

Original Article

Chinese herbal combination of Epimedium, Drynariae rhizoma, and Salvia miltiorrhiza extracts prevents osteoporosis in ovariectomized rats

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Abstract: Osteoporosis and related fragility fracture is a worldwide health problem for middle and advanced aged women. To find an alternative for the estrogen treatment, which often accompanies by severe side effects, we investigated the anti-osteoporosis effect of a traditional Chinese herbal combination of Epimedium, Drynariae rhizome and Salvia miltiorrhiza extracts in an *in vivo* ovariectomy rat osteoporotic model. Our results demonstrated that the bone mineral density, calcium content and microstructure of bone tissue were significantly enhanced by the herbal medicine, to a similar extent as the estrogen therapy and probably via promoting calcium deposition in the bone. This study of ours therefore provides a promising novel, natural alternative option for prevention and treatment of osteoporosis.

Keywords: Traditional Chinese medicine, bone health, osteoporosis, bone mineral density, rat

Introduction

Osteoporosis (OP), a progressive and systemic skeletal disease characterized by osteopenia and degeneration of the bone microstructure, increases the risk of fragility fractures [1]. In fact, OP and concomitant fragility fracture have become a worldwide problem posing a serious threat to the health of middle-aged and elderly people, especially postmenopausal women: it has been reported that one in three women older than 50 years has OP [2, 3]. Early diagnosis and prevention is the key to effective treatment of OP [4]. Currently, calcium supplement is a fundamental therapy to prevent low bone mineral density (BMD) and OP [5]. Low BMD decreases bone mass and bone strength, and the incidence of low BMD is significantly higher than OP in older-age groups [6]. All these demand special attention to the prevention and treatment of low bone mass.

Natural medicines, especially Chinese herbal medicines, are rich in anti-OP elements, and are increasingly attracting researchers to use them to develop alternative therapies [7]. In

classical documents of Traditional Chinese medicine (TCM), descriptions of conditions that are very similar to OP exist, along with proposed treatments, therefore, Chinese herbal medicines may provide potential benefits to improve bone health [8].

Traditional Chinese herbal medicines usually consist of more than 10 different herbs in one prescription. It is thought that fewer components containing TCM herbal medicines would be more suitable for long-term use in the prevention of OP, and would also help standardize and modernize TCM. In this study, we investigated the anti-OP effects of a TCM combination medicine with herbal extracts from Epimedium, Drynariae rhizoma and Salvia miltiorrhiza, which traditionally are most frequently used in the treatment of OP, in an ovariectomized *in vivo* rat model.

Materials and methods

Animals

Six-month-old SD rats (weighed 250 ± 20 g) were purchased from Shanghai Super-B&K La-

boratory Animal Corp. Ltd. (License number: SYXK (Shanghai) 2009-0069). The animal drug dosing and sacrifice procedures were in accordance with the Guidelines For the Ethical Treatment of Experimental Animals issued by the Ministry of Science and Technology in 2006.

Reagents

According to the TCM bone health enhancing principles for the preparation of herbal medicine [9, 10], the recipe included three Chinese herbal medicine extracts: Epimedium herb, Drynariae rhizoma and Salvia miltiorrhiza. For Epimedium, the effective component was 18% Icariin, which was obtained by grounding and sieving dry Epimedium, and extracting three times (1 h each time) using 10-15x volume of water. Three such extracts were combined after filtration, isolation and purification with D101 macroporous adsorption resin, dried and eluted with alcohol to acquire the 18%-Icariin Epimedium extract. Dry Drynariae rhizoma was grounded and sieved and extracted three times (1 to 2 h each time) with 10x volume of 30-75% ethanol at 50-70°C. Three such extracts were obtained after filtration and subjected to reduced pressure (vacuum) to obtain dried Drynariae rhizoma extract of 3% Naringin. Dry Salvia miltiorrhiza was grounded and sieved, and extracted twice (1 to 2 h each time) with 7-8x volume of 40-60% ethanol at 55-75°C. The two extracts were then combined after filtration, subjected to reduced pressure (vacuum) to acquire the dried extract of 14% Salvianolic Acid B. The purity of Icariin, Naringin and Salvianolic Acid B in the combination with three extracts was validated by high-performance liquid chromatography (HPLC). All herbal extracts were provided by Amway (China) R&D Center Co. LTD. Standard compounds Naringin (Lot: 140306), neoeriocitrin (Lot: 141-12108) and icariin (Lot: 150724), were purchased from Chemstrong Scientific Biotech. Co. Ltd. (SZ, China). The purity of these standards was detected using HPLC method (> 98%).

HPLC analysis of extracts

Principal components of three extracts were analyzed by HPLC. Briefly, Salvia miltiorrhiza extract (DS-E), Epimedium brevicornum extract (YYH-E) and Drynaria fortune extract (GSB-E)

were diluted with 70% ethanol by ultrasound to 1.42, 0.73 and 5.51 mg/mL, respectively. Standard compounds naringin, neoeriocitrin, icariin, salvianolic acid B and tanshinonella were diluted with 70% ethanol to 0.189, 0.150, 0.190, 0.284 and 0.006 mg/mL, respectively. These sample solutions were then filtered through 0.45 µm membrane for HPLC injections (10 µL).

The analyses of samples and standard compounds using reverse phase (RP)-HPLC were performed on a Waters 2695 HPLC instrument. A Diamonsil C18 column (5 µm, 4.6 × 250 mm) was selected for the experiment. The GSB-E was analyzed with a gradient elution from 2% B (100% acetonitrile) and 98% A (water contain 0.4% formic acid) to 9% B (92% A) in 8 min, then 18% B (82% A) in 22 min, to 27% B (73% A) in 42 min and then to 55% B (45% A) in 50 min at a flow rate 1.0 mL/min with a Photo-Diode Array (PDA) detector at 260 nm. The YYH-E was analyzed using the same solvent system under following condition: linear gradient from 10% B to 24% B for 10 min, to 25% B for 25 min, to 47% B for 35 min, to 64% B for 43 min, to 92% B for 60 min at a flow rate 1.0 mL/min at 260 nm, whereas DS-E was analyzed using the linear gradient from 8% B to 22% B for 12 min, to 25% B for 22 min, to 40% B for 42 min, to 74% B for 48 min, to 82% B for 58 min, to 85% B for 65 min then stand to 70 min at a flow rate 1.0 mL/min at 270 nm.

Establishment of the low bone mass rat model

Ovariectomized female rats were used for establishing the OP model as previously described [11]. To create the model, an intramuscular injection of 20000 U of gentamycin was administered for 3 days consecutively after the operation (Southwest Pharmaceutical Co. LTD.).

Animal grouping and administration of the herbal medicines

The rats were allowed to acclimatize for 5 days after the operation, then were randomized into 4 groups (10 rats/group): ovariectomy group (OVX group), OVX + estradiol group (positive control group), OVX + Epimedium herb/Drynariae rhizome/Salvia miltiorrhiza group (test group), and the sham group, which underwent a sham operation and was considered as the control group. As a result of complications

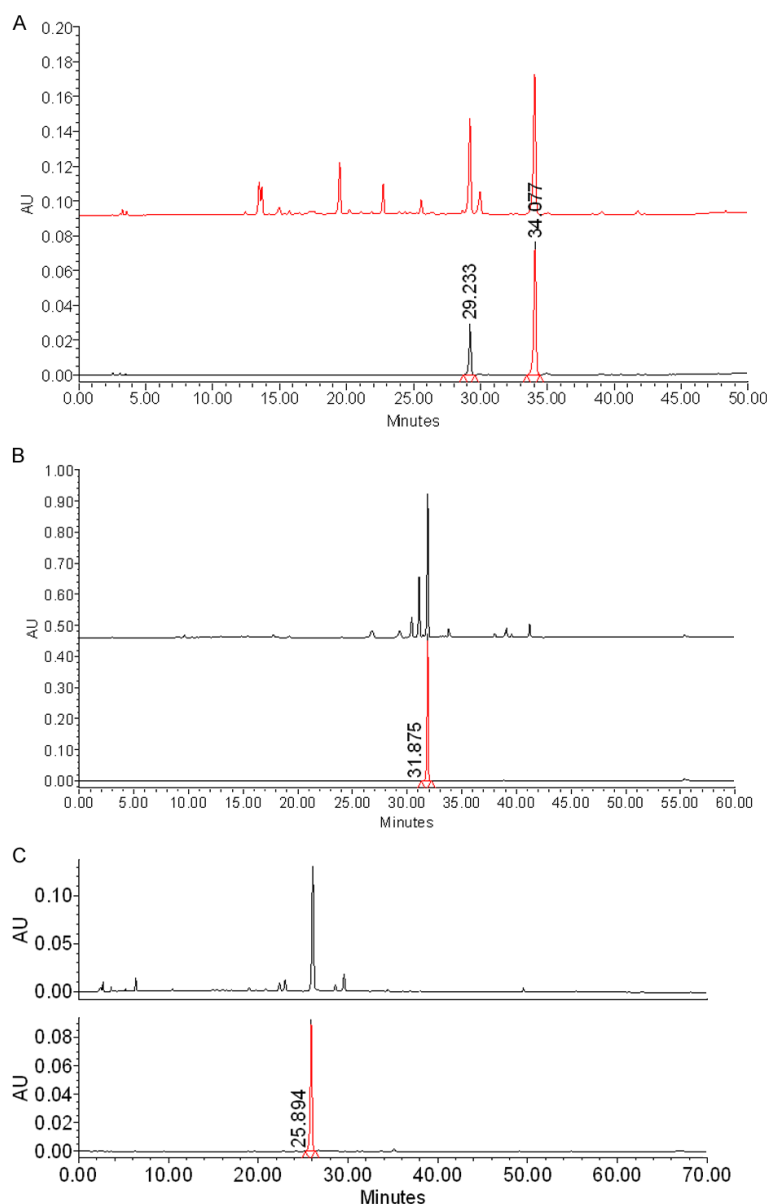


Figure 1. A. The HPLC fingerprint of GSB-E, detected at 260 nm. The peaks at Rt 29.23 min and 34.08 min were identified as neoeriocitrin and Naringin by comparing with authentic standard compounds. B. The HPLC fingerprint of YYH-E, detected at 260 nm. The peak at Rt 31.88 min was identified as icariin by comparing with authentic standard compound. C. The HPLC fingerprint of DS-E, detected at 270 nm. The peaks at Rt 25.89 min was identified as salvianolic acid B with authentic standard compound.

related to the surgery, the final number of animals in each group was as follows: 6 in the sham group, 9 in the OVX group, 10 in the positive control group, and 9 in the test group.

The experimental herbal compound solution was administered via oral gavage at the fifth day post-operation: each animal in the test group was given 3 ml of the compound once a day for

12 consecutive weeks. Distilled water with sodium carboxymethyl cellulose of the same volume was administered in the sham group and OVX group, and estradiol of the same volume was administered in the positive control group (dose, $0.105 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (Bayer Pharmaceutical Co. Ltd., Guangzhou). The dose used in the test group was $163.333 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, which was composed of $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of the Epimedium herb extract, $31.667 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of the Y extract, and $31.667 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of the Salvia miltiorrhiza extract.

Body weight and uterus weight

The animals were weighed once a week after the operation. After sacrifice, uteri of the animals was dissected out and weighed.

BMD measurement

The average BMD of the entire femur was determined using dual-energy X-ray absorptiometry (DEXA; Lunar Prodigy Advance; GE Health care, small animal scanning software). The region of interest (ROI) included the whole femur, femur head and neck. The measurements with repositioning of the bones were repeated for three times, the results of the BMD in the whole femur, femur head and neck were expressed as the mean value.

Preparation of decalcified tissue specimens and dyeing methods

The left tibia was completely dissected out, with the surrounding soft tissue removed. The tibia was then fixed with 4% paraformaldehyde overnight, before being placed in 8% EDTA-Na for de-calcification. Afterwards, the proximal tissue of the tibia was subject to dehydration,

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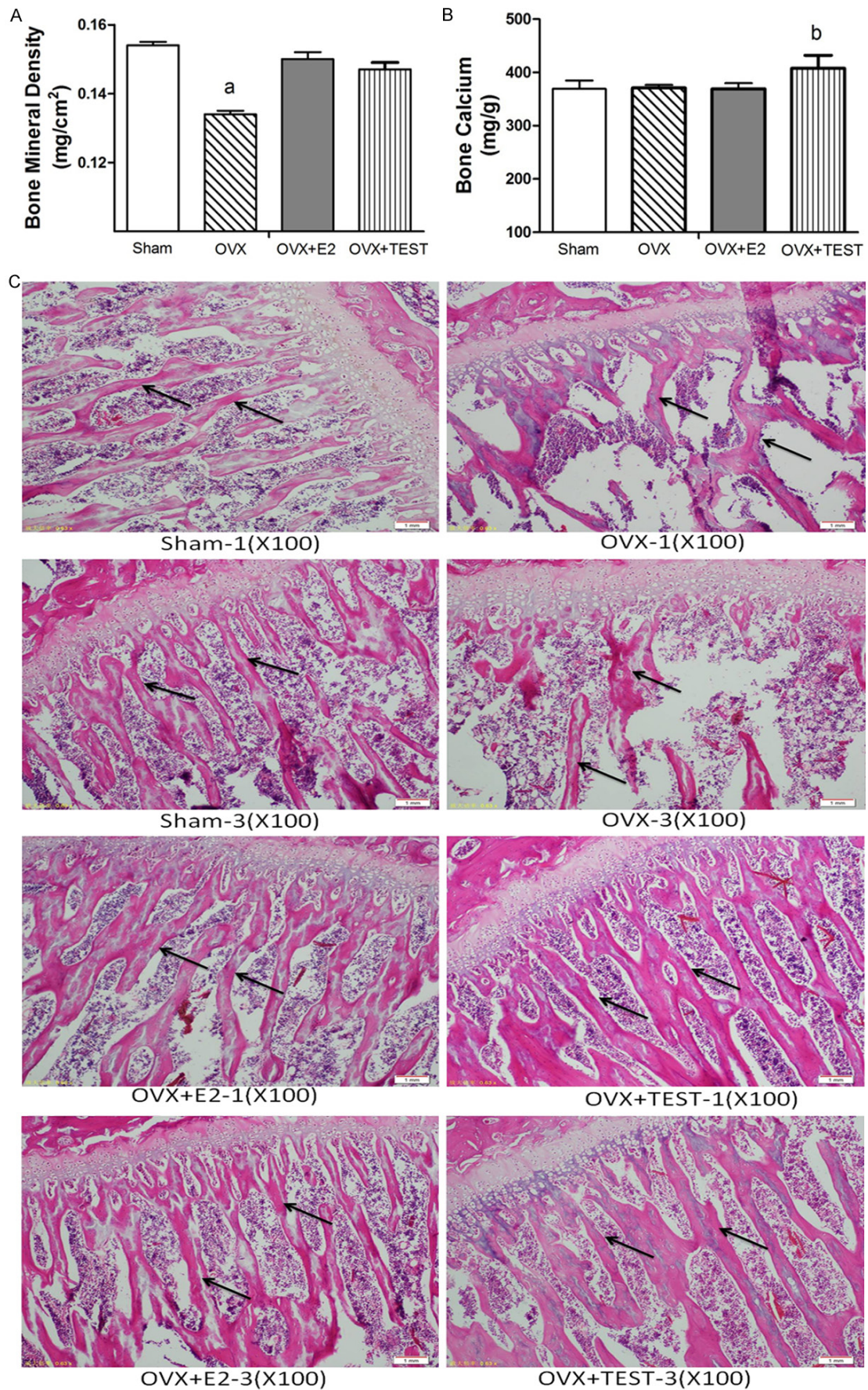


Figure 2. BMD, bone calcium content and tibial tissue microstructure in the rats treated for 12 weeks. A. BMD values, detected with dual-energy X-ray (g/cm^2 ; $n = 6-10$). B. Bone calcium content measured by atomic absorption method (mg/g ; $n = 6-10$). C. HE stained sections of metaphysis tissue at the proximal part of the rat tibia: the red streaks labeled with arrows represent bonetrabecula ($\times 100$). Scale bar, 1 mm (CoA). The values are expressed as the means of 6-10 animals, and the vertical bars represent the standard errors. The letters indicate the statistical significance as revealed by ANOVA (^a $P < 0.05$, OVX versus SHAM, OVX + E2, and OVX + TEST; ^b $P < 0.05$ OVX + TEST versus SHAM, OVX, and OVX + E2).

embedding and sectioning. The paraffin blocks were trimmed first when sectioning and fixed in a LEICA RM 2165 microtome, 6- μm serial sections of the proximal metaphysis of the tibia were cut and stained after drying in accordance with the conventional HE techniques.

Determination of the bone calcium content

(1) Ashing of the femur: The femoral specimen was dissected and the muscle portion was removed. The distal femur was drilled with a needle, and the bone marrow was washed away with saline and dried in a circulated hot air heat box (HeraeusSut 6060, Germany) at 120°C for 1 h. The volumetric bone mineral density (vBMD) (g/cm^3 , physical density) was then measured using an AG balance with density determination software (AG204; Mettler Toledo, Switzerland). The bone volume (cm^3) was calculated from the vBMD value. Then, the femur was degreased by a 2:1 chloroform-methanol mixture for 72 h, dried at 120°C for 6 h, and weighed. The ratio of dry weight to volume was then derived from the volume and weight measured before (g/cm^3). The femur was then placed in a muffle furnace (Shanghai SX2-4-10; Shanghai Dengfeng Electric) for ashing (at 800°C for 6 h) and weighed, after which the ratio of the dry weight to the volume was determined (g/cm^3) again. (2) Determination of the bone calcium content using the atomic absorption method: 100 mg of bone powder was mixed in a 50 ml solution of nitric acid and water at volume ratio of 1:1, heated at 120°C on an electrothermal board for 1 h, and then transferred to a 50-ml volumetric flask for further assay. The instrument used was an inductively coupled plasma emission spectrometer (ICP-AES) obtained from HITACHI, Japan (model, P-4010).

Statistical analysis

Data are presented as mean $\pm$ standard deviations. One-way analysis of variance (ANOVA) was performed using IBM SPSS Statistics

Version20 software, with $P < 0.05$ indicating statistical significance.

Results

HPLC fingerprinting identified three effective ingredients in the anti-OP TCM combination medicine

The HPLC fingerprint of three ingredients was showed in **Figures 1-3**. As in **Figure 1A**, the dominant component in GSB-E was identified as naringin at retention time (Rt) of 34.08 min and neoeriocitrin at RT 29.23 min. The contents of naringin and neoeriocitrin in GSB-E were about 4.0% and 3.2% using external standard calculation method. **Figure 1B** illustrated that the icariin at RT 31.88 min was the leading component in YYH-E. The content of icariin in YYH-E was approximately to 21.7%. **Figure 1C** demonstrated, the peak at RT 25.89 min was identified as salvianolic acid B, which reaches to 14.1% in DS-E. The tanshinonellA, a characteristic component in Salvia miltiorrhiza, was also detected in DS-E at RT 59.76 min with a low content of 0.02%.

The anti-OP TCM combination medicine didn't increase body weight

As shown in **Table 1**, after consecutive feeding for 3 months, the percentage of weight gain in the OVX + estradiol ("E2") rat group (42%) was significantly higher than that in the OVX group, while the percentage of weight gain in the OVX + Epimedium herb/Drynariae rhizome/Salvia miltiorrhiza ("TEST") group was lower at 29%.

The anti-OP TCM combination medicine increased bone mineral density and serum calcium, improving bone microstructure in rats

The BMD of the OVX group, 12 weeks after ovariectomy, was significantly lower than that of the control SHAM group, indicating that the model was successfully established [12] (**Figure 2A**). The BMDs of both the OVX + TEST and OVX + E2 groups were similar, and more

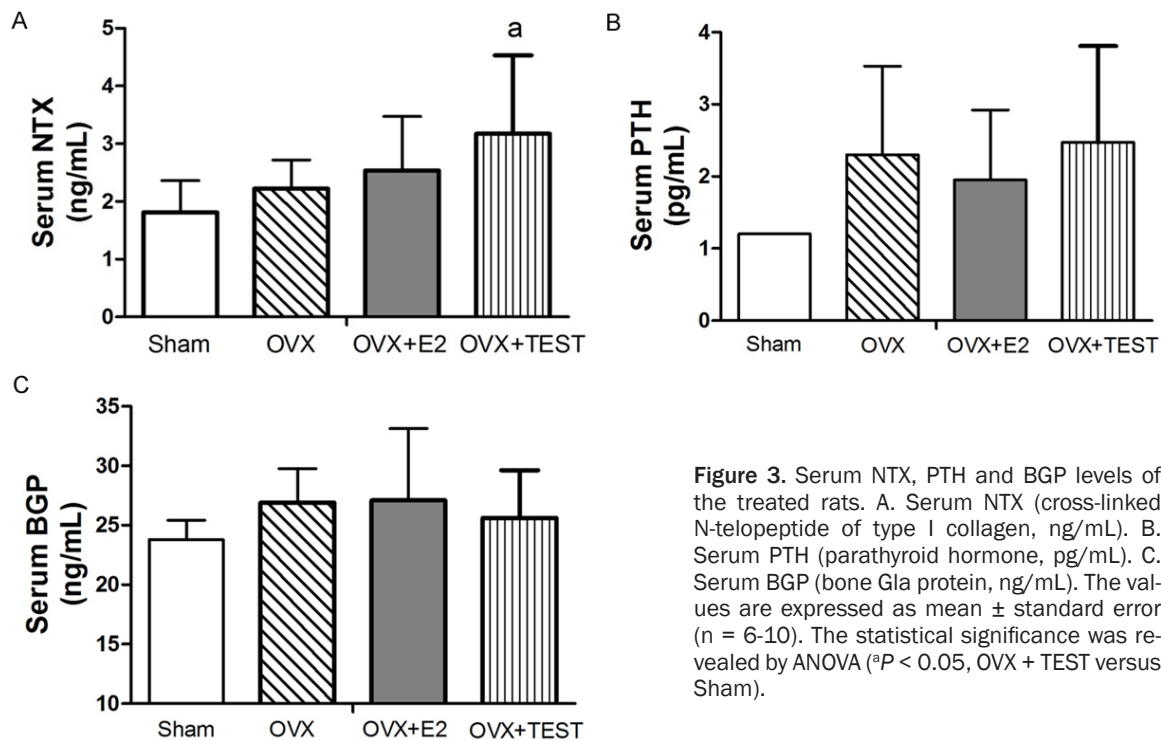


Figure 3. Serum NTX, PTH and BGP levels of the treated rats. A. Serum NTX (cross-linked N-telopeptide of type I collagen, ng/mL). B. Serum PTH (parathyroid hormone, pg/mL). C. Serum BGP (bone Gla protein, ng/mL). The values are expressed as mean $\pm$ standard error (n = 6-10). The statistical significance was revealed by ANOVA (^aP < 0.05, OVX + TEST versus Sham).

importantly, significantly higher than that of the OVX group. As shown in **Figure 2B**, in spite of no difference being observed among OVX, sham and OVX + E2 groups, the bone calcium content of the OVX + TEST group was significantly higher than the rest. Next, the tibial bone tissue microstructures of the animals were studied. The bone trabecula were tightly arranged in the control sham rats, but sparsely arranged in the OVX group 12 weeks after ovariectomy. Furthermore, in the OVX rats, number of trabecula was obviously decreased, often accompanied by visible fractures and big gaps (**Figure 2C**). However, the number of bone trabecula in the OVX + TEST and OVX + estradiol groups was significantly increased, and the morphology of these trabecular were relatively thick and densely arranged.

The anti-OP TCM combination medicine didn't alter levels of serum calcium, phosphorus, 25(OH)D, PTH (parathyroid hormone) and BGP (bone Gla protein)

Next, we found out, whilst all their serum calcium levels being significantly lower than that of the control sham group, there was no marked difference among the OVX, OVX + E2 and OVX + TEST groups (**Table 2**). The serum phosphorus

level was significantly lower in the sham group than the rest, while the OVX + E2 rats had highest levels of phosphorus among all the OVX groups (**Table 2**). The 25(OH)D levels didn't vary among four groups, neither of the PTH and GFP levels. Worth mentioning is that the level of NTX (cross-linked N-telopeptide of type I collagen) was significantly increased in OVX + TEST group, relative to the sham animals, but not to other groups (**Figure 3**).

Discussion

The ovariectomized rat model used in our study is a classic animal model for investigating the pathogenesis of postmenopausal OP and low bone mass, as well as the curative effects of herbal extracts. In such model, our results showed that the TCM treatment combining the Epimedium/Drynariae rhizoma/Salvia miltiorrhizaherbal extracts significantly improved BMD and bone calcium content, as evidenced by the increased number of bone trabeculae, without any impact on the levels of serum calcium, phosphorus, and vitamin D.

Many lines of evidence support a close relationship between body mass and BMD. Zhuang et al. reported that OVX model rats

Table 1. Rat body weight changes before and after treatments

Groups (n = 6-10)	Pre-treatment weight (g)	Post-treatment weight (g)	Percentage weight gain before and after administration
SHAM	250.00 ± 10.41	290.67 ± 21.70	16%
OVX	247.89 ± 12.42	327.00 ± 17.91	32%
OVX + E2	247.89 ± 12.42	353.00 ± 20.03 ^a	42%
OVX + TEST	262.56 ± 19.73	338.00 ± 34.76	29%

Note: Results are expressed as mean ± standard deviation. ^aP < 0.05, OVX versus OVX + E2 (significance values according to ANOVA).

Table 2. Serum calcium, phosphorus and 25(OH)D concentrations of treated rats

Groups (n = 6-10)	Serum calcium (mmol/L)	Serum phosphorus (mmol/L)	25(OH)D (ng/mL)
Sham	2.44 ± 0.04	2.13 ± 0.76	24.49 ± 18.89
OVX	2.32 ± 0.09 ^a	2.47 ± 0.16 ^a	28.67 ± 4.35
OVX + E2	2.33 ± 0.07	2.81 ± 0.51 ^b	23.75 ± 2.95
OVX + TEST	2.31 ± 0.13	2.38 ± 0.23 ^c	34.00 ± 15.71

Results are expressed as mean ± standard deviation. ^aP < 0.05, OVX versus SHAM; ^bP < 0.05, OVX + E2 versus OVX; ^cP < 0.05, OVX + TEST versus OVX + E2 (significance according to ANOVA).

gained weight along with decreased BMD, and that the administration of Chinese herbal medicine increased the BMD while slowing down the weight gain [13]. This is consistent with our study showing weight and BMD changes of the OVX rats. More interestingly, our anti-OP TCM combination medicine with Epimedium herb, Drynariae rhizoma and Salvia miltiorrhiza significantly alleviated the weight gain and BMD loss of OVX animals, to the extent of being similar or even better than estrogen treatment.

The main component in the Epimedium herb is a phytoestrogen that belongs to the flavonoid family, which plays a role in monitoring the function of the hypothalamus-pituitary-gonad axis and improving metabolism of the bone during menopause. With a relatively mild effect, it is suitable for long-term use to prevent and improve low bone mass, i.e. OP [10, 14]. Besides BMD, the bone calcium content of the combination medicine treated rats was markedly higher than that of the estrogen group, implying that the improved BMD was probably via an increase in the bone calcium. Phytoestrogen-like compounds have a similar structure as estrogen and binding affinity to estrogen receptors, that probably promotes the calcium absorption in the gut or calcium deposition in

bony tissue through the estrogen receptor pathway [15-18].

It has also been reported that Epimedium herb extract reduced loss of calcium and phosphorus in emasculate rats [19]. In our study, we then determined the levels of serum calcium, phosphorus and vitamin D, and found the difference too little to tell compared with the control group. Given the fact that the bone calcium content was increased by our combination medicine, the effective components of Epimedium herb, Drynariae rhizoma and Salvia miltiorrhiza extracts might induce an increase of calcium deposition in bone tissues. Of note, in our study we only measured the serum calcium levels at the treatment endpoint of 3 months, with data points missing during the treatment. Future investigation on this herbal medicine will be conducted to collect more serum samples to better understand its effect on absorption of dietary calcium, in a temporal manner. Our histopathology analysis suggested that the herbal combination medicine dramatically improved bone microstructure of OVX rats by increasing number of bone trabecula and trabecular connections. As the bone strength is positively associated with proper bone structure, the use of this herbal medicine could effectively prevent fractures caused by menopausal OP. We further determined the effects of combination herbal medicine on serum NTX, PTH and BPG levels in OVX rats. NTX, a degraded product of type I collagen, is an accurate marker for the levels of bone matrix collagen degradation [20]. Little difference was observed for serum NTX concentrations in the herbal combination treated OVX rats compared with the OVX group and estrogen group, suggesting the no effect on bone collagen degradation. Further tests ruled out any impact of this herbal concoction on serum BGP and PTH.

Given validated evidence establishing a clear association between estrogen deficiency and postmenopausal OP, Denmark has promoted the use of estrogen to prevent postmenopausal

OP and achieved promising results [21]. However, estrogen therapy has numerous severe side effects such as increased risk of endometrial and breast cancers [22]. Here, we demonstrated that the herbaceous plant extracts from Epimedium herb, Drynariae rhizoma and Salvia miltiorrhizain combination effectively improved the BMD and bone microstructure of OVX rats, probably via a mechanism of promoting calcium deposition in the bone. Meanwhile, this herbal combination also ameliorated the weight gain problem induced by ovariectomy, therefore proving to be a promising new alternative therapy for prevention and treatment of OP. Future effort will be dedicated to delineating the detailed mechanism of how the herbal combination affects bone metabolism, digestion and absorption of dietary calcium.

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Disclosure of conflict of interest

None.

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