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Original Article

The osteoprotective effect of *Herba epimedii* (HEP) extract *in vivo* and *in vitro*

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Herba epimedii (HEP) is one of the most frequently used herbs prescribed for treatment of osteoporosis in China. In the present study, the *in vivo* effects of HEP extract on bone metabolism were evaluated using 4-month-old ovariectomized (OVX) or sham-operated (Sham) female Sprague-Dawley rats orally administered with HEP extract (110 mg $kg^{-1}d^{-1}$), 17ß-estrogen (2 mg $kg^{-1}d^{-1}$) or its vehicle for 3 months. HEP extract significantly decreased urinary calcium excretion, suppressed serum alkaline phosphatase (ALP) activity and urinary deoxypyridinoline levels in OVX rats ($P < 0.05$ versus vehicle-treated OVX rats). Histomorphometric analysis indicated that HEP extract could prevent OVX-induced bone loss by increasing tibial trabecular bone area and decreasing trabecular separation in OVX rats ($P < 0.05$ versus vehicle-treated OVX group). The *in vitro* effects of HEP extract were also studied using rat osteoblast-like UMR 106 cells. HEP extract significantly stimulated cell proliferation in a dose-dependent manner ($P < 0.01$ versus vehicle-treated) and increased ALP activity at 200 μg ml^{-1} ($P < 0.01$ versus vehicle-treated) in UMR 106 cells. It modulated osteoclastogenesis by increasing osteoprotegrin (OPG) mRNA and decreasing receptor activator of NF-κB ligand (RANKL) mRNA expression, resulting in a dose-dependent increase in OPG/RANKL mRNA ratio ($P < 0.01$ versus vehicle-treated). Taken together, HEP treatment can effectively suppress the OVX-induced increase in bone turnover possibly by both an increase in osteoblastic activities and a decrease in osteoclastogenesis. The present study provides the evidence that HEP can be considered as a complementary and alternative medicine for treatment of postmenopausal osteoporosis.

Keywords: *Herba Epimedium* – osteoblast-like UMR 106 cells – osteoclastogenesis – osteoporosis – ovariectomized rat

Introduction

Chinese herbal medicine has been widely used for thousands of years for the treatment of fractures and joint diseases. The aerial part of *Herba epimedii* (HEP) is commonly used in traditional Chinese medicine for 'strengthening the *kidney*' (1,2). Based on theories of Chinese medicine, the *kidney* (different from the organ kidney in a modern sense) is responsible for the nourishment of bone and supports gonadal functions. Herbal formulas classified as kidney-tonifying are thus traditionally used in cases of bone diseases and gonadal dysfunction. Eight of the 16 species of the *Epimedium* genus have been used for centuries in traditional Chinese herbal formulations to treat a wide range of diseases, including osteoporosis (3).

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In the past, the development of herbal anti-osteoporosis formulas was pursued mainly by scientists in Asian countries, including China, Japan and Korea (4–6). However, as a result of recent evidence that estrogen replacement therapy (ERT) is associated with increased risk of breast, ovarian and endometrial cancer in postmenopausal women (7,8), it is now generally recognized that alternative approaches to the prevention and treatment of osteoporosis might be worth exploring.

HEP is one of the most frequently used herbs in formulas that are prescribed for the treatment of osteoporosis in China (9). Over the years, numerous scientific studies have been carried out in China on the *in vivo* as well as *in vitro* effects of this herb on bone and mineral metabolism. However, these studies have been largely inaccessible to international scholars since they were published in Chinese-language journals. Previous studies by various scientists in China have repeatedly shown that HEP extract, either as a single herbal extract or in a composite formula, could reduce bone loss in an ovariectomized (OVX) rat model (2,10–13) as well as in aged (14) or other rat (15,16) models. A short-term clinical study involving postmenopausal women (1) has also demonstrated that HEP extract could prevent bone loss and increase osteocalcin and E_2 levels. In addition, the total flavonoid fraction of HEP can improve bone mineral density, enhance the E_2 level and decrease the circulating IL-6 level in OVX rats (13). *In vitro* experiments showed that the flavonoid fraction of HEP could promote cell proliferation and increase alkaline phosphatase (ALP) activity in primary rat calvarial osteoblasts (17–19).

With the recent discovery of the receptor activator of NF-κB ligand (RANKL)–RANK interaction, the role of osteoblasts in osteoclast differentiation is now clearly defined (20–23). The binding of RANKL (the membrane-associated factor) on osteoblastic cells to RANK on the osteoclast cell surface results in the induction of osteoclast function. At the same time, the secretion of osteoprotegrin (OPG, the soluble decoy receptor of RANKL) (20,21,24) by osteoblasts can interfere with RANKL–RANK interactions, thereby modulating osteoclastogenesis. Thus, the effects of HEP on the expression of RANKL and OPG mRNA in UMR 106 cells can be studied to assess its potential effects on osteoclastogenesis.

In the present study, we aimed to systematically evaluate the *in vivo* and *in vitro* effect of HEP extract on bone and mineral metabolism. This study is designed to demonstrate the efficacy of using HEP extract in the treatment of osteoporosis as well as to delineate its molecular actions in modulating osteoblastic and osteoclastic activities.

Methods

Preparation of Crude Extracts of HEP

The dry aerial part of *Epimedium brevicornum Maxim* was purchased from Shenyang Northeast Drugstore and authenticated by Prof. Zerong Jiang, Shenyang Pharmaceutical University. It was extracted with boiling water three times. The decoction was then concentrated and spray-dried, giving a yield of extraction of ~10%. Analysis of the chemical composition of HEP found that flavonol glycosides, especially icarrin, were the major and active components (3,25–27). The flavonoid components of crude extract powder were analyzed according to the method given in the Chinese medicine pharmacopoeia (28). The standard solution contains 10 μg ml^{-1} of icarrin. Total flavonoid content was ~9.6 ± 0.3% of HEP crude extract. An HPLC spectrum of the HEP extract is shown in Fig. 1.

Animal Study

A total of 35 virgin Sprague–Dawley specific-pathogen-free (SPF) female rats (Laboratory Animal Center, Guangzhou University of Traditional Chinese Medicine) were used in this study. The rats were ~3 months old upon arrival, and 4 months old upon the commencement of herbal treatment. The rats were housed in cages under a 12 h light/12 h dark cycle at 22°C. Deionized water was provided to the animals *ad libitum*. During the study, OVX rats were pair-fed with normal diet based on the average weekly food consumption of the sham control group. The animals were weighed at bi-weekly intervals throughout the study period. Husbandry of the animals was based on the Guide for Care and Use of Laboratory Animals (29).

The animals were either sham-operated (sham, $n = 8$) or ovariectomized (OVX, $n = 27$). The OVX rats were randomly divided into three groups: vehicle treated (OVX, $n = 8$); estrogen treated (E_2, $n = 10$); and HEP extract treated (HEP, $n = 9$). The animals were monitored for 4 weeks before initiation of the therapeutic regime to allow them to recover from the operation. HEP extract powder was dissolved in deionized distilled water and was orally administered to rats at a dosage of 110 mg kg^{-1} d^{-1} for 3 months. The dosage of HEP for rats in this study was determined based on the dosage used in clinical trials and calculated using the dose conversion table between human and rats. Oral E_2 treatment was given at a dosage of 2 mg kg^{-1} d^{-1} for 3 months. Twenty-four hour urine samples were collected using separators by placing animals in metabolic cages during the last week. Urine samples were acidified with 2 ml of 1 M HCl and centrifuged at 1015 *g* for 10 min at 4°C to remove contaminating sediments; aliquots were stored at −20°C until they were assayed. After 3 month herbal treatments, the rats were killed under deep ether anesthesia, blood samples were collected and the uteri were weighed. Blood samples were allowed to clot at room temperature and the serum was separated by centrifuging at 1015 *g* for 20 min. Serum samples were stored at −70°C until analysis. Left femora and tibias were removed and wrapped in gauze saturated with physiological saline and stored at −20°C until use.

Biochemical Analysis of Serum and Urine Samples

The calcium (Ca) and phosphorus (P) concentrations of both serum and urine samples were measured using standard colorimetric methods with commercial kits (ZhongSheng BeiKong

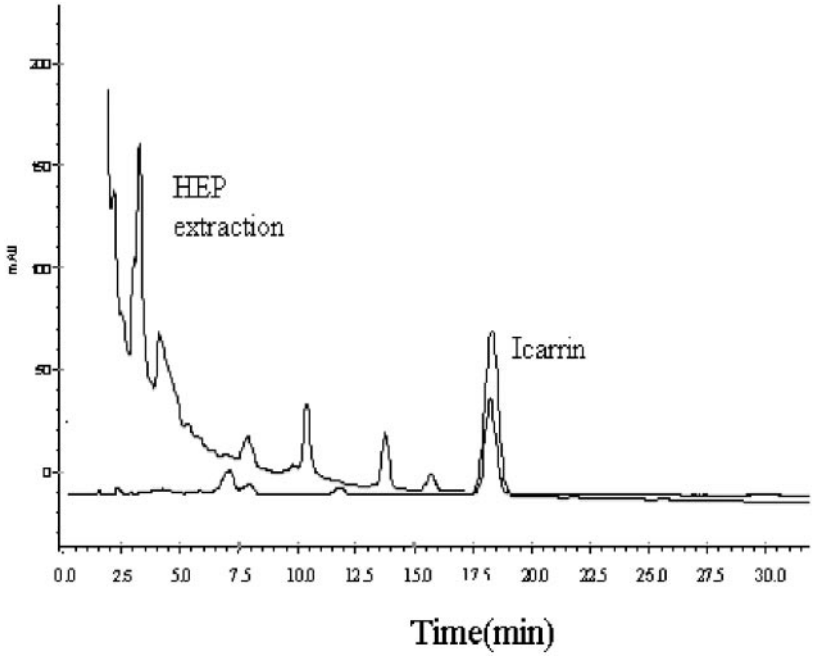

Figure 1. Reverse-phase HPLC for the quantification of icarrin in HEP. The mobile phase was MeOH:H_2O = 55:45 at 1 ml min^{-1}. Peaks eluting at 18.5 min are icarrin.

Bio-technology and Science Inc., China) and analyzed using an automatic analyzer (ALCYON 300i, Abbott Laboratories Ltd, USA). Urinary creatinine (Cr) was determined using the picric acid method (commercial kit from Shanghai KeHua DongLing Diagnostic Products Company Ltd, China). ALP activity was determined using a commercial ALP kit (Zhong-Sheng BeiKong Bio-technology and Science Inc., China). Urine deoxypyridinoline (DPD) level was assayed using a rat DPD enzyme-linked immunosorbent assay (ELISA) kit (Quidel Corporation, San Diego, CA, USA). The urinary Ca excretion rate was expressed as the ratio of urinary Ca to Cr level (Ca/Cr), and urinary DPD levels were expressed as the ratio of urinary DPD to Cr level (DPD/Cr).

Bone Histomorphometric Measurement

Left tibias were collected, cleaned by removal of adherent tissue and fixed in chloroform. Upon fixation, diaphyseal segments of the tibias were dehydrated, defatted in acetone followed by ether and then embedded in bioplastic. The blocks were sectioned at a thickness of 5 μm with a Reichert–Jung supercut microtome (Reichert–Jung, Heidelberg, Germany) and digitized using a Leica camera (MPS60, Leica Microsystems Welzlar GmbH, Bensheim, Germany). Sections containing the tibio-fibular junction were coupled to an Olympus AH-2 microscope (Olympus Corporation, Japan) and were determined using the Leica QW 550 image software (Leica, Bensheim, Germany). Cancellous bone was measured in the proximal tibial metaphysis at a standard sampling site in the secondary spongiosa, as described previously (30,31). Structural variables such as total tissue area (TV), trabecular area (TbAr) and trabecular bone surface (BS) were measured in the spongiosa within a conventional visual field window whose upper side was centered 1 mm below the growth plate–metaphyseal junction. TbAr% refers to the percentage of trabecular bone area within total tissue (metaphyseal) area. The trabecular thickness and separation were calculated according to structural variables, as previously described (32).

Culture of Rat Osteoblast-like UMR 106 Cells

UMR 106 cells (ATCC no. CRL-1661) were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1}. DMEM, FBS and penicillin–streptomycin–glutamine were purchased from Life Technologies Inc. (Carlsbad, CA, USA). At ~80% confluence, cells were seeded in a 96-well microtiter plate (Falcon, Becton-Dickison Franklin hakes, USA) or 24-well or 6-well plates at a density of 2500, 100 000 or 200 000 cells per well, respectively. Upon confluence, the culture medium was switched to serum-free DMEM for another 24 h. Cells were then treated with HEP extract at 50, 100 and 200 μg ml^{-1} for 24 or 48 h. HEP extract was prepared by dissolving 20 mg of total HEP extract in 1 ml deionized distilled water and diluting with DMEM to achieve the final concentration. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Proliferation Assay

The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT; Amersham Pharmacia Biotech, Little Chalfont, UK) assay was used as an indirect measure of growth, as

described previously (33). Briefly the medium was removed and replaced with 100 µl of MTT reagent (5 mg ml^{-1}) in PBS. The plates were incubated for 4 h at 37°C, and 100 µl DMSO was added to dissolve formazan crystals. The multi-well plates were shaken for 15 min and the signals were detected using a POLARstar Galaxy spectrophotometer (BMG Labtechnologies GmbH, Germany) at a wavelength of 570 nm. The results of the MTT assay were expressed as cell numbers upon conversion of absorbance into cell numbers using a calibration curve. The calibration curve was prepared by seeding an assigned number of UMR 106 cells into a 96-well plate for 24 h before adding MTT solution.

ALP Activity Assay

Cells were washed with ice-cold PBS and scraped in 10 nM of Tris–HCl solution containing 2 mM of $MgCl_2$ and 0.05% TritonX-100, pH 8.2. The cell suspension was sonicated on ice. Aliquots of supernatants were subjected to protein assay using a Bio-Rad kit (Hercules, CA, USA) according to Bradford's method, and ALP activity was measured using commercial ALP reagent (Starbio Laboratory, Sigma, Texas, USA). Briefly, the assay mixture containing 16 mM of *p*-nitrophenyl phosphate in 350 mM aminomethyl propanol AMPbuffer, pH 10.5, supplemented with 2 mM of $MgCl_2$ was incubated at 37°C. The amount of *p*-nitrophenol liberated was measured using an ALCYON Analyzer (ALCYON® 300i, Abbott Laboratories, USA).

RNA Extraction and RT–PCR

Total RNA was isolated from cells cultured in a 6-well plate using TRIzol reagent according to the manufacturer's instructions (Life Technologies Inc., Carlsbad, CA, USA). Total RNA (2 µg) was used to generate cDNA in each sample using SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) with 0.5 µg oligo$(dT)_{15}$ primers. One microliter of total cDNA was amplified in each PCR reaction mixture containing 0.5 µM of sense and antisense primers (Genemed Synthesis, Inc., South San Francisco, CA, USA) of selected genes (Table 1). The PCR reaction mixture (in a total volume of 20 µl) contained 1× *Taq* reaction buffer, 0.2 mM of deoxynucleoside triphosphate (dNTP), 1.5 mM of $MgCl_2$, 0.5 mM of each primer and 0.5 U of *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA). PCR amplification was performed on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, USA). The PCR was carried out as follows: denaturation program (94°C, 4 min), amplification for 30 cycles (94°C for 30 s; 55°C for 30 s; and 72°C for 60 s) and final extension at 72°C for 7 min. The PCR products were analyzed using agarose gel electrophoresis. Optical densities of ethidium bromide-stained DNA bands were quantified using Bio-Rad image scanning software and the mRNA expression levels were normalized to the expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. Primers for reverse transcription–polymerase chain reaction (RT–PCR)

Primer	Sequence	Product		
		Orientation	Size (bp)	Annealing temperature (°C)
OPG	GACGAGATTGAG-AGAACGAG	Sense	502	55
	GGTGCTTGACTTT-CTAGGTG	Antisense		
RANKL	TCAGGAGTTCCAG-CTATGAT	Sense	298	55
	CCATCAGCTGAAG-ATAGTCC	Antisense		
GAPDH	TACATTTTGCTGAT-GACTGG	Sense	202	55
	TGAATGGTAGGAG-CTTGACT	Antisense		

Statistical Analysis

Data are reported as mean ± SEM. Data were statistically analyzed by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$. Critical differences between means were evaluated by Dunnett's multiple comparison test set at $P < 0.05$.

Results

Body Weight and Uterine Weight in OVX Rats

As shown in Fig. 2, the body weights of the OVX and sham rats were not significantly different at the start of the study. One month after surgery, OVX rats weighed 11% more than sham rats ($P < 0.01$) despite the fact that these animals were pair-fed the same amount of food as the sham group. The body weight of the OVX group continued to be significantly higher throughout the study. Treatment with E_2 but not HEP extract prevented the OVX-induced weight gain (Fig. 2A). Uterine weight was significantly reduced in the vehicle-treated OVX group ($P < 0.05$, versus sham group) (Fig. 2B). Treatment of OVX rats with E_2 but not with HEP extract significantly stimulated the growth of the atrophic uterus ($P < 0.01$, versus vehicle-treated OVX group).

Serum and Urine Biochemical Markers in OVX Rats

The effects of HEP extracts on serum and urine biochemical markers are summarized in Table 2. OVX appeared to lower serum Ca and P levels in rats, but the changes did not reach statistical significance ($P =$ n.s., versus sham group); similarly, treatment of OVX rats with HEP extract or E_2 did not significantly alter serum Ca and P levels. Urinary Ca levels increased significantly in response to OVX ($P < 0.05$, versus sham group). Treatment with either HEP extract or E_2 prevented the OVX-induced increase in urinary Ca levels ($P < 0.01$, versus vehicle-treated OVX group). OVX appeared to lower

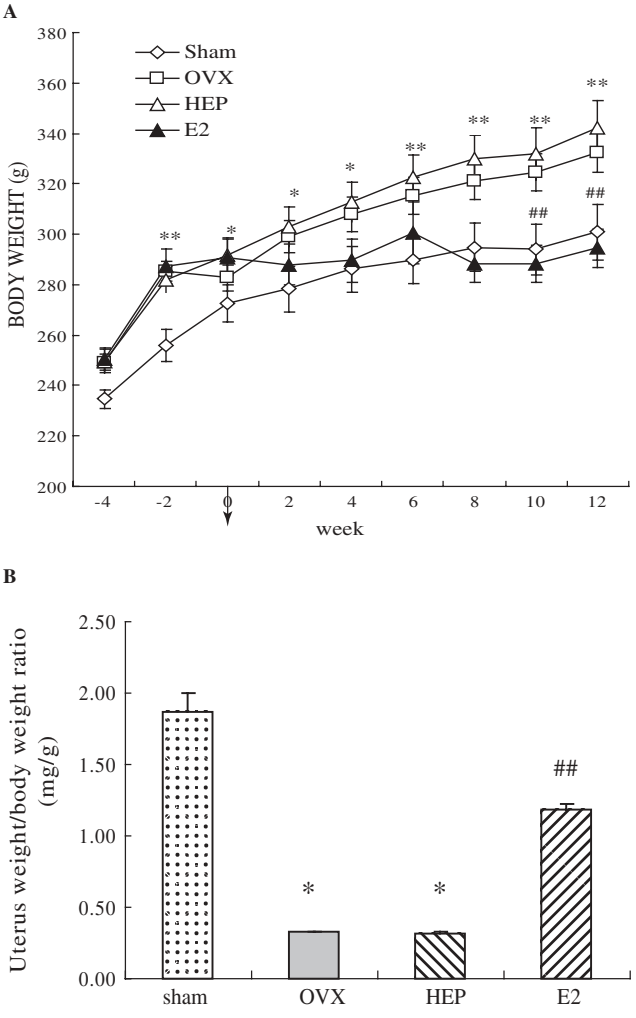

Figure 2. Effect of HEP extract on body weight and uterine weight in OVX rats. (**A**) Body weight was measured during the experimental period in the sham group (diamonds), OVX group (squares) and OVX rats with oral administration of HEP extract at 110 mg kg^{-1} d^{-1} (open triangles) or 17-β estradiol at 2 mg kg^{-1} d^{-1} (filled triangles). (**B**) Uterine weight was measured in the baseline group and 4 months after operation in the sham group, OVX group, OVX rats treated with 110 mg kg^{-1} d^{-1} of HEP extract and OVX rats treated with 2 mg kg^{-1} d^{-1} of 17-β estradiol. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, versus sham control; $^{\#\#}P < 0.01$, versus OVX group.

urinary P levels, but treatment of OVX rats with either HEP extract or E_2 appeared to raise urinary P levels; however, none of these changes reached statistical significance. These results indicate that treatment of OVX rats with either HEP extract or E_2 could reverse OVX-induced changes in Ca and P homeostasis in these animals.

The effects of HEP extract on bone formation and bone resorption *in vivo* were determined by the measurement of ALP activity and urinary DPD (a breakdown product of collagen during bone resorption) level. Serum ALP activity and urinary DPD levels increased in response to OVX ($P < 0.05$, vehicle-treated OVX group versus sham group), indicating an increase in the bone turnover rate in OVX rats. Treatment of OVX rats with HEP extract or E_2 suppressed the increase in serum ALP activity ($P < 0.05$ and $P < 0.01$, respectively, versus vehicle-treated OVX group). Similarly, the increase in urinary DPD level induced by OVX was prevented by treatment with either HEP extract or E_2 ($P < 0.05$, versus vehicle-treated OVX group). These results suggest that treatment of OVX rats with HEP extract as well as E_2 can prevent the OVX-induced increase in bone turnover rate.

Bone Architecture in Rat Tibia

The effects of HEP extract on rat trabecular bone architecture are shown in Fig. 3 and the measured structure variables are summarized in Table 3. As shown in Fig. 3, OVX altered the trabecular architecture in rat tibia, but E_2 or HEP extract reduced the alteration of the trabecular architecture induced by OVX. TbAr% decreased and trabecular separation (TbS) increased significantly in OVX rats ($P < 0.01$, versus sham group, Table 3), suggesting that OVX induced significant loss of trabecular bone in rat tibia. Treatment of OVX rats with either E_2 or HEP extract prevented the decrease in TbAr% and increase in TbS induced by OVX ($P < 0.05$, versus vehicle-treated OVX group, Table 3). The thickness of all slides was similar (as show in Table 3) during the comparison of trabecular bone area and trabecular separation among groups.

Proliferation and Differentiation of Rat Osteoblast-like UMR 106 cells

Treatment of UMR 106 cells with HEP extract for 24 or 48 h stimulated cell proliferation in a dose-dependent manner (Fig. 4A). HEP 100 and 200 μg ml^{-1} significantly increased osteoblastic cell numbers at 24 h by 1.4-fold ($P < 0.01$) and 1.6-fold ($P < 0.01$), respectively. A similar trend was observed at 48 h of treatment. To determine whether HEP could alter osteoblastic cell differentiation, its effect on ALP activity was studied. Treatment of UMR 106 cells with HEP extract for 24 h stimulated ALP activity in a dose-dependent manner (Fig. 4B). ALP activity in UMR 106 cells was significantly increased by 1.5-fold ($P < 0.01$) with 200 μg ml^{-1} HEP extract. These results indicate that HEP extract can induce UMR 106 cell proliferation and differentiation in a dose-dependent manner.

mRNA Expression of OPG and RANKL in Rat Osteoblast-like UMR 106 cells

As shown in Fig. 5A, HEP extract significantly increased OPG mRNA expression in UMR 106 cells in a dose-dependent manner ($P < 0.01$). On the other hand, the expression of RANKL mRNA in these cells was significantly downregulated by treatment with 200 μg ml^{-1} of HEP extract ($P < 0.05$) (Fig. 5B). The ratio of mRNA expression of OPG to RANKL (OPG/RANKL) was calculated to assess its effect on osteoclastogenesis. As shown in Fig. 5C, the OPG/RANKL ratio in UMR 106 cells increased when they were treated with HEP extract at 100 μg ml^{-1} ($P < 0.05$) and 200 μg ml^{-1} ($P < 0.01$).

Table 2. Effects of HEP extract on serum and urinary biochemical markers in OVX rats

Group	Dose (mg kg^{-1} d^{-1})	Serum Ca (mg l^{-1})	Serum P (mg l^{-1})	Urinary Ca/Cr (mg/mg)	Urinary P/Cr (mg/mg)	Serum ALP (U l^{-1})	Urinary DPD/Cr (nmol mg^{-1})
Sham	—	11.0 ± 0.2	3.18 ± 0.11	0.81 ± 0.13	5.65 ± 0.54	74.0 ± 10.5	4.9 ± 0.6
OVX	—	10.4 ± 0.1	3.01 ± 0.08	1.35 ± 0.15*	5.22 ± 024	101. ± 9.8*	8.7 ± 0.8*
E_2	2	10.8 ± 0.4	3.13 ± 0.16	0.51 ± 0.13##	6.43 ± 1.02	48.8 ± 2.7##	6.3 ± 1.1#
HEP	110	10.7 ± 0.2	2.98 ± 0.23	0.45 ± 0.06##	5.68 ± 0.24	79.2 ± 6.9#	5.9 ± 0.9#
F-value		0.526	1.093	11.563	0.702	8.493	4.316
P		>0.05	>0.05	<0.05	>0.05	<0.05	<0.05

Measurement of serum Ca, P, ALP and urinary Ca, P, DPD levels in the sham- and OVX-operated rats upon E_2 and HEP treatment. The rats were orally administrated with HEP extract (110 mg kg^{-1} d^{-1}), 17-β estradiol (E_2, 2 mg kg^{-1} d^{-1}) or its vehicle for 3 months. Serum and urine samples were collected and analyzed as described in 'Methods'. Urinary Ca, P and DPD levels were corrected with the urinary Cr level and expressed as a ratio to the Cr level. Data were expressed as mean ± SEM.
*$P < 0.05$; **$P < 0.01$, versus sham control.
#$P < 0.05$; ##$P < 0.01$, versus OVX group.

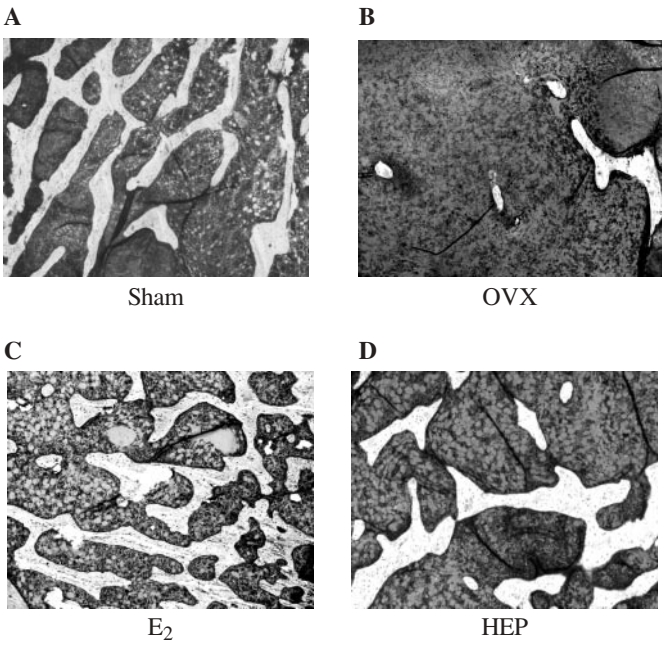

Figure 3. Bone histomorphometric analysis of rat tibia. The images of the metaphyseal trabeculae of the sham (**A**), OVX (**B**), E_2 (**C**) and HEP (**D**) groups.

Table 3. Effect of ovariectomy, estradiol and HEP on trabecular bone architecture in the proximal tibial metaphysis

Group	Dose (mg kg^{-1} d^{-1})	TbAr (%)	TbS (μm)	TbTh (μm)
Sham	—	40.8 ± 10.1	229.7 ± 28.8	55.8 ± 4.0
OVX	—	13.8 ± 8.2**	728.5 ± 199.5**	55.3 ± 6.5
E_2	2	21.0 ± 6.1#	297.9 ± 58.5#	59.8 ± 4.1
HEP	110	20.9 ± 5.8#	396.6 ± 41.1#	56.6 ± 8.3
F-value		10.369	5.684	1.012
P		<0.05	<0.05	>0.05

Measurement of trabecular bone architecture in proximal tibial metaphysis in the sham- and OVX-operated rats upon E_2 and HEP treatment. The rats were orally administrated with HEP extract (110 mg kg^{-1} d^{-1}), 17-β estradiol (E_2, 2 mg kg^{-1} d^{-1}) or its vehicle for 3 months. Rat tibia were collected and analyzed as described in 'Methods'. The percentage of trabecular area (TbAr%), trabecular separation (TbS) and trabecular thickness (TbTh) were determined. Data were expressed as mean ± SEM.
*$P < 0.05$; **$P < 0.01$, versus sham control.
#$P < 0.05$; ##$P < 0.01$, versus OVX group.

The increase in OPG/RANKL ratio suggests that HEP can inhibit osteoclastogenesis by decreasing the direct interaction between RANKL expressed on osteoblast and RANKL expressed on osteoclast cell surface.

Discussion

The current approach to the design of anti-osteoporotic drugs is directed along the two basic processes of bone remodeling. These are agents aimed at preventing bone resorption (estrogen, calcitonin, bisphosphonates, calcium, vitamin D, raloxifene) and agents that stimulate bone formation (fluoride, anabolic steroids) (34). Among these, ERT used to be a popular regime for prevention and treatment of postmenopausal osteoporosis. However, recent evidence suggests that ERT is associated with increased risk of breast, ovarian and endometrial cancer (7,8). In addition, the most frequently used anti-osteoporotic drugs are developed in affluent countries and the costs are too high to benefit the ordinary people in developing or even developed countries. Thus, alternative approaches for managing osteoporosis are needed.

HEP and other *kidney*-tonifying Chinese herbal medicines have been widely used in China for thousands of years to treat bone disease. These herbal medicines will undoubtedly continue to be used as a cost-effective alternative to commercial pharmaceutical products by the Chinese. However, the international acceptance of these herbal extracts as an alternative therapeutic regime for the management of osteoporosis is hampered by poor understanding of their actions *in vivo* and *in vitro*, an ill-defined mechanism for their actions and their unidentified active ingredients.

In the present study, the *in vivo* and *in vitro* effects of HEP extract on bone and mineral metabolism were systematically evaluated. Our results indicate that oral administration of

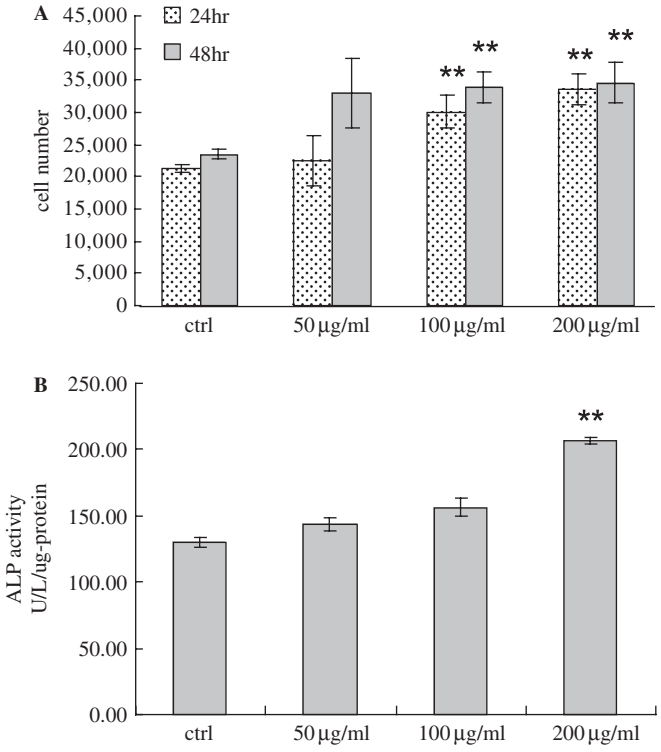

Figure 4. Effects of HEP extracts on cell proliferation and ALP activity in rat osteoblast-like UMR 106 cells. (**A**) Cell proliferation of UMR 106 cells upon HEP extract treatment. UMR 106 cells were seeded in a 96-well plate at a density of 2500 cells per well. After starvation for 24 h, different concentrations (50, 100 and 200 μg ml^{-1}) of HEP extract were added, and treated for 24 or 48 h. Vehicle-treated cells served as control (ctrl). Cell number was determined by MTT assay. Results were obtained from two independent experiments and expressed as mean ± SEM. $^{**}P < 0.01$, versus control for $n = 6$. (**B**) ALP activity of UMR 106 cells upon HEP extract treatment. UMR 106 cells were seeded in 24-well plates at a density of 100 000 cells per well. After starvation, 50, 100 and 200 μg ml^{-1} of HEP were added, and treated for 24 h before ALP activity determination. Vehicle-treated cells served as control (ctrl). The ALP activity was corrected for the amount of protein used and expressed as U l^{-1} μg^{-1}. Results were obtained from two independent experiments and expressed as mean ± SEM. $^{**}P < 0.01$ versus control for $n = 3$.

HEP extract for 3 months lowered serum ALP activity, urinary Ca excretion and urinary DPD levels in OVX rats, suggesting that HEP extract can prevent an OVX-induced increase in bone turnover rate in rats. In addition, HEP extract could increase trabecular bone area as well as decrease trabecular separation in rat tibia. These osteoprotective effects were similar to those of E_2 as reported in the present study as well as in studies by others (35). Despite the similarity of their effects in bone, our results showed that HEP extract did not mimic the effect of E_2 on body and uterus weight. The latter is of particular interest as it indicates that HEP extract exerts its beneficial effects on bone without inducing potentially harmful proliferative effects in reproductive tissues.

Our *in vitro* studies further demonstrated direct beneficial effects of HEP extract on bone cells. It was found to stimulate osteoblastic cell proliferation and differentiation in UMR 106 cells. These results are in agreement with both *in vivo* and

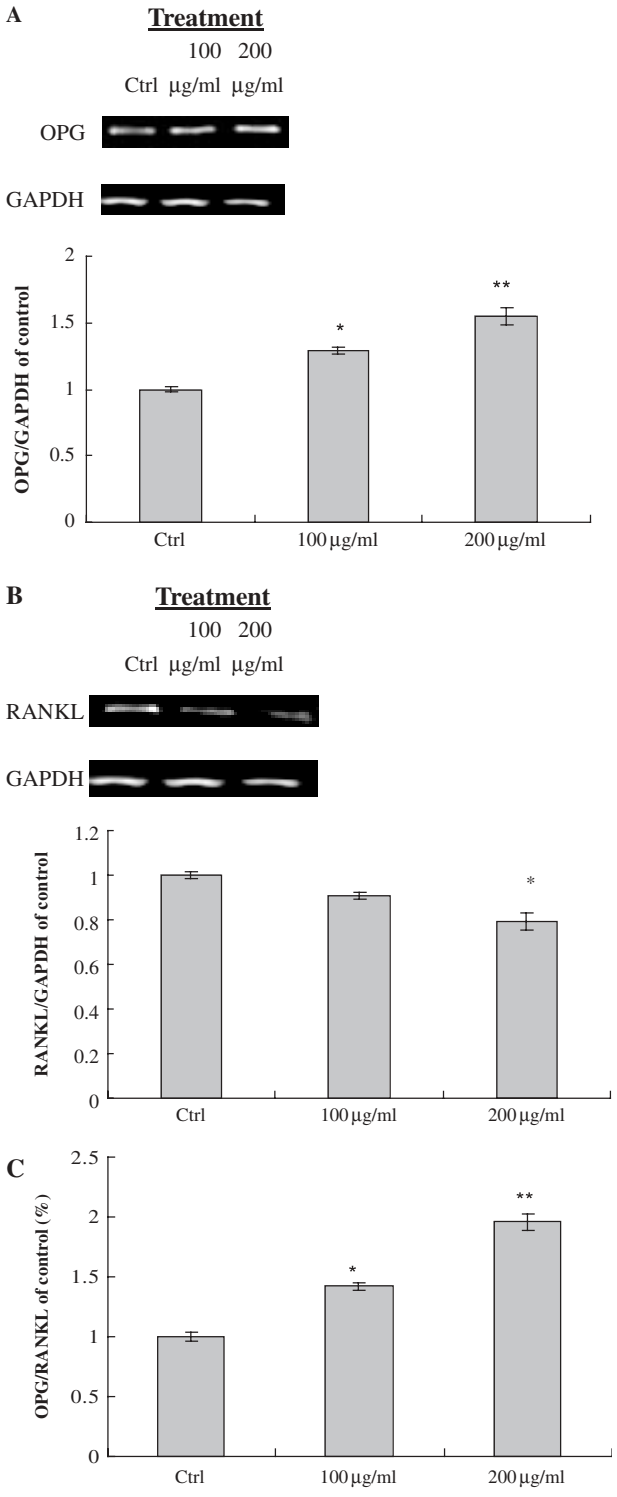

Figure 5. Effect of HEP on OPG and receptor activator of NF-κB ligand (RANKL) mRNA expression in rat osteoblast-like UMR 106 cells. UMR 106 cells were treated with 100 μg ml^{-1} and 200 μg ml^{-1} HEP extract or its vehicle (ctrl) for 48 h. Total RNA was isolated and subjected to semi-quantitative RT–PCR analysis of OPG (**A**) and RANKL (**B**) mRNA expression under the conditions described in 'Methods'. (**C**) The ratio of OPG/RANKL was normalized by an internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were obtained from two independent experiments and expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, versus control for $n = 3$.

in vitro studies reported by others (5–19). In addition, our study is the first to report that HEP extract stimulated the expression of OPG mRNA but suppressed the expression of RANKL mRNA in the UMR 106 cell line, resulting in a dose-dependent increase in the OPG/RANKL ratio. These results suggest that HEP extract might inhibit osteoclastogenesis via the modulation of the OPG/RANKL system in osteoblastic cells.

This study was originally designed to determine whether the HEP extract used in our *in vivo* study could demonstrate osteoprotective effects *in vitro*, namely stimulation of osteoblastic cell proliferation and inhibition of osteoclastogenesis via its action on osteoblasts. However, it should be noted that limitations exist in using herbal extracts for *in vitro* studies. The HEP extract, which contains numerous compounds (both active and inactive), is normally processed by the gastrointestinal tract and metabolized before the active ingredients exert effects directly on osteoblastic cells. In addition, HEP extract might contain other substances that interfere with the biological actions of the active ingredient on cell lines. Thus, a study of the effects of the total extract of HEP *in vitro* might not be ideal. Future studies are needed to identify the active ingredient(s) in the HEP extract that are responsible for the osteoprotective effects in OVX rats.

In summary, the present study clearly demonstrates the *in vivo* efficacy of using HEP extract to prevent OVX-induced increase in bone turnover rate and to restore the loss of trabecular bone architecture in OVX rats. Moreover, the *in vitro* data indicated that the observed increase in trabecular bone area and decrease in bone turnover rate by HEP extract in OVX rats might be mediated by its direct action of stimulating bone formation and inhibiting bone resorption. Both *in vivo* and *in vitro* studies provide evidence that HEP extract is a promising alternative and complementary therapeutic agent for the management of postmenopausal osteoporosis. However, in order to develop HEP extract in the international scientific community as an alternative regime for the treatment of bone diseases, more research will be needed to identify the active ingredients in HEP extract as well as the mechanism that mediates the action of HEP extract *in vivo*.

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