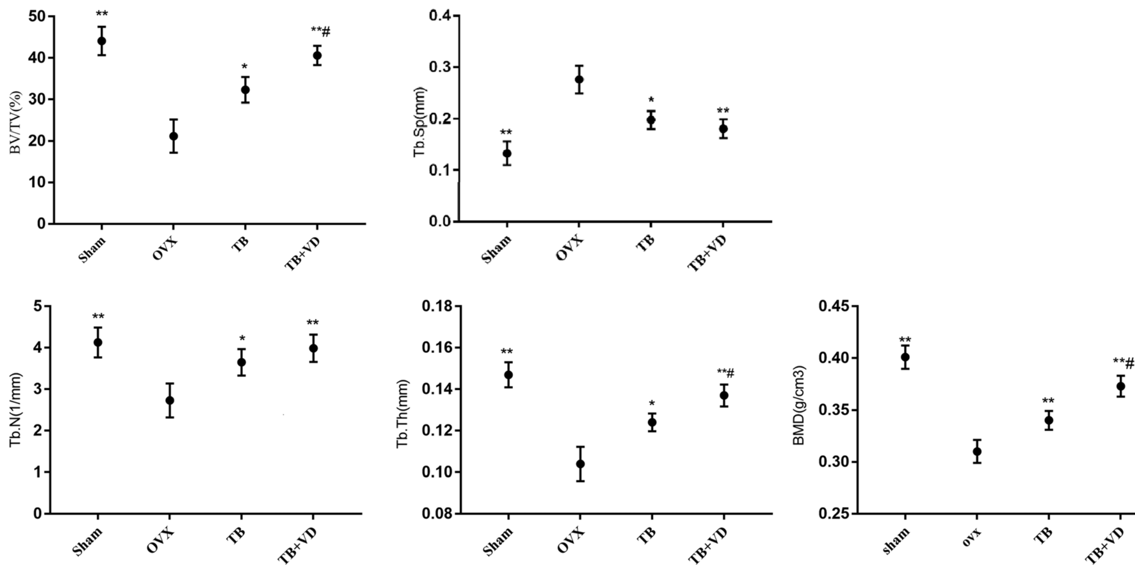


Fig. 3 Comparison of MicroCT parameters of lumbar vertebrae L_6 in each group (BV/TV: ratio of bone volume over tissue volume. Tb.N: number of bone trabeculae; Tb.Th: bone trabecular thickness; Tb.Sp: bone trabecular space; BMD: bone mineral density. Compared with the OVX group: * $P < 0.05$, ** $P < 0.01$. Compared with the TB group: # $P < 0.05$, ## $P < 0.01$).

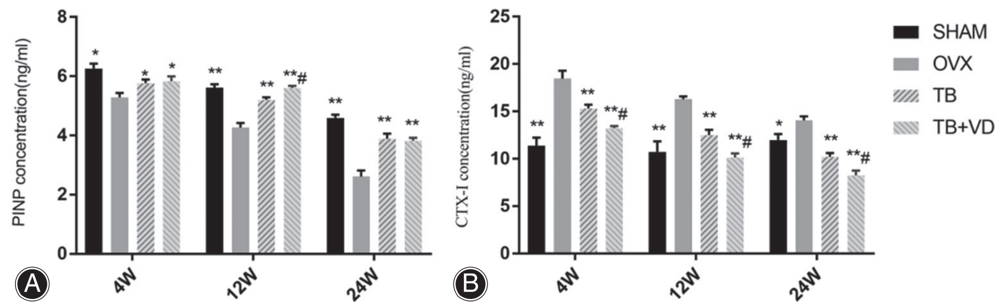


Fig. 4 The serum bone metabolism indexes of each group at different time points (compared with the OVX group: * $P < 0.05$, ** $P < 0.01$. Compared with the TB group: # $P < 0.05$, ## $P < 0.01$).

Bionic Tiger-Bone Powder Increased the Wet Weight of Gastrocnemius Muscle

The wet weight of gastrocnemius muscle in osteoporotic rats was significantly lower than that in the normal group. Compared with the OVX group, the weight of gastrocnemius muscle in the TB and TB + VD groups was increased, but which were only significant at week 12. The weight of gastrocnemius muscle in osteoporotic rats increased slightly after 4 weeks of single or combined treatment, but did not reach statistical significance. At week 24, drug intervention had no obvious effect on the weight of gastrocnemius muscle (Fig. 5). Increasing attention has been paid to the study of osteoporosis and sarcopenia, and studies have shown that the skeletal muscle mass is positively correlated with osteoporosis[17]. The wet weight changes of gastrocnemius muscle in this study provide evidence to support the effects of bionic

tiger-bone powder on strengthening muscles and bones; however, the mechanisms need to be further investigated.

Bionic Tiger Bone Meal Promoted Bone Formation Through BMP-2/ Smad1/5 /RUNX2 Signaling Pathway, and Inhibited Bone Destruction Through OPG/RANKL/ RANK Signaling Pathway

Total proteins of femurs in the Sham, OVX and TB groups were extracted for gel electrophoresis at week 24. The expressions of BMP-2, P-SMAD1/5, RUNX2 and OPG in bone tissues of the OVX group were significantly lower than that in the Sham group, while the expression of RANKL was increased. After 24 weeks of bionic tiger-bone powder treatment, the expressions of BMP-2, P-SMAD1/5, RUNX2 and OPG were significantly higher than that in the OVX group, the expression of RANKL protein was downregulated and the ratio of OPG/RANKL was decreased (Fig. 6).

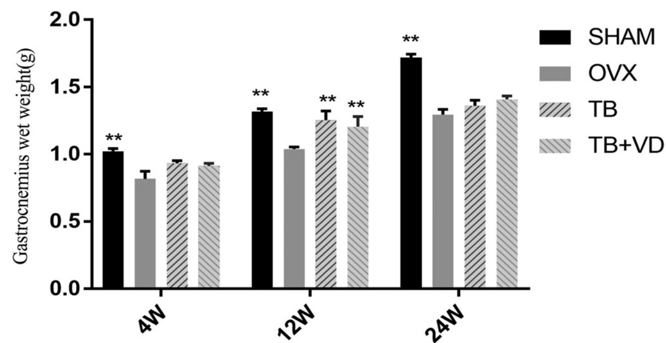
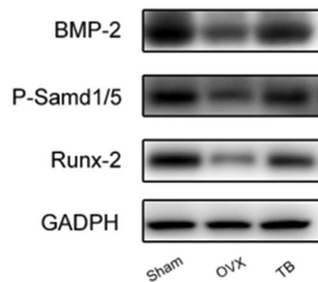


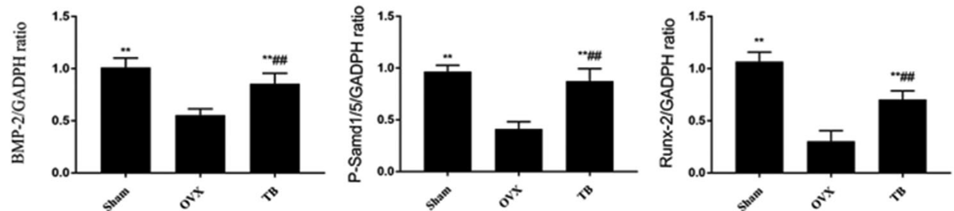
Fig. 5 The wet weight of gastrocnemius muscle at different time points in each group. (Compared with the OVX group: * $P < 0.05$, ** $P < 0.01$).

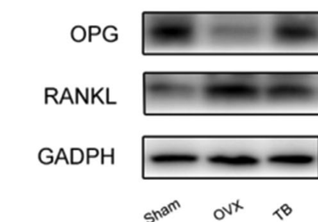
Discussion

Osteoporosis is a bone metabolism disease associated with a bone turnover imbalance induced decrease of bone mass and bone mineral density[18]. Two categories of osteoporosis have been identified: primary and secondary. Postmenopausal osteoporosis (PMOP) is the most common form of osteoporosis[5]. In this study, a model of estrogen deficiency-induced osteoporosis was established by ovariectomy in rats. This is a commonly used model of postmenopausal osteoporosis in females.

Tiger bone is a traditional Chinese medicine that consists of inorganic substances such as calcium and phosphorus, and organic substances such as collagen, amino acids, lipids, and polysaccharides[9]. Clinical studies reported that tiger bones improved bone mineral density and alleviated symptoms such as pain and fatigue in the treatment of osteoporosis[11]. In this study, we found that the hydroxyproline content in bone after 4, 12 and 24 weeks of bionic tiger-bone powder treatment was 25.45%, 37.78% and 48.22% higher than that of the OVX group, respectively (Fig. 1A). Masson's trichrome staining of the proximal end of femurs showed that the collagen content of bone in the two treatment groups was significantly higher than that in the OVX group. Furthermore, the broken bone collagen structure was repaired, and remodeling of damaged collagen was observed indicating repair (Fig. 1B). There was no significant difference in bone collagen content between the combined treatment group and the bionic tiger-bone powder group, suggesting the role of bionic tiger-bone powder in improving







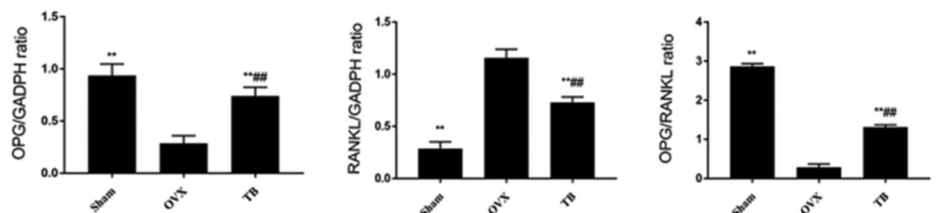


Fig. 6 Semi-quantitative measurement by gel electrophoresis of the contents of BMP-2/Smad/Runx2 and OPG/RANKL/RANK pathway related proteins in left femurs from the Sham, OVX and TB groups after 24 weeks of intervention (Compared with the OVX group: * $P < 0.05$, ** $P < 0.01$).

the structure and content of bone collagen. This result is consistent with the effect of bionic tiger-bone powder confirmed by Han and Wang, *et al.* in their *in vitro* experiment on the expression of type 1 collagen in osteoblasts[10, 17].

The basic function of bone is to resist mechanical stress and absorb pressure, and bone strength depends on the quantity and quality of bone tissues[18]. MicroCT is currently used to quickly evaluate the morphology and structure of bone trabeculae[19]. In this study, MicroCT detection of trabecular structure and bone mineral density showed that compared with the Sham group, the bone volume fraction, the number of bone trabeculae and bone mineral density in the OVX group was significantly lower, and the bone trabecular spacing was significantly higher ($P < 0.05$), indicating that ovariectomy led to the loss of trabecular in cancellous bone and the destruction of bone structure. Compared with the OVX group, the bone volume fraction, the trabecular thickness, the number of bone trabeculae, the bone trabecular spacing and bone mineral density were significantly improved in the two treatment groups ($P < 0.05$). Combined treatment with vitamin D further increased the proportion of bone tissue, the trabecular thickness and bone mineral density ($P < 0.05$). This indicated that the long-term application of bionic tiger-bone powder might significantly improve the bone microstructure of lumbar vertebrae in rats. Furthermore, the combination of vitamin D and bionic tiger-bone might further improve the bone volume fraction and trabecular thickness compared with bionic tiger-bone powder alone.

Biomechanical tests can directly detect the mechanical properties of bones[20]: the fracture load represents the force that causes fractures, and the fracture displacement is the distance of the discharge head movement before fracture occurs. The results of a three-point bending test showed that compared with the Sham group, ovariectomy resulted in a significant decrease in fracture load and fracture displacement of femurs in the OVX group ($P < 0.05$). After 4, 12 or 24 weeks of intervention with bionic tiger-bone powder and combined treatment, the fracture load and fracture displacement of femurs in rats were increased ($P < 0.05$). Compared with drug treatment alone, the combined treatment increased the fracture load of the femur after 24 weeks ($P < 0.05$). The biomechanical strength of bone is closely related to the risk of fracture. The results of the three-point bending test showed that bionic tiger-bone powder improved the fracture resistance of bone, and long-term treatment combined with vitamin D further improved the bone structure.

The BMP-2/Smad/Runx2 signaling pathway is osteogenesis-related. BMP-2 transmits signals to cells by binding to two serine/threonine kinase receptors (BMPR-I and BMPR-II) on the surface of target cells. After binding to BMPR-II, BMPR-I can specifically bind to the complex and undergoes self-phosphorylation. BMPR-I then activates Smad1/5 by phosphorylation, and P-Smad1/5 binds to Smad4 and enters the nucleus to regulate the transcription of specific genes. As a co-regulator, Smad interacts with Runx2 and is thus involved in the expression of phenotypic genes and differentiation of osteoblasts. Runx2 and Smad regulate the expression of collagen in osteoblasts, and Runx2 can bind directly to OSE2 to activate the transcription of osteocalcin genes. Smad1 and Smad5 bind to OSE2 and activate osteocalcin gene promoters only with the help of Runx2[21], a transcription factor belonging to the Runx family, which contains three members: Runx1, Runx2 and Runx3. Runx2 plays an important role in regulating bone metabolism. It was reported that Runx2 is the earliest and most specific marker involved in osteogenesis and is a regulator of chondroblast and osteoblast differentiation[13]. In this study, total protein extraction from the Sham, OVX and TB groups for gel electrophoresis showed that the expressions of BMP-2/Smad/Runx2 signaling pathway proteins (including BMP2, p-Smad1/5 and Runx2) decreased after ovariectomy. However, the levels of these proteins were increased after bionic tiger-bone powder treatment. Therefore, we think that bionic tiger-bone powder has a potential effect on osteogenesis, which is through the BMP-2/Smad/Runx2 signaling pathway.

OPG/RANKL/RANK is a classical osteoclast-associated pathway. Bone marrow stroma cells or bone marrow cells secrete RANKL and OPG, and RANKL binds to osteoclast precursor cells or RANK on their surface to promote osteoclast formation and bone resorption. The competitive binding of OPG to RANKL prevents the binding between RANKL and RANK and inhibits osteoclast formation and bone resorption[22]. The OPG/RANKL ratio has an important role in normal bone remodeling and bone mass stability[23], and an increase of this ratio indicates a reduced number and decreased activity of osteoclasts. The results of this study showed that after 24 weeks of intervention, the OPG level in bone tissues of the OVX group was significantly lower than that of the Sham group, and the RANKL level was significantly increased. This indicated that osteoclasts were in an active state of differentiation and maturation. Furthermore, 24 weeks of bionic tiger-bone powder intervention increased the OPG content, decreased the RANKL content and increased the OPG/RANKL ratio. This study suggests that bionic tiger-bone powder inhibits bone resorption in osteoporotic rats *via* the OPG/RANKL/RANK signaling pathway.

The results of this study confirmed that bionic tiger-bone powder promotes bone formation through the BMP2/Smad/Runx2 signal transduction pathway, inhibits bone resorption through the OPG/RANKL/RANK pathway, repairs bone collagen structure, increases collagen content, and improves bone microstructure and bone biomechanical strength. The combination of bionic tiger-bone powder with vitamin D can be used as an adjuvant treatment.

References

1. Teitelbaum SL. Bone resorption by osteoclasts. Science, 2000, 289: 1504–1508.
2. Marcus R, Majumder S. The nature of osteoporosis. In: Marcus R, Feldman D, Kelsey J, eds. Osteoporosis. London: Academic Press, 2001; 3.
3. World Health Organization. Guidelines for the Preclinical Evaluation and Clinicaltrials in Osteoporosis. Geneva: WHO, 1998.
4. Sharma C, Mansoori MN, Dixit M, *et al*. Ethanolic extract of Coelogyne cristata Lindley (Orchidaceae) and its compound coelogin promote osteoprotective activity in ovariectomized estrogen deficient mice. Phytomedicine, 2014, 21: 1702–1707.
5. Li Y, Lü SS, Tang GY, *et al*. Effect of Morinda officinalis capsule on osteoporosis in ovariectomized rats. Chin J Nat Med, 2014, 12: 204–212.
6. Andersen CY, Kristensen SG. Novel use of the ovarian follicular pool to postpone menopause and delay osteoporosis. Reprod Biomed Online, 2015, 31: 128–131.
7. Xu F, Ding Y, Guo Y, *et al*. Anti-osteoporosis effect of epimedium via an estrogen-like mechanism based on a system-level approach. J Ethnopharmacol, 2016, 177: 148–160.
8. Shen J-J. The prevention and treatment of osteoporosis by traditional Chinese medicine. Nei Mongol J Tradi Chin Med, 2012, 03: 77–78.
9. Wei L-x, Zhang B-c, Du Y-z. The analysis of hydroxyproline in Sailonggu and Hugu bone. AMI ACI & BIO RES, 2001, 17: 13–14.
10. Na H, Tan-Bin W. Effect of bionic tiger-bone powder on rat osteoblasts proliferation and type 1 collagen expression. Chinese J Tissue Eng Res, 2012, 16: 201–205.
11. Zhang J, Wu LS, Sun S. Clinical study on effect of JINTIANGE capsules for treatment of primary osteoporosis. Chin J Osteopor, 2005, 11: 25–27.
12. Vlacic-Zischke J, Hamlet SM, Friis T, *et al*. The influence of surface microroughness and hydrophilicity of titanium on the up-regulation of TGFβ/BMP signaling in osteoblasts. Biomaterials, 2011, 32: 665–671.
13. Komori T. Regulation of bone development and extracellular matrix protein genes by Runx2. Cell Tissue Res, 2010, 339: 189–195.
14. Crockett JC, Mellis DJ, Scott DI, Helfrich MH. New knowledge on critical osteoclast formation and activation pathways from study of rare genetic diseases of osteoclasts: focus on the RANK/RANKL axis. Osteoporos Int, 2011, 22: 1–20.
15. Kimura S, Saito M, Kida Y, Seki A, Isaka Y, Marumo K. Effects of raloxifene and alendronate on non-enzymatic collagen cross-links and bone strength in ovariectomized rabbits in sequential treatments after daily human parathyroid hormone (1–34) administration. Osteoporos Int, 2017, 28: 1109–1119.
16. Reffitt DM, Ogston N, Jugdaohsingh R, *et al*. Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro. Bone, 2003, 32: 127–135.
17. Verschueren S, Gielen E, O'Neill TW, *et al*. Sarcopenia and its relationship with bone mineral density in middle-aged and elderly European men. Osteoporos Int, 2013, 24: 87–98.
18. Parfitt AM. Bone remodeling and bone loss: understanding the pathophysiology of osteoporosis. Clin Obstet Gynecol, 1987, 30: 789–811.
19. Laib A, Kumer JL, Majumdar S, Lane NE. The temporal changes of trabecular architecture in ovariectomized rats assessed by MicroCT. Osteoporos Int, 2001, 12: 936–941.
20. Leppänen OV, Sievänen H, Järvinen TL. Biomechanical testing in experimental bone interventions—may the power be with you. J Biomech, 2008, 4: 1623–1631.
21. Pratap J, Lian JB, Stein GS. Metastatic bone disease: role of transcription factors and future targets. Bone, 2011, 48: 30–36.
22. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res, 2000, 15: 2–12.
23. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys, 2008, 473: 139–146.