Tanshinone prevents cancellous bone loss induced by ovariectomy in rats[1]

Liao CUI[2,3], Tie WU[3], Yu-yu LIU[3], Yi-feng DENG[4], Chun-mei AI[4], Huai-qing CHEN[2]

[2]Institue of Biomedical Engineering, West China Medical Center, Sichuan University, Chengdu 610041, China;
[3]Department of Pharmacology, [4]Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical College, Zhanjiang 524023; China

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ABSTRACT

AIM: To investigate the skeletal effects of total tanshinone in ovariectomized rats by analyzing cancellous bone histomorphometry of fourth lumbar vertebrae (LV4) and proximal tibial metaphyses (PTM). **METHODS:** Four-month-old Sprague-Dawley female rats were sham-operated and treated with vehicle or ovariectomized and treated with either vehicle, total tanshinone (200 $mg \cdot kg^{-1} \cdot d^{-1}$, equivalent to 35 $\mu g \cdot kg^{-1} \cdot d^{-1}$ of tanshinone II A and 16 $mg \cdot kg^{-1} \cdot d^{-1}$ of cryptotanshinone), or 17α-ethynylestradiol (30 $\mu g \cdot kg^{-1} \cdot d^{-1}$ as positive treatment group) starting one day post-surgery for 10 weeks. Double *in vivo* fluorochrome labeling was administered to all rats. The undecalcified longitudinal LV4 and PTM sections were cut and stained with Goldner's Trichrome (4-μm thickness) or unstained (8-μm thickness) for the bone histomorphometric analysis. **RESULTS:** A significant decrease in trabecular bone volume (BV/TV) and trabecular number (Tb.N) and a significant increase in osteoclast surface (OCS/BS) and mineralizing surface (MS/BS) were found in both LV and PTM of vehicle-treated OVX rats compared with sham controls. Tanshinone completely prevented the decreases in BV/TV and Tb.N and the increase in OCS/BS in the LV4, and partially prevented the decreases in BV/TV and Tb.N in the PTM of OVX rats. In addition, tanshinone increased trabecular thickness (Tb.Th) whereas it did not alter MS/BS. Moreover, tanshinone had no effect on uterine weight and body weight of OVX rats. Estrogen treatment increased BV/TV and Tb.N and decreased OCS/BS, but, also markedly decreased MS/BS and increased uterine weight in OVX rats. **CONCLUSION:** The current study demonstrated that the adequate supply of tanshinone prevented OVX-induced cancellous bone loss in rats through inhibition of elevated bone resorption.

INTRODUCTION

Tanshinone is the fat soluble extract of Danshen (*Salvia miltiorrhiza* Bunge), a traditional herbal medicine, which has been widely used in clinical practice for the prevention of cardiac diseases, arthritis and other inflammation-related disorders based on its pharmacological actions in multiple tissues[1]. The known bioactive compounds in fat soluble extract of danshen are tanshinone IIA and cryptotanshione[2]. Recently, the skeleton effects of danshen have been under investigation. Studies have shown that the original herbal medicine of danshen accelerated fracture healing in

[1] Project supported by the Guangdong Natural Science Foundation, No 020554.
[2] Correspondence to Dr Liao CUI. Phn 86-759-238-8405. Fax 86-759-223-4434. E-mail cui_liao@yahoo.com
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rats[3,4] and, prevented femoral head necrosis induced by glucocorticoid treatment in human[5] and inhibited the increase of bone resorption markers in postmenopausal women[6]. However, there are no studies have been done to demonstrate quantitatively the effects of bioactive compounds of Danshen on bone in animal models of osteoporosis. Therefore, the aim of this study was to investigate the skeletal effects of tanshinone by applying quantitative analysis of cancellous bone histomorphometry in ovariectomized rat, an animal model for postmenopausal bone loss.

MATERIALS AND METHODS

Drugs and reagents Total tanshinone (Hebei Xinglong Pharmaceutical Co China, Lot 010316), containing 17 % of tanshinone II A and 8 % of cryptotanshinone, respectively (assayed by HPLC); 2-fluoro-17alpha-ethynylestradiol, calcein, tetracycline hydrochloride, iron hematoxylin, anhydrous ferric, beibrich scarlet, acid fuchsin, and fast green were all purchased from Sigma Chemical Co, USA; methyl methacrylate (Beijing Chemical Factory), dibutyl phthalate (Guangdong Shantou Chemical Co) and benzoyl peroxide (Hubei University Chemical Factory) were purchased as indicated.

Animals and study protocol Thirty-two 4-month-old Spragus-Dawley female rats, weighing 293±20 g, were acclimated to local vivarium conditions (temperature 24-26 ºC, humidity 67 %) and allowed free access to water and diets containing 1.33 % calcium, 0.95 % phosphorus and 30 IU % vitamin D3. The experiment was conducted according to SPF (special pathogen free) animal care-approved protocols, Animal Center of Guangdong Medical College, Zhanjiang, China. Eight rats were sham-operated and treated with vehicle (deionized water) as aging control (Sham+Veh). The remaining rats were bilaterally ovariectomized and randomly divided into three groups with 8 per group. They were treated with either vehicle (OVX+Veh), total tanshinone at dose of 200 $mg \cdot kg^{-1} \cdot d^{-1}$ (equivalent to 35 $mg \cdot kg^{-1} \cdot d^{-1}$ of tanshinone II A and 16 $mg \cdot kg^{-1} \cdot d^{-1}$ of cryptotanshinone (OVX+Tan), or 17α-ethynylestradiol at dose of 30 $\mu g \cdot kg^{-1} \cdot d^{-1}$ as positive treatment group (OVX+EE) for 10 weeks. Rats received treatments po starting from one day after surgeries. For *in vivo* fluorochrome labels, tetracycline (20 $mg \cdot kg^{-1}$) and calcein (10 $mg \cdot kg^{-1}$) were sc injected to the rats on days -14, -13 and days -4, -3 before death. At necropsy, rats were killed by cardiac puncture under anesthesia. The uterus were removed and weighed.

Cancellous bone histomorphometry The fourth lumbar vertebrae (LV4) and the right proximal tibial metaphysis (PTM) were opened to expose the marrow cavity using an isomet low speed saw (Buechler LTD, USA) and fixed in 10 % phosphate buffered formalin for 24 h. They were then dehydrated in ethanol, defatted in xylene and embedded undecalcified in methyl methacrylate[7]. The frontal sections were cut at 4-μm and 10-μm thickness with microtome (Leica RM2155, Germany). The 4-μm section was stained with Goldner's Trichrome staining for static histomorphometric measurements, the unstained 10-μm sections were used for dynamic histomorphometric analyses[8].

A semi-automatic digitizing image analysis system (Osteometrics, Inc Cecatur, GA, USA) was used for quantitative bone histomorphometric measurements. The studied region of LV4 was cancellous bone between 0.5 mm distal to two ends of growth plate. The studied region of PTM was cancellous bone between 1 and 4 mm distal to the growth plate-epiphyseal junction. The respectively omitted 0.5 and 1 mm region to the growth plate of LV4 and PTM was to exclude the primary spongiosa as illustrated in Fig 1. Static measurements included total tissue area (TV), trabecular area (TbAr), trabecular bone surface (BS) and osteoclast surface (OCS). Dynamic measurements include single-labeled surface (sLS), double labeled surface (dLS) and interlabel width (IntWi). These parameters were used to calculate trabecular bone volume (BV/TV, %), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp), percent osteoclast surface (OCS/BS), percent mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR) per unit of bone volume (BFR/BV), BFR per unit of bone tissue area (BFR/TV), and BFR per unit of bone surface (BFR/BS) as previously described[8,9].

Statistic analysis Data were expressed as mean±SD. The statistical differences among groups were evaluated using variance (ANOVA) with Fisher's PLSD test. $P<0.05$ was considered significant.

RESULTS

Effects of OVX and drug treatment on body weights and uterine weights Body weights were significantly increased and uterine weights were significantly decreased in vehicle-treated OVX rats compared with sham controls at 10 weeks post-surgery. Tanshinone treatments had no effect on body weights

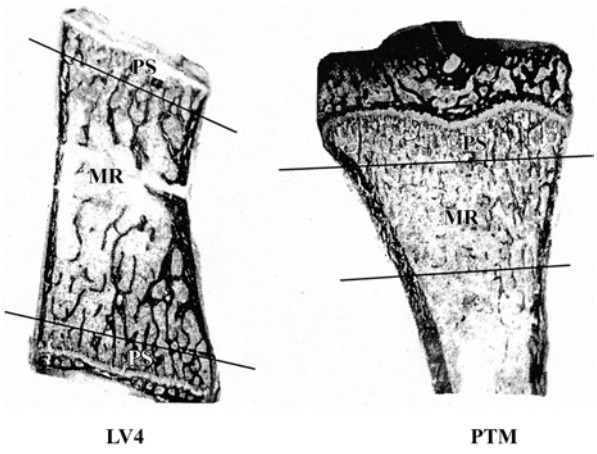

Fig 1. Illustration of the measurement regions (MR) of LV4 and PTM. Note the MR excluding the primary spongiosa (PS).

Tab 1. Effects of tanshinone on body weights and uterine weights. *n*=8. Mean±SD. $^{c}P<0.01$ *vs* Sham. $^{f}P<0.01$ *vs* OVX.

Parameters	Body weight/g (endpoint)	Uterine weight/g
Sham+Veh	318.1±16.2	0.27±0.07
OVX+Veh	375.6±27.3^{c}	0.04±0.01^{c}
OVX+Tan	388.7±37.1^{c}	0.05±0.02^{c}
OVX+EE	349.4±36.0^{f}	0.17±0.06^{f}

Sham: sham operated rats treated with vehicle; OVX: ovariectomized rats treated with vehicle; OVX+Tan: ovariectomized rats treated with tanshinone; OVX+EE: ovariectomized rats treated with 17α-ethynylestrodiol.

and uterine weights while EE treatment decreased body weight and increased uterine weight significantly in OVX rats compared with vehicle treatment (Tab 1).

Effects of OVX and drug treatment on cancellous bone of LV4 BV/TV was significantly decreased (-20 %, $P<0.01$) in the vehicle-treated OVX rats relative to sham-operated rats. A significant decrease in TbN and increase in TbSp and OCS/BS, and a trend for increased MS/BS, BFR/BS and BFR/BV were also observed in the vehicle-treated OVX rats when compared with sham-operated controls. Tanshinone treatment completely prevented OVX-induced bone loss as BV/TV was increased by 33 % compared with vehicle-treated OVX rats and by 6 % compared with sham-operated control. Treatment of OVX rats with tanshinone increased Tb.Th ($P<0.01$) and decreased OCS/BS ($P<0.01$), but had no effect on MS/BS, BFR/TV and BFR/BV when compared with vehicle treatment. Treatment with EE significantly increased BV/TV and TbN, and decreased OCS/BS in OVX rats compared with vehicle treatment. In addition, EE treatment significantly decreased MS/BS, BFR/BV and BFR/BS in these animals (Fig 2A, 3 and Tab 2).

Effects of OVX and drug treatment on cancellous bone of PTM A marked bone loss (BV/TV by -74 %, $P<0.01$) was seen in the vehicle-treated OVX rats when compared with sham controls. This bone loss was accompanied with a significant decrease in TbN and increase in TbSp, OCS/BS and bone formation indices such as MS/BS, MAR, BFR/BS and BFR/BV. The following changes were observed in tanshinone-treated OVX rats: 1) BV/TV was significantly higher than that in vehicle-treated OVX rats (+84 %, $P<0.01$), but it was still significant lower than that in sham controls; 2) TbTh was significantly increased compared

Tab 2. Effects of tanshinone on selected histomorphometric changes of LV4. *n*=8. Mean±SD. $^{c}P<0.01$ *vs* Sham. $^{f}P<0.01$ *vs* OVX.

Parameters	Trabecular number/mm^{-1}	Trabecular separation/μm	Mineral apposition rate/μm·d^{-1}	Bone formation rate/BS (μm/d×100)	Bone formation rate/BV (%/year)	Bone formation rate/TV (%/year)
Sham+Veh	3.39±0.28	235.7±22.2	0.30±0.14	1.8±1.0	19.6±12.8	3.7±1.9
OVX+Veh	2.73±0.42^{c}	315.4±64.3^{c}	0.39±0.17	3.3±1.7	33.9±15.8	5.6±1.6
OVX+Tan	3.19±0.38^{f}	249.4±40.5^{f}	0.38±0.06	3.5±0.9	31.6±7.3	6.8±2.1
OVX+EE	3.17±0.06^{f}	260.5±39.4^{f}	0.32±0.07	1.7±0.4	17.4±5.5^{f}	3.3±1.2

Sham: sham operated rats treated with vehicle; OVX: ovariectomized rats treated with vehicle; OVX+Tan: ovariectomized rats treated with tanshinone; OVX+EE: ovariectomized rats treated with 17α-ethynylestrodiol.

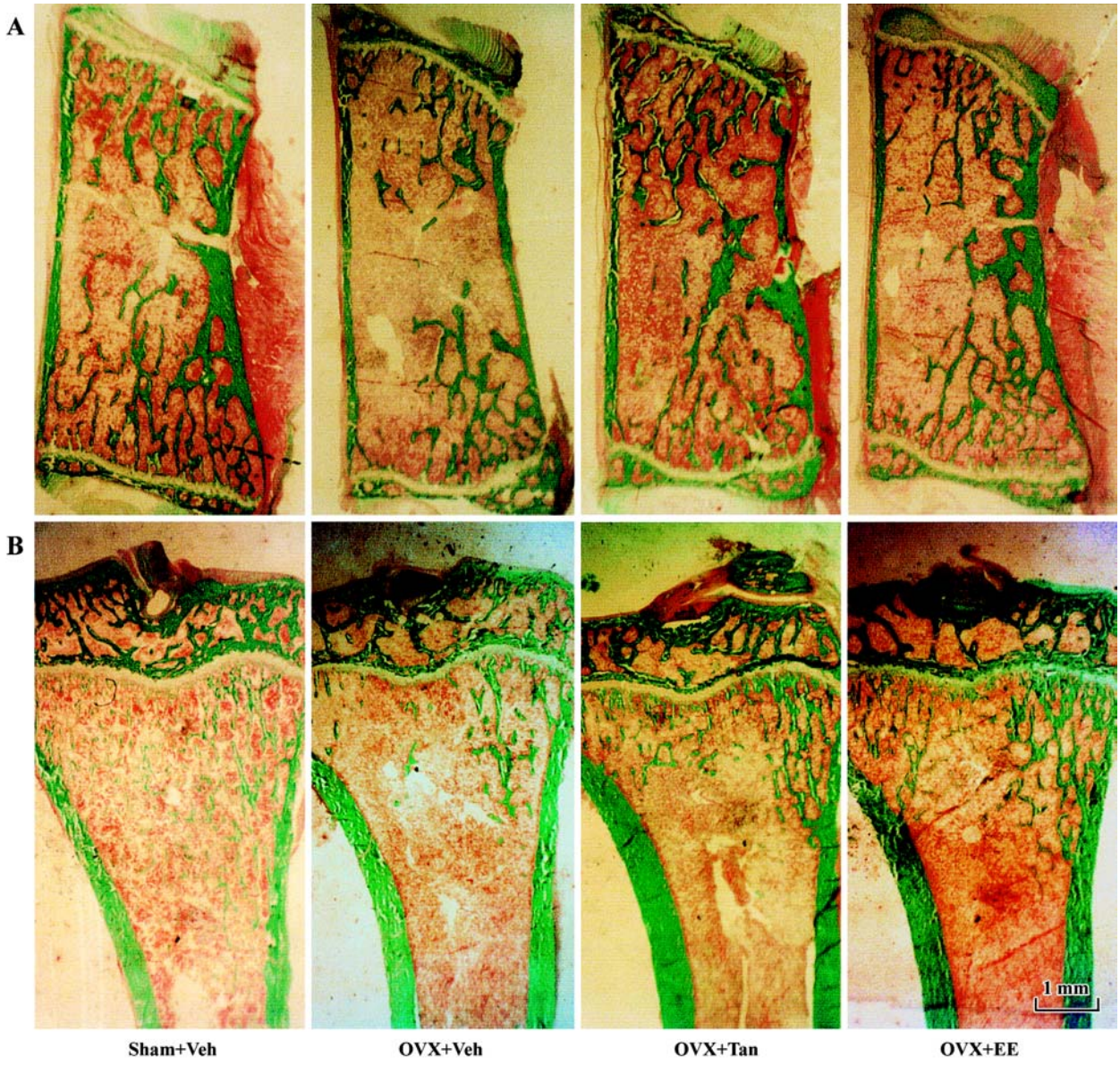

Fig 2. Microphotographs of the sagittal sections of fourth lumber vertebrae (LV4, A) and longitudinal sections of right proximal tibial metaphysis (PTM, B) from sham control (Sham+Veh), OVX control (OVX+Veh), and OVX rats treated with total tanshinone (OVX+Tan) and 17α-ethynylestradiol (OVX+EE) for 10 weeks. Less trabecular bone and poor trabecular structure are seen in the OVX rats compared with sham control. Tan and EE treatment completely prevent cancellous bone loss in LV4 but partially prevent cancellous bone loss in PTM. Goldner's Trichrome staining, A) original magnification×20; B) original magnification ×12.

with both sham and OVX rats treated with vehicle; 3) OCS/BS was significantly lower than that in vehicle-treated OVX rats but still significantly higher than that in sham rats; 4) MS/BS and BFR/BS were not different from vehicle-treated OVX rats whereas MAR and BFR/BV were significantly decreased compared with vehicle-treated OVX rats. Similar to tanshinone treatment, EE partially prevented bone loss at the PTM of OVX rats. However, it significantly decreased MS/BS and BFR/BV and had no effect on TbTh in OVX rats (Fig 2B, 3 and Tab 3).

DISCUSSION

Our study for the first time demonstrated that total tanshinone prevented cancellous bone loss induced by estrogen deficiency in OVX rats. It is well known[10] and also confirmed by HPLC that the total tanshinone mainly contain tanshinone II A and cryptotanshinone. Although it cannot be completely excluded that the pos-

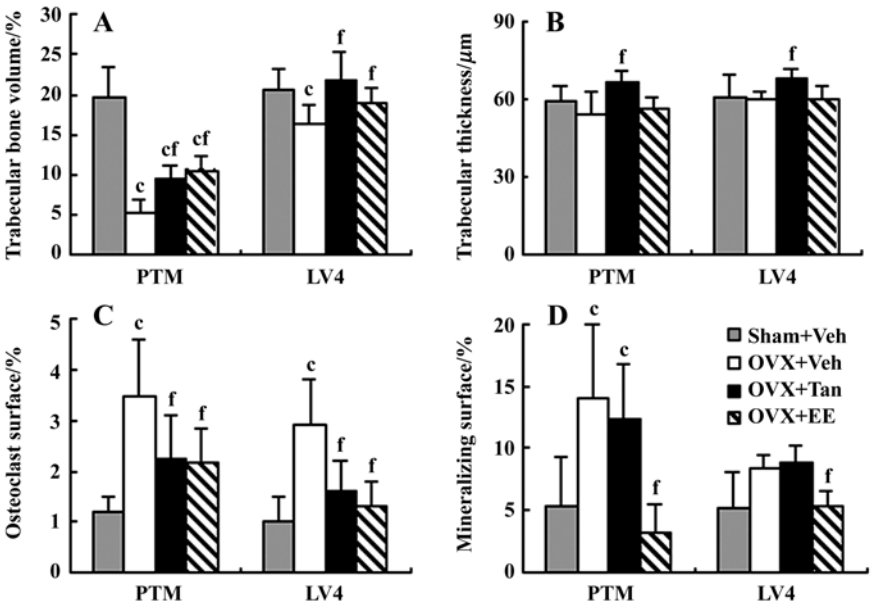

Fig 3. Effects of sham controls (Sham+Veh), ovariectomized rats treated with vehicle (OVX+Veh), ovariectomized rats treated with tanshinone (OVX+Tan) and ovarietomized rats treated with 17α-ethynylestradiol (OVX+EE) on selected bone histomorphometry of trabecular bone volume (A), trabecular thickness (B), osteoclast surface (C) and mineralizing surface (D) in proximal tibiae metaphysis (PTM) and in fourth lumbar vertebrae (LV4). *n*=8. Mean±SD. $^{c}P<0.01$ *vs* Sham. $^{f}P<0.01$ *vs* OVX.

Tab 3. Effects of tanshinone on selected histomorphometric changes of PTM. *n*=8. Mean±SD. $^{c}P<0.01$ *vs* Sham. $^{f}P<0.01$ *vs* OVX.

Parameters	Trabecular number/mm^{-1}	Trabecular separation/μm	Mineral apposition rate/μm·d^{-1}	Bone formation rate/BS (μm/d×100)	Bone formation rate/BV (%/year)	Bone formation rate/TV (%/year)
Sham+Veh	3.36±0.58	247.1±55.1	1.49±0.21	8.24±6.47	81.5±60.8	15.5±10.5
OVX+Veh	1.01±0.47^{c}	1159±555^{c}	2.14±0.4^{c}	29.2±18.3^{c}	321.3±166.2^{c}	15.9±7.9
OVX+Tan	1.42±0.31	667.3±167.2^{cf}	1.41±0.24^{f}	17.9±9.54^{c}	164.5±86.9^{cf}	15.9±9.58
OVX+EE	1.61±0.52^{f}	613.2±182.2^{cf}	1.16±0.21^{cf}	5.08±3.37^{f}	57.7±38.6^{f}	4.60±3.74^{cf}

Sham: sham operated rats treated with vehicle; OVX: ovariectomized rats treated with vehicle; OVX+Tan: ovariectomized rats treated with tanshinone; OVX+EE: ovariectomized rats treated with 17α-ethynylestradiol.

sibility of other compounds in total tanshinone have effects on bone, our findings support additional studies to further examine tanshinone II A and cryptotanshinone for their biological activities on bone and their potentials for the managements of osteoporosis. The dose of total tanshinone given to the rats in this study was equivalent to the dose (3 g per day) used for the treatment of bone marrow inflammation in human, based on the dose translating factor (0.018) between human and rat[11].

The significant cancellous bone loss induced by estrogen depletion in OVX rats shown in the current study was consistent with previous studies[8,12], This bone loss in OVX rats was more rapid at the appendicular bone site (PTM) than at axial bone site (LV), a phenomenon was also noted by Ke *et al*[13]. The markedly increased bone turnover rate as indicated by increased BFR/BV in PTM was believed to be the cause of faster bone loss at this bone site after OVX. Treatments of OVX rats with tanshinone completely prevented bone loss and restored bone mass above sham level in LV, but only partially prevented bone loss in

PTM. The more favorable effect of tanshinone on LV may be due to the slower rate of bone loss at this bone site than PTM after OVX. Higher doses of tanshinone may be required to completely prevent aggressive bone loss at bone sites like the PTM of OVX rats. The crude extracts of Danshen have been shown to inhibit osteoclast activity *in vitro*[14,15] and the main bioactive component tanshinone II A has been shown to inhibit osteoclast activities *in vitro* in our pilot study. Consistent with these results, data from the current study showed that tanshinone significantly decreased osteoclast surface in OVX rats. Taken together, these data suggest that the protective effects of tanshinone on bone in OVX rats are likely due to a suppression of the increased bone resorption by estrogen depletion.

Unlike well-known antiresorptive agents estrogen, as shown in this study, which inhibit bone resorption accompanying with a subsequent decrease of bone formation through coupling mechanism[16], tanshinone treatment only decreased mineral apposition rate but not mineralizing surface at the PTM of OVX rats. In addition, all bone formation indices such as mineralizing surface, mineral apposition rate, and bone formation rates were not altered by tanshinone treatment at the LV in OVX rats. Furthermore, tanshinone also thickened the trabecula whereas estrogen did not have such effect as shown in the current study. These results indicate that tanshinone does not inhibit osteoblast bone formation occurred on bone surface while inhibiting bone resorption thus causes a positive bone balance resulting in bone gain. Whether tanshinone stimulate osteoblasts and bone formation *in vivo* needs further investigation although the original herbal medicine of Danshen has been shown to stimulate the differentiation of osteoblast-like MC3T3-E1 cells *in vitro*[17]. The potential stimulatory effects of tanshinone on osteoblasts suggest additional studies designing to evaluate the efficacy of tanshinone in animal models with low bone turnover such as aging and glucocorticoid-induced osteopenia.

Clinical studies have indicated that estrogen replacement therapy (HRT) significantly increases the risk for breast and endometrial cancers in postmenopausal women and has other undesirable side effects[18], which reduce the compliance of HRT in postmenopausal women. Different from estrogen, tanshinone, had no effect on uterine weight while protecting against bone loss induce by estrogen depletion in OVX rats as shown in the current study. This finding suggests that tashinone treatment may potentially provide safety advantage over HRT in clinical applications.

In conclusion, our study demonstrated that tanshinone prevented cancellous bone loss induced by ovariectomy in rats. The inhibition of elevated bone resorption is likely the main mechanism of such bone protective effects.

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