TRADITIONAL CHINESE MEDICINE IN ORTHOPAEDICS

Combination Treatment with Whole Body Vibration and a Kidney-tonifying Herbal Fufang Prevent Osteoporosis in Ovariectomized Rats

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Objective: To assess the ability of whole body vibration (WBV) with the kidney-tonifying herbal Fufang (Bushen Zhuanggu Granules, BZG) to prevent osteoporosis in ovariectomized rats.

Methods: Fifty 6-month-old female Sprague Dawley rats were divided into five groups: sham-operated (SHAM), ovariectomized (OVX), OVX with WBV (OVX + WBV), OVX with BZG (OVX + BZG), OVX with both WBV and BZG (OVX + WBV + BZG). The SHAM group received normal saline. After 12 weeks of treatment, the rats were killed, their serum concentrations of osteopontin (OPN), receptor activator of nuclear factor kappa-B ligand RANKL and bone turnover markers assayed and bone mineral density (BMD), histomorphometry and bone strength evaluated.

Results: Concentrations of OPN were significantly lower in the SHAM, OVX + WBV and OVX + WBV + BZG groups at 12 weeks, whereas concentrations of RANKL had decreased significantly in the SHAM, OVX + WBV, OVX + BZG and OVX + WBV + BZG groups. In the OVX + WBV, OVX + BZG and OVX + WBV + BZG groups the amount of bone turnover had been significantly antagonized. Compared with OVX group, BMD, % trabecular area (Tb.Ar), number of trabeculae (Tb.N) and assessed biomechanical variables were higher in OVX+WBV group, whereas and BMD, %Tb.Ar, Tb.N, maximal load and yield load were higher in the OVX + BZG group. All tested indices were significantly lower in the OVX + WBV and OVX + BZG groups than in the OVX + WBV + BZG group.

Conclusion: Either WBV or BZG alone prevents OVX-induced bone loss. However, BZG enhances the effect of WBV by further enhancing BMD, bone architecture and strength.

Key words: Kidney-tonifying herbal; Osteopontin; Osteoporosis; Ovariectomy; Whole body vibration

Introduction

Postmenopausal osteoporosis (PMO), a skeletal disease in which estrogen deficiency results in bone resorption outpacing bone formation, is characterized by systemic disruption of bone microarchitecture that leads to fragility fractures^{1,2}. Therefore, suppressing excessive bone resorption is the main

strategy for treating PMO. The commonest anti-resorptive agents, alendronate and estrogen, are effective but sometimes have adverse effects, such as atypical femoral fractures, jaw necrosis and breast cancer^{3,4}. Thus, it is necessary to develop effective and safe therapies for preventing and treating PMO.

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Low-magnitude high-frequency mechanical stimulation, such as whole body vibration (WBV), has been shown to have great anabolic potential, increasing bone strength and bone mineral density (BMD) as well as decreasing the amount of bone turnover in both ovariectomized (OVX) rats and postmenopausal women⁵⁻⁹. Many studies have shown that WBV attenuates receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclast differentiation *in vitro* and inhibits osteoclastogenesis^{10,11}. RANKL is the key mediator of osteoclast formation and function^{12,13}. In addition, WBV can also improve bone strength and BMD in OVX rats by down-regulating the amount of circulating RANKL⁶. Thus WBV, a non-pharmacological therapy, has attractive advantages in that it is a non-invasive and easy-to-apply approach for PMO⁷⁻⁹.

Recently, a five-year multi-center clinical study confirmed that the kidney-tonifying herbal preparation Fufang, which contains epimedium-derived phytoestrogen, is effective and safe for preventing PMO and reducing the incidence of fragility fractures¹⁴. This kidney-tonifying herbal preparation, in the form of Bushen Zhuanggu granules (BZG; Yi-Fang Pharmacy Company of Guangdong Province, Lishui, China), reportedly alleviates bone loss induced by estrogen decline without obvious adverse events in postmenopausal women¹⁴.

However, it has not yet been established whether BZG combined with WBV has a cumulative effect in in OVX rats. Our purpose was to investigate whether combined treatment with BZG and WBV enhances the preventive effect of the latter on bone loss in OVX rats.

Materials and Methods

Animals and Ethics Statement

Fifty 6-month-old female Sprague Dawley rats weighting 286.36 ± 28.87 g were purchased from the Laboratory Animal Center of Southern Medical University (SCXK-Guangdong-2011-0015; Guangzhou, China). The rats were acclimatized in standard cages for one week under a 12-hour light-dark cycle and allowed free access to water and rodent diet. This study was performed according to the guidelines for care and use of laboratory animals, published by the USA National Institutes of Health¹⁵. All surgeries and anesthesia in this study were approved by the Ethical Committee of the General Hospital of Guangzhou Military Command of the People's Liberation Army.

Experimental Design

In this study, ovariectomies were performed on 40 Sprague Dawley rats through a dorsal approach under general anesthesia with intraperitoneal 10% chloral hydrate injection (3.3 mL/kg)⁶. Sham operations in which a small amount of adipose tissue near the ovaries was excised were performed on 10 Sprague Dawley rats. All animals recovered uneventfully from surgery. Four days after surgery, the OVX rats were randomly divided into four groups to guarantee the balanced distribution of weight in each group: OVX, OVX + WBV, OVX + BZG and OVX + WBV + BZG, 10 rats per group. The OVX only and

Sham groups received normal saline. Rats in the OVX + WBV and OVX + WBV + BZG groups underwent WBV, which was performed using an experimental vibration platform (Juvent 1000, Juvent Medical, Somerset, NJ, USA) set at a frequency of 30–35 Hz and an acceleration of 0.3 g twice a day for 20 min with a 5 min rest at the mid-point (10 mins on, 5 mins off, 10 mins on) for 5 days/week for 12 weeks¹⁶⁻¹⁹. The parameters above were confirmed by the Guangdong Medical Devices Quality Surveillance and Test Institute (NO: JK083007).

Fufang BZG was formulated based on the combination theory of herbs in Chinese medicine for balancing “Yin-Yang” and strengthening “Muscle-Bone”^{20,21} and contained seven herbal compounds; namely, *Herba epimedii*, *Rehmannia glutinosa*, *Dioscorea batatas*, *Cornus officinalis*, *Cinnamomum cassia*, *Drynaria fortunei* and *Morinda officinalis*¹⁴. All these herbs were decocted and concentrated into a thick paste, crushed into granules after vacuum drying, and finally packed to form Bushen Zhuanggu granules, that is, Fufang BZG (NO: JFB-(Z-1)-2009; Yi-Fang Pharmacy Company of Guangdong Province) for oral administration. BZG was given to rats in the OVX + BZG and OVX + WBV + BZG groups for 12 weeks in a dose of 2.5 mg/kg per day by gavage using a stomach tube, this dose having been determined by previous *in vitro* studies^{22,23}.

During the experimental period, all rats' body weights were measured using a JJ500 electronic scale (STIFCC, Changshou, China) weekly and doses administered adjusted accordingly. At the end of the 12 weeks, the rats were anesthetized with intraperitoneal 10% chloral hydrate injection (3.3 mL/kg) and blood specimens obtained from the abdominal aorta to measure serum osteopontin (OPN), RANKL, procollagen I N-terminal peptide (PINP) and C-terminal cross-linked telopeptides of type I collagen (CTX) by enzyme-linked immunosorbent assay (ELISA). Samples were collected from the left femurs and tibiae, wrapped in warm saline gauze after all the soft tissue had been removed and stored at -20°C for BMD determination, histological evaluation and biomechanical testing.

Serum Analysis

Whole blood was centrifuged for 15 min at 1000 g to separate serum, which was stored at -80°C until the following assays were performed. The serum concentrations of OPN, RANKL, the anabolic marker PINP and bone resorption marker CTX were measured using an ELISA commercial Kit (Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions. The sensitivities of the kit for OPN, RANKL, PINP and CTX were 7.8 pg/mL, 15.63 pg/mL, 15.6 pg/mL and 3.9 pg/mL, respectively. They had the same intraassay (<8%) and interassay (<10%) precision.

Bone Mineral Density Examination

The BMDs of the left femurs were measured by using a hand-regional high resolution instrument especially designed for performing dual energy X-ray absorptiometry on small animals (Hologic, Bedford, MA, USA). The left femurs were divided into three regions of interest (ROI); namely, the proxi-

TABLE 1 Effect of WBV + BZG on body weight, BMD and biomechanical properties in OVX rats

Indexes	SHAM	OVX	BZG	WBV	WBV + BZG
Body weight (g)					
Before the experiment	282.9 ± 44.0	288.5 ± 34.5	289.2 ± 35.8	288.0 ± 14.0	283.3 ± 13.8
4 weeks after treatment	285.4 ± 32.9	313.5 ± 36.7	305.3 ± 56.9	303.3 ± 13.2	297.6 ± 22.4
8 weeks after treatment	292.1 ± 18.5	338.1 ± 42.2*	326.5 ± 36.3	316.0 ± 12.1	307.9 ± 13.5
12 weeks after treatment	305.9 ± 16.1	353.0 ± 30.3†*	342.0 ± 35.5†	333.7 ± 33.9†	316.5 ± 16.3†
BMD (g/cm ²)					
Intact femur	0.330 ± 0.011	0.269 ± 0.031*	0.302 ± 0.009*†	0.305 ± 0.005*†	0.328 ± 0.014†¶**
Proximal femur	0.307 ± 0.017	0.256 ± 0.017*	0.282 ± 0.009*†	0.286 ± 0.009*†	0.303 ± 0.009†¶**
Femoral shaft	0.355 ± 0.012	0.283 ± 0.014*	0.327 ± 0.010*†	0.329 ± 0.007*†	0.336 ± 0.013*†
Distal femur	0.326 ± 0.012	0.275 ± 0.011*	0.295 ± 0.012*†	0.309 ± 0.011*†	0.312 ± 0.013†¶**
Biomechanical properties					
Maximal load (N)	125.85 ± 10.63	77.75 ± 9.24*	97.59 ± 8.26*†	102.82 ± 11.35*†	116.14 ± 11.75†¶**
Yield load (N)	112.30 ± 11.25	67.52 ± 13.18*	88.84 ± 13.11*†	92.63 ± 10.46*†	107.47 ± 9.08†¶**
Stiffness (N/mm)	127.26 ± 3.94	73.02 ± 9.77*	91.60 ± 9.38*†	99.75 ± 7.58*†	112.16 ± 13.13†¶**

Data are presented as mean ± SD. †, $P < 0.05$ versus before the experiment; *, $P < 0.05$ versus SHAM; ‡, $P < 0.05$ versus OVX; ¶, $P < 0.05$ versus BZG; **, $P < 0.05$ versus WBV.

mal femur (ROI-1), femoral shaft (ROI-2) and distal femur (ROI-3). ROI-1 was defined as a site just proximal to the lower edge of the lesser trochanter, ROI-2 as a 2 cm long site between the inferior edge of the lesser trochanter and the superior edge of the femoral condyle and ROI-3 as a site just distal to the junction of the femoral condyle cancellous bone and femoral diaphysis cortical bone. After measuring BMD, the left femurs were stored in warm saline for 3 hours for subsequent biomechanical testing.

Histological Evaluation

The left proximal tibiae were decalcified, dehydrated, rinsed with running water, made transparent with benzoic acid methyl ester and then embedded in paraffin wax. Sections 5 µm thick were cut and stained with hematoxylin–eosin for routine histomorphologic analysis. Morphologic changes were evaluated with an image autoanalysis system (Olympus BX41, Tokyo, Japan). Morphologic variables were calculated using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Silver Spring, MD, USA) and expressed according to the recommendations of the American Society for Bone and Mineral Research nomenclature committee²⁴. Trabecular bone was measured 3 mm away from the proximal growth plate. All values are presented as the mean of three measurements in three nonconsecutive sections²⁴. The image analysis system automatically determined the measuring areas, and the structural variables are presented as percentage of trabecular area (%Tb.Ar) and number of trabeculae per mm (Tb.N).

Biomechanical Testing

After thorough hydration of the left femoral samples, the mechanical properties of the femoral shafts were tested to failure via three-point bending using a Bose ElectroForce 3520 biological material testing system Bose Corporation at the following address: 10250 Valley View Road, Suite 113 Eden Prairie, MN 55344 USA (MN, USA) with a speed of 5 mm/min

on the middle of femoral shaft on the anterior surface²⁵. Load and deformation data were recorded and calculations performed using WinTest Dynamic Mechanical Analysis software. A load versus deformation graph was plotted for each femur and maximal load and yield load determined from the gradient of the curve. The ratio of load/deformation represents the bending stiffness (N/mm)²⁶.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD) and analyzed by SPSS 13.0 software. One-way ANOVA and Tukey's post-hoc test were performed for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Body Weight

Results are shown in Table 1. Before the experiment, there was no significant difference in mean weight (286.36 ± 28.87 g) of rats among the five groups ($P = 0.993$). The body weights of rats in the OVX, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups had increased considerably by the 12th week compared with the baseline ($P < 0.05$ for all groups); no significant change in weight had occurred in the SHAM group ($P = 0.533$). In addition, the average weight was significantly higher in the OVX group than in the SHAM group in the eighth and 12th weeks ($P = 0.046$ and $P = 0.033$, respectively).

Serum Analysis

Table 2 summarizes the percentage differences in serum concentrations of assessed compounds according to group.

Figure 1 shows that the serum concentration of OPN was significantly lower in the SHAM, OVX + WBV and OVX + WBV + BZG groups than in the OVX group ($P < 0.001$ for all).

TABLE 2 Percentage change in serum OPN, RANKL and bone turnover markers for each group (%)

Comparison	OPN	RANKL	P1NP	CTX
OVX versus SHAM	88.2*	114.0*	119.9*	76.3*
OVX + BZG versus SHAM	74.4*	32.7	6.9	42.6*
OVX + WBV versus SHAM	18.1	37.6	16.0	32.3*
OVX + WBV + BZG versus SHAM	13.9	8.8	2.6	14.9
OVX + BZG versus OVX	-7.3	-38.0†	-51.4†	-19.1†
OVX + WBV versus OVX	-37.2†	-40.4†	-47.3†	-25.0†
OVX + WBV + BZG versus OVX	-39.5†	-49.2†	-53.4†	-34.9†
OVX + WBV versus OVX + BZG	-32.3‡	-3.9	8.5	-7.2
OVX + WBV + BZG versus OVX + BZG	-34.7‡	-18.1	-4.1	-19.4‡
OVX + WBV + BZG versus OVX + WBV	-3.6	-14.8	-11.6	-13.2¶

*, $P < 0.001$ versus SHAM; †, $P < 0.001$ versus OVX; ‡, $P < 0.001$ versus OVX + BZG; ¶, $P < 0.05$ versus OVX + WBV.

The results for the SHAM, OVX + WBV and OVX + WBV + BZG groups compared with the OVX + BZG group ($P < 0.001$ for all) paralleled these. The OVX + WBV and OVX + WBV + BZG groups had higher serum concentrations of OPN ($P = 0.138$ and $P = 0.371$, respectively) than the SHAM group; however, these differences are not statistically significant. No difference was found between the OVX and OVX + BZG groups ($P = 0.380$).

The concentrations of RANKL were significantly lower in the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups than in the OVX group ($P < 0.001$ for all groups; Fig. 2). Although no significant differences between the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups were identified, the concentrations were a little higher

in the OVX + BZG, OVX + WBV and OVX + WBV + BZG groups than in the SHAM group ($P = 0.073$, $P = 0.177$ and $P = 0.952$, respectively); these differences are not statistically significant.

Serum PINP concentrations were significantly lower in the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups than in the OVX group ($P < 0.001$ for all groups; Fig. 3A); however, no significant differences between the SHAM, OVX + BZG, OVX + WBV, and OVX + WBV + BZG groups were identified. As for the PINP concentrations, the CTX concentrations were significantly lower in the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups than in the OVX group ($P < 0.001$ for all groups; Fig. 3B). However, CTX concentrations were significantly higher in the

Fig. 1 Graph showing mean $\pm$ SD values for serum OPN concentrations 12 weeks after treatment. Serum samples were taken from the rats of the SHAM, OVX, OVX + BZG (BZG), OVX + WBV (WBV), and OVX + WBV + BZG (WBV + BZG) groups 12 weeks after treatment. *, $P < 0.001$ versus SHAM group; #, $P < 0.001$ versus OVX group; Δ, $P < 0.001$ versus BZG group.

Fig. 2 Graph shows mean $\pm$ SD values for serum RANKL concentrations 12 weeks after treatment according to treatment group. Serum samples were taken from the rats of the SHAM, OVX, OVX + BZG (BZG), OVX + WBV (WBV), and OVX + BZG (WBV + BZG) groups 12 weeks after treatment. *, $P < 0.001$ versus SHAM group; #, $P < 0.001$ versus OVX group.

Fig. 3 Graph showing mean $\pm$ SD values for serum bone turnover markers (A) PINP and (B) CTX concentrations 12 weeks after treatment. Serum samples were taken from the rats of the SHAM, OVX, OVX + BZG (BZG), OVX + WBV (WBV), and OVX + WBV + BZG (WBV + BZG) groups 12 weeks after treatment. *, $P < 0.001$, versus SHAM group; #, $P < 0.001$ versus OVX group; Δ, $P < 0.001$ versus BZG group; ▲, $P < 0.001$ versus WBV group.

OVX + BZG and OVX + WBV groups than in the SHAM group ($P < 0.001$ for both) and there was an identical trend when the OVX + BZG and OVX + WBV groups were compared with the OVX + WBV + BZG group ($P < 0.001$ and $P < 0.05$, respectively). No difference was found between the SHAM and OVX + WBV + BZG groups ($P = 0.126$).

Bone Mineral Density Analysis

The BMD in the intact femurs and different regions of them was significantly higher in the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups than in the OVX group ($P < 0.01$ for all groups; Table 1). However, the values were significantly lower in the OVX + BZG and OVX + WBV groups than in the SHAM group ($P < 0.05$ for both). Compared with the SHAM group, the OVX + WBV + BZG group showed 0.6% ($P > 0.05$), 1.3% ($P > 0.05$), 5.4% ($P < 0.01$) and 4.3% ($P > 0.05$) lower BMD values at the intact femur, proximal femur, femoral shaft and distal femur, respectively. The OVX + WBV + BZG group BMD values were higher by 8.6% in the intact femur ($P < 0.01$), 7.4% in the proximal femur ($P < 0.01$), 2.8% in the femoral shaft ($P > 0.05$), and 5.8% in BMD at distal femur ($P < 0.05$) than in the OVX + BZG group. Compared with the OVX + WBV group, BMD values in the intact femur, proximal femur, femoral shaft and distal femur in the OVX + WBV + BZG group were higher by 7.5%, 5.9%, 2.1% and 1.0%, respectively ($P < 0.05$, $P < 0.05$, $P = 0.115$ and $P < 0.05$, respectively). Treatment with WBV alone resulted in a slight but not significant improvement in BMD in the intact femur and its regions compared with the OVX + BZG group.

Histomorphometric Analysis

Results of histomorphometric analysis were shown in Fig. 4 for the tibial proximal metaphyseal region. OVX group had a 80.2% decrease in %Tb.Ar ($P < 0.001$; Fig. 5A) and a 78.4% decrease in Tb.N ($P < 0.001$; Fig. 5B) compared with SHAM group. Compared with OVX group, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups significantly increased

Fig. 4 Histologic sections of proximal metaphyses of the tibiae of (A) SHAM (B) OVX, (C) OVX + BZG, (D) OVX + WBV, (E) OVX + WBV + BZG groups stained with hematoxylin-eosin, $\times 40$.

Fig. 5 Graphs showing mean $\pm$ SD values for (A) Tb.Ar, % and (B) Tb.N, 1/mm. Bone specimens were obtained from the rats of the SHAM, OVX, OVX + BZG (BZG), OVX + WBV (WBV) and OVX + WBV + BZG (WBV + BZG) groups 12 weeks after treatment. *, $P < 0.05$ versus SHAM group; #, $P < 0.05$ versus OVX group; Δ, $P < 0.05$ versus BZG group; ▲, $P < 0.05$ versus WBV group.

%Tb.Ar (104.8%, $P = 0.008$; 160.0%, $P < 0.001$; and 292.7%, $P < 0.001$, respectively) and Tb.N (120.5%, $P = 0.002$; 185.7%, $P < 0.001$; and 301.3%, $P < 0.001$, respectively). OVX + WBV + BZG group showed an increase in %Tb.Ar (91.7% and 51.0%, $P < 0.001$ for both) and an increase in Tb.N (82.0% and 40.4%, $P < 0.001$ for both) compared with OVX + BZG or OVX + WBV group. Compared with OVX + BZG group, OVX + WBV group showed slight improvement both in %Tb.Ar and Tb.N although it's not statistically significant (26.9%, $P = 0.348$ and 29.6%, $P = 0.197$, respectively). However, these data were still lower in OVX + WBV + BZG group than in SHAM group ($P = 0.004$ and $P = 0.243$, respectively). Taken together, these findings indicated that combination treatment with WBV and BZG can improve bone architecture in OVX mice.

Biomechanical Properties Analysis

Results are shown in Table 1. Compared with the SHAM group, the OVX group showed dramatic reductions in maximal load (−38.2%, $P < 0.001$), yield load (−39.9%, $P < 0.001$), and stiffness (−42.6%, $P < 0.001$). Compared with the

OVX group, the OVX + BZG, OVX + WBV and OVX + WBV + BZG groups showed significantly increased maximal loads (25.5%, 32.2% and 49.4%, respectively; $P < 0.01$ for all groups), yield load (31.6%, 37.2% and 59.2%, respectively; $P < 0.01$ for all groups) and stiffness (25.4%, 36.6% and 53.6%, respectively; $P < 0.001$ for all groups). However, the maximal load, yield load and stiffness in the OVX + BZG group were lower than those in the OVX + WBV + BZG group (−16.0%, −17.3% and −18.3%, respectively; $P < 0.01$ for all) and there was an identical trend when the OVX + WBV and OVX + WBV + BZG group were compared (−11.5%, −13.8% and −11.1%, respectively; $P < 0.05$ for all). The OVX + WBV + BZG group had 11.9% less stiffness than with the SHAM group ($P = 0.006$). No significant differences in maximal load, yield load or stiffness were identified between the OVX + BZG and OVX + WBV groups ($P = 0.788$, $P = 0.947$, $P = 0.298$, respectively). Similarly, no differences in maximal load or yield load were found between the SHAM and OVX + WBV + BZG groups ($P = 0.238$ and $P = 0.881$, respectively).

Discussion

In the present study, we found that a combination of WBV and the kidney-tonifying herbal preparation Fufang (BZG) had a synergistic effect on reversing negative changes in bone mass, bone architecture and mechanical properties in rats with ovariectomy-induced estrogen-deficiency. Furthermore, combination treatment with WBV and BZG resulted in significantly lower in serum concentrations of OPN, RANKL and amount of bone turnover than were found in the OVX only group.

The importance of biomechanical signals in determining bone morphology and strength has long been recognized^{27–29}. Various non-drug mechanical interventions that utilize the benefits of mechanical signals have been designed as countermeasures to osteoporosis. WBV simulates a primary natural form of mechanical loading, which plays a key role in maintaining bone mass and bone strength and reducing bone turnover in both OVX rats and postmenopausal women^{5–9}. The response of bone tissues to mechanical stimuli depends on various factors, including the magnitude, duration and rate of the stimuli. Dynamic or cyclic mechanical stimuli have been shown to have great anabolic potential in OVX rats or a functional disuse model^{27,29,30}. Our previous five-year multicenter study on the herbal preparation Fufang in the form of BZG, in which epimedium is the main component, demonstrated its beneficial effects in preventing bone loss and reducing the risk of fragility fractures in postmenopausal women¹⁴. Two recent clinical trials have shown the effect of the oral herbal preparation Xianlinggubao on mitigation of postmenopausal bone loss. The main component of this preparation is also epimedium, which produces an anti-osteoporosis effect in postmenopausal women mainly by reducing the concentrations of bone resorption marker and maintaining concentrations of bone formation marker^{31,32}. Furthermore, the effects of individual treatments such as WBV or other herbal Fufang preparations have also been extensively studied^{5–7,27,33–35}. However, there are no published studies investigating the effect

of WBV combined with herbal Fufang on bone metabolism in OVX rats. The current study has shown that a combination of WBV and BZG therapy significantly increases BMD through increasing the percentage of trabecular area and number of trabeculae, and also enhances bone mechanical strength by increasing maximal force, yield force and stiffness. The BMD of the femur was determined by dual energy X-ray absorptiometry using a device especially designed for small animals. This enabled us to determine total and region of interest BMD values. Maximal force (the force that causes bone fracture), yield force (the force that creates irreversible bone damage) and stiffness (ability to tolerate bone distortion) are the most widely used biomechanical variables for measuring bone strength²⁶. We found that femoral BMD, percentage of trabecular area, number of trabeculae and biomechanical variables were significantly less in the OVX than the SHAM group and that this was reversed by 12 weeks of combination treatment with WBV and BZG; that is, the femoral BMD, %Tb.Ar, Tb.N, maximal force, yield force and stiffness caused by ovariectomy all increased. In addition to BMD in the femoral shaft and %Tb.Ar in the proximal tibia, other bone metabolism variables such as BMD, Tb.N, and biomechanical variables were superior in the OVX + WBV + BZG group compared with the WBV or BZG groups, and were identical to those in the SHAM group. Furthermore, rats with WBV or BZG therapy alone also showed significant improvement in bone metabolism variables and bone biomechanical properties; however, those in the WBV or BZG groups were inferior to those in the SHAM group.

There is, however, controversy regarding the effect of WBV on bone metabolism and bone healing in OVX rats. Some studies have reported that the beneficial effects of WBV therapy alone on bone mineral metabolism and capacity of promoting fracture healing are significantly superior to those of OVX rats^{8,16,27,36–38}; whereas others have found no difference^{39–41}. It was worth noting that reported differences may be attributable to variations in the targeted skeletal site, applied frequency and magnitude, intervention time, type of vibration platform and so on. In addition, two recent studies have shown that the synergistic treatment effects of WBV and alendronate in improving trabecular architecture⁴² and of WBV with estradiol and raloxifene in promoting fracture healing in OVX rats⁴³. Our findings are similar to those reported by Ferreri *et al.*²⁷, Sehmisch *et al.*³⁶ and Judex *et al.*³⁸.

Osteopontin, a secreted phosphorylated glycoprotein, is found in bone tissues and peripheral blood in humans^{44–46}. Recently, many studies have suggested that OPN is involved in negatively regulating bone mass in OVX rats and postmenopausal women^{45–47}. Several studies have demonstrated that OPN is one of the pivotal mechanical stress signaling mediators required for unloading-induced bone loss^{48,49}. More importantly, unloading-induced osteoclast recruitment and increased RANKL expression can be reversed in OPN knockout mice⁵⁰. Thus, OPN is the key regulator of RANKL-induced osteoclastogenesis under the condition of unloading. In addition,

WBV also inhibits RANKL-induced osteoclastogenesis^{10,11} and down-regulates expression of serum RANKL in OVX rats⁶.

Based on these data concerning OPN and RANKL, our main hypothesis, that WBV has a positive effect on bone anabolic in OVX rats by directly down-regulating the expression of circulating OPN, was confirmed by our findings. We found that serum OPN and RANKL concentrations were significantly higher in the OVX than in the SHAM group. However, serum OPN concentrations in rats treated with WBV alone or WBV combined with BZG were significantly lower than in those treated with BZG alone. No significant difference was observed between the OVX + BZG and OVX groups. Additionally, serum RANKL concentrations in the OVX + BZG group were significantly lower than those in the OVX group. No significant differences were observed between the OVX + BZG, OVX + WBV and OVX + WBV + BZG groups. All of these findings help in understanding the mechanism by which WBV combined with BZG decreases osteoporosis; namely, by reducing the expression of serum OPN and RANKL. BZG may also promote bone formation in OVX rats by directly decreasing the expression of circulating RANKL. This is in line with the findings of Chen *et al.*, who reported that herbal epimedium extract suppresses mouse osteoclastogenesis by reducing RANKL expression in marrow cells⁵¹.

OVX induces osteoporosis of a high turnover type in which bone resorption and bone formation are increased, particularly the former. PINP is a bone anabolic marker and CTX a bone resorption marker. In our study, serum PINP and CTX concentrations were significantly higher in the OVX group than in the SHAM group. No significant differences were found between the SHAM, OVX + BZG, OVX + WBV and OVX + WBV + BZG groups. Serum CTX concentrations in the OVX group were superior to those in the WBV or BZG groups. However, serum CTX concentrations in the OVX + WBV + BZG group were inferior to those in the WBV or BZG groups and similar to those in the SHAM group, showing that WBV combined with BZG therapy had a greater effect on bone resorption than WBV or BZG alone.

According to our findings, either WBV or BZG therapy alone can reduce osteoporosis by increasing BMD, bone architecture and bone strength; however, these tested indexes were inferior to those in the SHAM group rats. However, a combination of these two modalities did prevent bone loss in OVX rats by decreasing the expression of circulating OPN; the effects of the combination were similar to the SHAM group. Therefore, combination therapy with WBV and BZG may be an attractive option for treating PMO.

In conclusion, the current study indicates that either WBV or BZG intervention alone prevents bone loss in OVX rats. However, BZG enhances the effect of WBV by further enhancing the BMD, bone architecture and bone strength. We should note, however, that because we did not fully elucidate the possible mechanism(s), further studies are needed to explore whether circulating OPN is not merely an observed phenomenon but is truly necessary for the therapeutic effect of WBV combined with BZG.

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