Increase in Bone Mass and Bone Strength by *Sambucus williamsii* HANCE in Ovariectomized Rats

Fang XIE,^{a,c} Chun-Fu WU,^a Yan ZHANG,^{a,c} Xin-Sheng YAO,^{*,a} Pik-Yuen CHEUNG,^{b,c} Albert Sun-Chi CHAN,^{b,c} and Man-Sau WONG^{*,b,c}

^a Department of Pharmacology, Shenyang Pharmaceutical University; Shenyang, 110016, China; ^b Open Laboratory of Chirotechnology of the Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University; Hong Kong SAR, China; and ^c State Key Laboratory of Chinese Medicine and Molecular Pharmacology; Shenzhen, 518057 China.

Received July 19, 2005; accepted June 19, 2005

Herbal *Sambucus williamsii* HANCE (SWH) is a folk medicine with a long history of safe use for treatment of bone fractures and joint diseases in China. The present study was designed to investigate if SWH extract could be used for treatment of postmenopausal osteoporosis. SWH extracts (30 or 60 mg/100 g body weight/d) were orally administrated to four-months-old ovariectomized (OVX) rats for 3 months. SWH extracts did not alter weight gain and uterus weight in OVX rats. SWH extracts significantly increased serum Ca levels ($p < 0.05$, vs. OVX control group) as well as decreased urinary Ca excretion ($p < 0.01$, vs. OVX control group) in OVX rats. The upregulation of serum alkaline phosphatase, serum osteocalcin as well as urinary deoxypyridinoline levels by OVX was suppressed by treatment with SWH extracts in rats ($p < 0.05$, vs. OVX control group). SWH extract increased the stiffness of femur at both dosage ($p < 0.05$, vs. OVX control group) and increased tibial bone mineral density at 60 mg/100 g body weight/d ($p < 0.05$, vs. OVX control group) in OVX rats. Our results indicate that orally administrated SWH extracts can decrease urinary calcium excretion and bone turnover rate in OVX rats, resulting in positive effects on biomechanical strength of bone and bone mineral density. This study is the first to report that SWH could be considered as a potential candidate for management of postmenopausal osteoporosis. Then *in vitro* experiments were performed to determine the potential molecular mechanism of the anti-osteoporotic effect of SWH. Results suggested that chloroform fraction and ethyl acetate fraction of SWH can inhibit osteoclastogenesis osteoclast by modulating the expression of osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) mRNA in osteoblastic UMR 106 cells. Both of them increased OPG mRNA and decreased RANKL mRNA expression, resulting in a dose-dependent increase in OPG/RANKL mRNA ratio ($p < 0.01$, vs. vehicle-treated). Taken together, SWH treatment can effectively suppress the OVX-induced increase in bone turnover and its effects might be mediated by a decrease in osteoclastogenesis.

Key words *Sambucus williamsii*; ovariectomized rat; bone mineral density; bone strength OPG; RANKL

Osteoporosis is a rapidly growing global health problem. It is characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in skeletal fragility and susceptibility to fractures especially the wrist, spine and hip.^{1,2)} Current approach for the design of anti-osteoporotic drug is directed along two basic processes of bone remodeling including agents that targeting at preventing bone resorption (estrogen, calcitonin, bisphosphonates, calcium, vitamin D, raloxifene) as well as agents that stimulating bone formation (fluoride, anabolic steroids).^{3–9)}

Among them, estrogen replacement therapy (ERT) was used to be a popular regime for prevention and treatment of postmenopausal osteoporosis. However, recent evidence suggest that ERT is associated with increased risk of development of breast, ovarian and endometrial cancers.^{10–13)} In addition, the most frequently used anti-osteoporotic drugs are developed in affluent countries and the costs are too high to benefit a large population in the developing or even the developed countries for prevention and treatment of osteoporosis. Thus, alternative approach for management of osteoporosis is needed.

Chinese herbal medicine has been widely used in clinical practice to treat bone disease for thousands of years and will undoubtedly continue to be used as a cost-effective alternative to commercial pharmaceutical products by traditional users. The genus *Sambucus*, widely distributed in Europe,

Asia and North Africa, has been used as folk medicine in analgesic, antiviral, anti-inflammatory, homeostatic, and diuretic drugs which act on bruises, fractures and edema.^{14–16)} Li *et al.*¹⁷⁾ reported that extract of the stem of *Sambucus sieboldiana* (SS) inhibited bone resorption in organ culture. Oral administration of ethyl acetate (EtOAc) fraction of SS to ovariectomized (OVX) rat prevented the decrease in bone mineral density of the lumbar vertebra. These results suggested that EtOAc fraction of SS could suppress bone resorption *in vitro* and *in vivo* and that *Sambucus* is a herb with potential for prevention and treatment of osteoporosis. In China stem and ramulus of *Sambucus williamsii* HANCE (SWH) has been used for centuries for treatment of inflammation¹⁸⁾ and bone fractures and joint diseases.¹⁹⁾

In the present study, we aimed to investigate *in vivo* effects of herba SWH extract on postmenopausal osteoporosis using OVX rat model. Biochemical markers of bone turnover, bone strength and bone mineral density changes would be determined.^{20–22)} Our results indicated that orally administrated SWH extracts can decrease urinary calcium excretion and bone turnover rate in OVX rats, resulting in positive effects on bone biomechanical strength mineral density.

With the recent discovery of receptor activator of NF- κ B ligand (RANKL)–RANK interaction, the role of osteoblast in osteoclast differentiation is now clearly defined.²³⁾ Binding of RANKL (the membrane associated factor) secreted by os-

teoblastic cells to RANK on osteoclast cell surface results in induction of osteoclast function. Whereas, the secretion of osteoprotegerin (OPG, the soluble decoy receptor of RANKL)²³ by osteoblast can interfere with RANKL–RANK interactions, thereby modulating osteoclastogenesis. Thus, the study of the effects of SWH fraction on the expression of RANKL and OPG mRNA in osteoblastic UMR 106 cells can be used for assessing its potential effects on osteoclastogenesis.

The present study is designed to demonstrate the efficacy of using SWH extract in treatment of osteoporosis as well as to delineate its molecular actions in anti-resorption activities.

MATERIALS AND METHODS

Extraction and Fraction of *Sambucus williamsii* HANCE (SWH) SWH was collected in Shenyang Province located in the Northeast region of China in July, 2003 and was identified according to the method as listed in Chinese Bencao¹⁹ with the help of Prof. Zerong Jiang, Shenyang Pharmaceutical University. The identity of SWH was confirmed by the analysis of its major ingredients including pentacyclic triterpenoid, phenol acid and derivatives and protein. A summary of the major active constituents of SWH is listed in Table 1. Dry stem with ramulus of SWH (30 kg) was extracted in 240 l of 60% alcohol for 3 h by heating and retrieving method for three times. The extract was filtered and decompress concentrated at 50 l to produce a dense plaster. The extract was then freeze-dried and a total of 1.2 kg powder was obtained. Then lyophilized extract was suspended in water and leached by successive extraction with chloroform (CHCl₃) or EtOAc to give CHCl₃ or EtOAc-fractions, respectively.

Animals A total of 46 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 three months old upon arrival, and four months old upon the commencement of treatment. Rats were bilaterally ovariectomized using dorsal approach. A single longitudinal skin incision was made on the dorsal midline at the level of the kidneys. The ovaries were exposed and removed together with the surrounding fat, oviduct and a small portion of the uterus. Animals of the control group underwent sham operations, during which the ovary was exposed but left intact. The surgery was done under anesthesia, using ether. Rats were housed in cages under a 12/12 light/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. Husbandry of the animals was based on Guide for Care and Use of Laboratory Animals.

***Sambucus williamsii* HANCE (SWH) Extract Treatment** The animals were either sham-operated (Sham, *n*=8) or ovariectomized (OVX, *n*=38). OVX rats were randomly divided into four groups: OVX-control group (OVX, *n*=8); estrogen administration group (E₂, *n*=10, 2 mg/kg body weight/d); two SWH extract administration groups SWH30 (*n*=10, 30 mg/100 g body weight/d) and SWH60 (*n*=10, 60 mg/100 g body weight/d). Drug treatment started four weeks after the surgery and administrated orally for three

Table 1. Major Potential Active Constituents in *Sambucus williamsii* HANCE

Types of compound	Contents (g)	Percentage of total crude extracts (%)
Steroids	1.04	0.36
Triterpenoids	1.16	0.41
Lignans	0.2	0.07
Phenol acids	0.35	0.12
Others	0.1	0.004

Table 2. Effect of SWH Extract on Biomechanical Properties of Left Femur in Ovariectomized (OVX) Rats

Group	Maximum load (N)	Energy absorption (J)	Stiffness (N/mm)
Sham	158.1±3.3	88.0±5.9	453.7±33.8
OVX	145.0±5.6*	58.1±4.7*	412.8±22.4*
SW30	140.6±2.5	62.6±5.4	502.6±28.2 [#]
SW60	148.1±6.0	74.5±8.0	465.8±21.5 [#]
E ₂	153.3±6.0	73.8±5.8	532.2±32.2 ^{##}

Young female Sprague-Dawley rats were either sham-operated (Sham, *n*=8) or ovariectomized (OVX, *n*=38). OVX rats were randomly divided into five groups and subjected to treatment at four-months of age: OVX-control (OVX, *n*=8); 2 mg/kg body weight/d estrogen administration (E₂, *n*=10); 30 mg/100 g body weight/d of SWH extract administration (SW30, *n*=10); 60 mg/100 g body weight/d of SWH extract administration (SWH60, *n*=10). The biomechanical properties of left femur were determined by three-point bending test. Values are expressed as mean±S.E.M. **p*<0.05 vs. sham control. [#]*p*<0.05, ^{##}*p*<0.01 vs. OVX group.

months. The rats were then sacrificed for determining the parameters interested.

Sample Collection Rats were placed in the metabolic cages before sacrifice and allowed to have 24 h to accommodate to the new environment. Urine samples were then collected for 24 h and acidified with 2 ml of 1 M HCl, centrifuged at 1015 *g* for 5–10 min at 4 °C to remove contaminating sediments, and aliquots were stored at –20 °C until they were assayed. After sacrifice, blood samples were taken from the abdominal aorta and serum obtained was stored at –70 °C before biochemical analysis. Uterus was collected and weight was recorded. Left femora and tibiae were removed and wrapped in gauzes saturated with physiological saline and stored at –20 °C before analysis.

Biochemical Assay of Serum and Urine Samples Serum and urine samples were analyzed as follow: Ca concentration of both serum and urine samples were measured by standard colorimetric methods using commercial kits (ZhongSheng BeiKong Bio-technology and Science Inc, PRC) and analyzed by an automatic analyzer ALCYON 300i (Abbott Laboratories Ltd., U.S.A.). Urinary creatinine was determined by picric acid method (Commercial kit from Shanghai KeHua DongLing Diagnostic Products Company Limited, PRC). Serum ALP activity was determined by using commercial alkaline phosphatase kit (ZhongSheng BeiKong Bio-technology and Science Inc, PRC). Serum osteocalcin was assayed using a rat osteocalcin ELISA kit (Biomedical Technologies Inc., U.S.A.). Urine deoxypyridinoline (DPD) level was analyzed using a rat DPD ELISA kit (Quidel Corporation., San Diego, U.S.A.). Urinary Ca excretion rate was expressed as the ratio of urine Ca to creatinine (Cr) level (Ca/Cr) while urinary DPD levels were expressed as urine DPD to creatinine level (DPD/Cr).

Trabecular Bone Mineral Density Determination Using

Table 3. Primers for Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primer	Sequence	Product		
		Orientation	Size (bp)	Annealing temperature (°C)
OPG	GACGAGATTGAGAGAACGAG GGTGCTTGACTTTCTAGGTG	Sense Antisense	502 bp	55
RANKL	TCAGGAGTTCAGCTATGAT CCATCAGCTGAAGATAGTCC	Sense Antisense	298 bp	55
GAPDH	TACATTTTGCTGATGACTGG TGAATGGTAGGAGCTTGACT	Sense Antisense	202 bp	55

Peripheral Quantitative Computed Tomography (pQCT)

Left tibiae were scanned with a pQCT system (Densiscan 2000; SCANCO, Basserdorf, Switzerland). The metaphyseal region of the left proximal tibia was scanned at a site of 2.5 mm from the proximal articular surface. Scans were analyzed using a threshold of 280 mg/ccm for delineation of the marrow cavity and an area of cortical and trabecular regions. Trabecular bone densities were measured using pQCT.

Three-Point Bending Test An electro-mechanical (universal) testing machine (Model 5566, Instron, High Wycombe, U.K.) was used in conjunction with Instron Merlin software (version 4.03) for all tests. The left femur was cleaned to remove its surrounding soft tissue and stored at -20°C until analysis. The outer two supporting points were fixed 20 mm apart with a single central point positioned at the midpoint of the specimen. All loading points were 3 mm in diameter. The central loading point was displaced, and the load and displacement were recorded until the specimen was broken. Bending stiffness was then derived from the slope of the linear region of the resulting load *versus* displacement curve.

Culture of Rat Osetoblast-Like UMR 106 Cell UMR106 cells (ATCC no. CRL-1661), as a common osteoblast model for bone metabolism study *in vitro*, were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin 100 unit/ml and streptomycin 100 $\mu\text{g}/\text{ml}$. DME, Penicillin–Streptomycin–Glutamine and FBS were purchased from Life Technologies Inc. (Carlsbad, California). At about 80% confluence, cells were seeded in a 6 well-plate (Falcon, Becton-Dickison) at a density of 200000 cells/well. Upon confluence, culture medium was shifted to serum-free DMEM for another 24 h. Cells were then treated with CHCl_3 fraction at 12.5 $\mu\text{g}/\text{ml}$, 25 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ or EtOAc fraction at 25 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ for 24 h. Both fractions were prepared by dissolving 20 mg fractions into 1 ml DMSO 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 .

RNA Extraction and Reverse Transcription Polymerase Chain Reaction Total RNA was isolated from cells cultured in 6-well-plate by using TRIzol reagent according to the manufacturer's instructions (Life Technologies Inc., Carlsbad, CA, U.S.A.). Total RNA (2 μg) was used to generate cDNA in each samples using SuperScriptII reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, U.S.A.) with 0.5 μg oligo(dT)₁₅ primers. One microliter of total cDNA was amplified in each PCR reaction mixture that con-

tains 0.5 μM of sense and antisense primers (Genemed Synthesis, Inc., South San Francisco, U.S.A.) of selected genes (Table 3). The PCR reaction mixture (in a total volume of 20 μl) contained $1\times$ Taq reaction buffer, 0.2 mM of deoxynucleoside triphosphate (dNTP), 1.5 mM of MgCl_2 , 0.5 μM of each primer, and 0.5 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, U.S.A.). PCR amplification was performed on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, U.S.A.). The condition of PCR was carried out as follow: 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 by Bio-Rad image scanning software and the mRNA expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

Statistical Analysis Data were reported as mean $\pm$ S.E.M. Significance of differences between group means was determined by One-Way ANOVA. p -values <0.05 was considered statistically significant. Dunnett's procedure was used to determine the individual group difference at $\alpha=0.05$.

RESULTS

Body Weight and Uterine Weight Effects of SWH treatment on weight gain and uterine weight in OVX rats were shown in Fig 1. OVX treatment significantly increased weight gain of rats by 11% ($p<0.01$, vs. Sham) at one month after surgery despite the fact that all the animal groups were pair-fed. The weight gain of OVX group continued to be significantly higher than the sham-operated group throughout the study ($p<0.01$). SWH extract did not alter weight gain in OVX rats, while E_2 treatment reduced the weight gain in OVX rats ($p<0.01$, vs. OVX, Fig. 1A). In addition, OVX treatment significantly reduced uterine weight ($p<0.01$, vs. Sham, Fig. 1B). SWH treatment did not alter uterine weight in OVX rats while E_2 treatment stimulated atrophic uterine growth significantly. These results indicated that SWH extract did not behave as E_2 in reducing body weight gain and inducing uterine growth in OVX rats.

Biochemical Parameters of Serum and Urine Samples Effects of SWH treatment on serum and urine Ca levels are shown in Fig. 2. OVX decreased serum Ca levels ($p<0.05$, vs. Sham). Such a decrease could be restored by treatment with E_2 ($p<0.05$, vs. OVX) or SWH ($p<0.05$, vs. OVX) (Fig. 2A). On the other hand, urine Ca level was significantly increased

Fig. 1A. Effects of SWH on Body Weight in Ovariectomized (OVX) Rats

Three-months old Sprague-Dawley rats were either sham-operated ($\square$) (Sham, $n=8$) or ovariectomized (OVX, $n=38$). OVX rats were randomly divided into four groups and were subjected to drug treatment at four-months of age: OVX-control ($\blacktriangle$) (OVX, $n=8$); estrogen administration (2 mg/kg body weight/d E_2 , $\square$, $n=10$); 30 mg/100 g body weight/d of SWH extract administration (SWH30, $\circ$, $n=10$); 60 mg/100 g body weight/d of SWH extract administration (SWH60, $\bullet$, $n=10$). Data were expressed as mean $\pm$ S.E.M. * $p<0.05$, ** $p<0.01$ vs. sham group. ## $p<0.01$ vs. OVX group.

Fig. 1B. Effects of SWH on Uterine Weight in Ovariectomized (OVX) Rats

Uterine weight was measured in sham-operated group (Sham), OVX group, and OVX rats treated with 30 or 60 mg/100 g body weight/d of SWH extract (SW30, SW60), and OVX rats treated with 2 mg/kg body weight/d of estrogen (E_2). Data were expressed as mean $\pm$ S.E.M. ** $p<0.01$ vs. sham group. ## $p<0.01$ vs. OVX group.

(A)

(B)

Fig. 2. Effects of SWH Extract on Serum (A) and Urinary Calcium (B) Level in OVX Rats

Young female Sprague-Dawley rats were divided into 5 groups: sham-operated group (Sham), OVX group, and OVX rats treated with 30 or 60 mg/100 g body weight/d of SWH extract (SW30, SW60), and OVX rats treated with 2 mg/kg body weight/d of estrogen (E_2). Rats were treated for three months and serum and urinary calcium levels were measured. Data were expressed as mean $\pm$ S.E.M. * $p<0.05$ ** $p<0.01$, vs. sham control. # $p<0.05$, ## $p<0.01$ vs. OVX group. Urinary Ca level was expressed as urine Ca/creatinine ratio.

(A)

(B)

(C)

Fig. 3. Effects of SWH Extract on Serum ALP Activity (A), Serum Osteocalcin (B), and Urinary Deoxypyridinoline/Creatinine Ratio (C) in OVX Rats

Young female Sprague-Dawley rats were divided into 5 groups: sham-operated group (Sham), OVX group, and OVX rats treated with 30 or 60 mg/100 g body weight/d of SWH extract (SW30, SW60), and OVX rats treated with 2 mg/kg body weight/d of estrogen (E_2). Data were expressed as mean $\pm$ S.E.M. * $p<0.05$ vs. sham control. # $p<0.05$, ## $p<0.01$ vs. OVX group.

in response to OVX ($p<0.01$, vs. Sham, Fig. 2B). Nevertheless, SWH treatment could significantly reduced the increase of urine Ca level by OVX in a manner similar to that by E_2 ($p<0.01$, vs. OVX, Fig. 2B).

Effects of SWH treatment on bone markers are shown in Fig. 3. Serum alkaline phosphatase (ALP)²⁴) and osteocalcin²⁵) levels are the two major bone markers for assessment of bone formation rate, while urinary deoxypyridinoline (DPD) level is a major bone marker for assessment of bone resorption rate.²⁵) OVX treatment significantly increased serum ALP activity ($p<0.05$, vs. Sham, Fig. 3A). SWH treatment at both dosages, 30 mg/100 g body weight/d and 60 mg/100 g body weight/d significantly reduced serum ALP activities in OVX rats to a level similar to that of the sham-operated group ($p<0.05$, vs. OVX). E_2 treatment at a dosage of 2 mg/kg body weight/d significantly reduced serum ALP activity in OVX rats ($p<0.01$, vs. OVX) to a level much lower than the sham-operated group ($p<0.05$).

OVX treatment significantly increased serum osteocalcin (Fig. 3B) and urinary DPD/creatinine levels (Fig. 3C) ($p<0.05$, vs. Sham), indicating the induction of high bone turnover rate in rats by OVX. Treatment of OVX rats with

Fig. 4. Peripheral Quantitative Computed Tomography (pQCT) Analysis of Rat Tibia

Young female Sprague-Dawley rats were divided into 5 groups: sham-operated group (Sham), OVX group, and OVX rats treated with 30 or 60 mg/100 g body weight/d of SWH extract (SWH30, SWH60), and OVX rats treated with 2 mg/kg body weight/d of estrogen. Rats were treated for three months and trabecular bone mineral density was measured. Data were expressed as mean $\pm$ S.E.M. ** $p < 0.01$ vs. sham control; # $p < 0.05$, ## $p < 0.01$ vs. OVX group.

SWH extract resulted in significant reduction in serum osteocalcin level in the 30 and 60-mg/100 g body weight/d treated groups by 20% ($p < 0.05$, vs. OVX) and 27% ($p < 0.05$, vs. OVX), respectively. Similarly, SWH treatment significantly reduced urinary DPD excretion rate in OVX rats by 28% ($p < 0.05$, vs. OVX) and 22% ($p < 0.05$, vs. OVX), respectively. Effects of SWH on serum osteocalcin (Fig. 3B) and urinary DPD (Fig. 3C) levels in OVX rats were similar to that of E_2 , in which the increase in bone turnover rate by OVX were suppressed.

pQCT Analysis of Left Tibia pQCT analysis of proximal tibia was performed to determine if SWH treatment could increase bone mineral density (BMD), thereby protecting against bone loss induced by OVX. As shown in Fig. 4, there was a 33% reduction in trabecular BMD in OVX control group as compared to sham-operated group at four months post-surgery ($p < 0.05$, Fig. 4.) Treatment of OVX rats with SWH extract (60 mg/100 g body weight/d) or E_2 for four months increased trabecular BMD of tibia ($p < 0.05$ or $p < 0.01$ vs. OVX respectively). However, no change in BMD was observed in SWH (30 mg/100 g body weight/d) treated group ($p > 0.05$ vs. OVX), suggesting that the protective effect of SWH on BMD in OVX rats was dose-dependent.

Biomechanical Testing of Bone To determine if SWH treatment could improve bone strength, three-point bending test of femur was performed. As shown in Table 2, OVX treatment significantly reduced the maximum load, energy and stiffness of femur by 8%, 34%, and 10% respectively ($p < 0.05$ vs. Sham). Treatment with SWH or E_2 significantly prevented the reduction in bone stiffness induced by OVX (Table 2). SWH or E_2 treatment appeared to demonstrate positive effect on the maximum load and energy of femur in OVX rats, however, the effects did not reach significant differences (Table 2).

Effects on mRNA Expression of Osteoprotegerin (OPG) and Receptor Activated NF- κ B Ligand (RANKL) in Rat Osteoblast-Like UMR106 Cells

As shown in Fig. 5A, $CHCl_3$ fraction of SWH extract significantly increased OPG mRNA expression in UMR 106 cells and at the same time down-regulated the expression of RANKL mRNA in these cells ($p < 0.01$ vs. control). The ratio of mRNA expression of OPG to RANKL (OPG/RANKL) was then calculated for assessing its effect on osteoclastogenesis. $CHCl_3$ fraction significantly increase OPG/RANKL ratio in UMR 106 cells in a dose-dependent

Fig. 5. Effect of $CHCl_3$ Fraction (A) and EtOAc Fraction (B) on Osteoprotegerin (OPG) and Receptor Activator of NF- κ B Ligand (RANKL) mRNA Expression in Rat Osteoblast-Like UMR106 Cells

(Top panel) UMR106 cells were treated with 12.5, 25, 50 μ g/ml of $CHCl_3$ Fr. and its vehicle (ctrl) for 24 h. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis of OPG and RANKL mRNA expression. (Bottom panel) The ratio of OPG/RANKL was normalized by the expression level of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were obtained from two independent experiments and expressed as mean $\pm$ S.E.M. ** $p < 0.01$ vs. control, for $n = 6$.

manner ($p < 0.01$ vs. control). As shown in Fig. 5B, EtOAc fraction of SWH extract can only increase OPG mRNA expression at a dosage of 100 μ g/ml, but it significantly decrease RANKL mRNA expression in UMR106 cells at both 25 and 100 μ g/ml. The ratio of OPG/RANKL was significantly suppressed by EtOAc fraction in a dose-dependent manner ($p < 0.01$ vs. control).

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

DISCUSSION

The Chinese herbal medicine has been widely used in the orthopedic clinical practice for thousands years for the treatment of fractures and joint diseases.²⁶⁾ The philosophy of Chinese medicine is to restore balances at all effective levels by prescribing herbal drugs in combinations that work syner-

gistically in an inter-complementary way. From a modern scientific point of view, they may demonstrate effects on 1) the enhancement of positive bone metabolism by modulating the hormonal system²⁷⁾; 2) dietary calcium absorption²⁸⁾; and 3) stimulation of bone formation and inhibition of bone resorption.^{29–32)} In the present study we demonstrated for the first time that the extract of *Sambucus williamsii* HANCE (SWH) prevent the reduction of bone mass and bone strength induced by estrogen deficiency in rats without substantial effects on the uterus.

To systematically evaluate the efficacy of SWH for treatment of postmenopausal osteoporosis, the internationally accepted OVX rat model was used in the present study. Our study confirmed previous studies by others²⁰⁾ that OVX decreased serum Ca level, increased urinary Ca excretion, increased serum levels of ALP and osteocalcin as well as urinary DPD excretion in female rats. These results suggest that OVX alter calcium metabolism and cause significant increase in bone turnover rate. Treatment of OVX rats with SWH extract suppressed urinary Ca excretion rate, serum levels of ALP and osteocalcin, as well as urinary DPD excretion rate in a manner similar to that by E₂, suggesting that SWH extract could restore Ca homeostasis and prevent the increase in bone turnover rate induced by OVX.

Apart from evaluating the effect of SWH on biochemical markers, its effects on tibial bone mineral density (BMD) as well as on biomechanical properties of femur in OVX rats were also investigated. pQCT analysis in the present study indicated that OVX caused significant decrease in BMD in rat proximal tibia. Treatment of OVX rats with either 60 mg/100 g body weight/d of SWH extract or E₂ significantly reduced the decrease in trabecular BMD in tibia, but did not completely prevent the bone loss as induced by OVX. In addition, as revealed by three-point bending experiment, OVX induced significant change in biomechanical properties of rat femur, including maximum load, energy absorption and stiffness. Treatment of OVX rats with either SWH extract or E₂ significantly increased stiffness of femur, suggesting that they could improve the biomechanical properties of femur in OVX rats.

Based on the observations of the *in vivo* study, the ability of SWH extract to prevent OVX-induced reductions in bone mass and bone strength appear to be mediated through its inhibitory effect on bone resorption and bone turnover. The *in vitro* study using rat osteoblast-like UMR 106 cells provided further support for the potential action of SWH extract on bone. UMR106 cells, as a common osteoblastic cell model, was used in current study.³³⁾ We found that UMR106 cells can secrete the cytokines modulating osteoclastogenesis such as OPG and RANKL. Our results are the first to report that CHCl₃ and EtOAc fraction derived from SWH stimulated the expression of OPG mRNA but suppressed the expression of RANKL mRNA in UMR106 cell line, resulting in a dose-dependent increase in OPG/RANKL ratio. These results suggest that SWH extract might inhibit osteoclastogenesis *via* the modulation of OPG/RANKL system in osteoblastic cells.

Taken together, the effects of SWH extract reversed the negative bone balance induced by OVX, thereby preventing bone loss and preserving bone strength. And its potential mechanism was suppressing osteoclastogenesis by modulating OPG or RANKL expression.

Our results clearly showed that the effects of SWH extract on biochemical marker, BMD as well as bone strength in OVX rats were very similar to those of E₂. To determine if SWH extract behaved as E₂ in other tissues in OVX rats, its effect on uterine growth was studied. The results showed that OVX induced uterine atrophy in female rats and treatment of OVX rats with E₂, but not SWH extract, increased the weight of uterus. Thus, the results indicate that treatment of OVX rats with SWH extract for three months could reduce bone loss without affecting uterine growth. The latter is of particular importance as recent findings from the Women's Health Initiative Trial suggests that hormone replacement therapy (HRT) increases the risk of breast cancer, endometrial cancer, ovarian cancer, stroke, myocardial infarction, and thromboembolic events.^{34,35)} Our results suggested that SWH extract might be a safe alternative treatment to HRT in prevention of postmenopausal osteoporosis.

The properties of SWH reported in the present study are similar to those of genistein, a well known phytoestrogen isolated from soy. Previous studies demonstrated that low dose of genistein protected against bone loss without significantly increasing uterine weight in OVX rats.^{36–40)} However, as shown in Table 1, isoflavones or genistein are not the major constituents of SWH extract. Thus, it is of great significance to identify the active constituents in SWH extract that are responsible for its protective effects on bone. One potential candidate might be lignan, a well known phytoestrogen⁴¹⁾ that constitutes 0.07% of the total crude extract of SWH (Table 1). Lignans are widespread in foodstuff such as cereals, fruits and vegetables and have not been studied as thoroughly as isoflavones.⁴²⁾ A study by Ward W *et al.* reported that administration of lignan secoisolariciresinol diglycoside (SDG) to adolescent female offspring could increase bone strength.⁴³⁾ A novel precursor to the mammalian lignan-entrolactone showed potent antioxidant activity and decreased breast tumors without estrogenic activity in the uterine growth assay.⁴⁴⁾ Besides, the phenolic constituents might be the other potential candidate, for Li *et al.*¹⁷⁾ have demonstrated that some phenol acid compounds from stem of *Sambucus sieboldiana* (SS) demonstrated significant inhibitory effect on bone resorption and decreased the reduction of BMD in OVX mice. Based on these studies, we hypothesized that lignans, or/and phenol acid compounds might be the active constituents of SWH extract that are responsible for its beneficial effects on bone metabolism.

In conclusion, ovariectomy for four months led to significant increase in bone turnover resulting in dramatic decrease in BMD and bone stiffness. Treatment with SWH extract can prevent bone loss at the trabecular bone-rich sites and promote bone strength in rat femur. The improvement of bone properties by SWH extract appears to be mediated by the inhibition of bone resorption and bone turnover in OVX rats. And the *in vitro* experiments suggest that SWH extract might inhibit bone resorption by suppressing osteoclastogenesis *via* modulation of OPG/RANKL system. Most importantly, treatment with SWH extract improves bone properties without increasing the risk of E₂ on uterine growth. The present study, therefore, suggest that SWH might be a promising alternative therapeutic regime for management of postmenopausal osteoporosis.

Acknowledgements This work was supported by the Areas of Excellence Scheme Established under the University Grants Committee of the Hong Kong Special Administrative Region, China (AOE/P-10/01) and the Earmarked Research Grant Council, (B-Q767,3-ZF89), HKSAR.

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