Effects of *Cynanchum wilfordii* on osteoporosis with inhibition of bone resorption and induction of bone formation

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Abstract. *Cynanchum wilfordii* Hemsley has been used for the treatment of musculoskeletal diseases in traditional Republic of Korean medicine. The present study investigated the effects of *C. wilfordii* water extract (CW) on postmenopausal osteoporosis. Female mice were used and randomly assigned into a normal group and three ovariectomized (OVX) groups: OVX with vehicle (OVX + vehicle); OVX with 17β-estradiol (E2; 10 μg/kg/day); and OVX with CW (1 mg/kg/day). Oral administration of CW or E2 intraperitoneal injection began 9 weeks after OVX and continued for 3 weeks. Following sacrifice, bone histology, bone mineral density (BMD) and bone mineral content (BMC) of the femur were observed. Serum osteocalcin concentration was analyzed. In addition, the expression levels of osteoprotegerin (OPG) and osterix were evaluated in human osteoblast-like Saos-2 cells. In the lateral and medial epicondyles of the CW-administrated group, dense and well-formed bone marrow cells with reduced bone marrow pores were observed. CW decreased the number of tartrate resistant acid phosphatase-positive multinucleated osteoclasts. BMD and BMC were increased following increased serum osteocalcin levels by CW treatment. The expression levels of OPG and osterix were upregulated by CW treatment *in vitro*. The results suggested that *C. wilfordii* has an advantageous effect on osteoporosis and possesses the potential to be used in osteoporosis treatment.

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Introduction

Osteoporosis is a major public health problem, affecting over 200 million individuals worldwide (1). It is reported that ~40% of Caucasian postmenopausal women exhibit osteoporosis and the prevalence is expected to continue to escalate in the near future with an increasingly elderly population (2). In postmenopausal women, there is inordinate bone resorption relative to bone redeposition, caused by hormone deficiencies (3). Postmenopausal osteoporosis is directly linked to the decline in estrogen (E2), which can increase the generation and activity of osteoclasts (4).

As E2 is a well-documented factor for bone maintenance and hormone replacement therapy (HRT) has been demonstrated to possess beneficial effects on postmenopausal osteoporosis (5). HRT significantly decreased vertebral fracture risk and increased bone mineral density (BMD) in postmenopausal women with osteoporosis (6). However, severe adverse effects of long-term HRT have been reported, including breast and endometrial cancer (7,8). Depending on the effects of suppressed bone turnover, bisphosphonates are widely used for osteoporosis following marked reductions in HRT prescribing. Despite showing marked anti-resorptive effects, troublesome side effects of bisphosphonates have been reported, including atrial fibrillation, esophageal cancer, atypical femoral fractures and osteonecrosis of the jaw (9-11). Due to these limitations of osteoporosis therapies, there has been growing interest in alternative therapies from natural sources (12).

Cynanchum wilfordii root has been used in traditional Korean medicine for the treatment of geriatric and musculoskeletal disease including hair graying, impotence and muscle/bone weakness (13). Several studies have suggested that *C. wilfordii* has ameliorative effects on hypertension, hypercholesterolemia, gastric disorders and tumors (14-17). A study (18) identified that a herbal formula containing extracts of *C. wilfordii* attenuates various menopausal symptoms including hot flushes, insomnia, night sweats and vaginal dryness without any influence on the female hormone levels.

However, the anti-osteoporotic effects of *C. wilfordii* have not been established. The present study investigated the anti-osteoporotic effects of *C. wilfordii* water extract (CW) and its mechanisms in ovariectomized (OVX)-induced osteoporosis mice and in human osteoblast-like Saos-2 cells.

Materials and methods

Preparation of CW. The roots of *C. wilfordii* Hemsley was obtained from Jung-do Herb, Co. Ltd. (Guri, Korea). A total of 20 g of *C. wilfordii* were extracted with 200 ml distilled water for 24 h at room temperature (RT). The extract was concentrated in a rotary vacuum evaporator, and designated CW (yield: 13.7%). A voucher specimen (CW-W100) was deposited at the department of Convergence Korean Medical Science, Kyung Hee University.

For quantification of CW, 2,4-dihydroxyacetophenone was used as a standard. The content of 2,4-dihydroxyacetophenone was measured using a high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD; Agilent 1100 series; Agilent Technologies, Inc., Santa Clara, CA, USA). The extract was dissolved in 70% methanol and sonicated for 30 min. After filtering through a 0.45 μm filter membrane, an aliquot was injected in HPLC analysis. The column used was a Shiseido Capcell Pak C18 (250x4.6 mm, 5 μm; Shiseido Co., Ltd., Tokyo, Japan). The mobile phase consisted of 0.05% acetic acid in water and acetonitrile with 1.0 ml/min of flow rate at 30˚C. As demonstrated in Fig. 1, the concentration of 2,4-dihydroxyacetophenone in CW was 13.689 μg/ml (0.091%).

Ovariectomy-induced animals and in vivo treatment. A total of 36 female ICR mice aged 6 weeks (Raon Bio Animal, Inc., Yong-in, Korea) were provided free access to a standard chow diet (Orient Co. Ltd., Seongnam, Korea) and tap water. They were housed in a controlled environment (22±2˚C, a relative humidity of 50±5% and a 12 h light:dark cycle). The animal studies were conducted in accordance with the rules and regulations established by the Institutional Animal Ethics Committee of the Kyung Hee University [KHUASP (SE) -15-079].

After acclimatization for 1 week, the mice, with the exception of the normal group, were surgically ovariectomized (OVX), then recovered and osteoporosis induced for 9 weeks. They were randomly divided into three groups (OVX + vehicle, OVX + E2, and OVX + CW). 17β-estradiol (E2; 10 μg/kg/day) was injected intraperitoneally to the OVX + E2 group as a positive control and 1 mg/kg/day CW orally administrated to OVX + CW group. Normal and OVX + vehicle mice were orally administrated vehicle (in PBS containing 1% DMSO). All mice were treated 5 times per week for 3 weeks, then sacrificed. The body weight was measured weekly. The blood sample was collected by cardiac puncture.

Bone histopathology for hematoxylin and eosin (H&E) and tartrate resistant acid phosphatase (TRAP) staining. The epicondyles were removed and immediately fixed in 10% formalin for 18 h. Prior to dehydration, bone tissues were demineralized in 0.1 M ethylenediaminetetraacetic acid for 1 month. The sections of epicondyle were cut at a 5 μm thickness and stained with H&E or an Acid Phosphatase, Leukocyte TRAP kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Stained tissues were observed using Leica Application Suite microscope software (version 3.2.276.2; Leica Microsystems, Buffalo Grove, IL, USA).

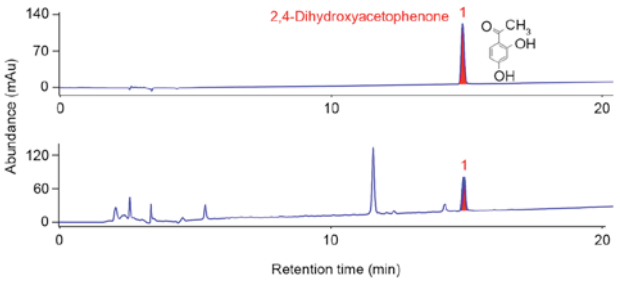

Figure 1. Standardization of CW using high-performance liquid chromatography. Chromatograms of standard 2,4-dihydroxyacetophenone (upper panel) and CW (lower panel). CW, *C. wilfordii* water extract.

Measurement of bone mineral content (BMC) and BMD. Subsequent to sacrifice, the proximal femur was collected and cleaned without attached muscles and connective tissue. The sample was stored at -80˚C in PBS until analysis. Dual-energy X-ray absorptiometry with a PIXImus instrument (Lunar Corp., Madison, WI, USA) was used for the determination of BMC and BMD.

Serum analysis. The collected blood was centrifuged at 12,000 x g for 30 min at RT and the supernatant stored at -80˚C until use. The concentration of serum osteocalcin was measured using Mouse Gla-osteocalcin high sensitive EIA kit (TaKaRa Bio, Inc., Otsu, Japan) according to the manufacturer's protocol.

Cell culture and treatment. Saos-2 cells (human osteosarcoma cell line; Korean Cell Line Bank, Seoul, Korea) were cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in 5% humidified CO_2 atmosphere. Saos-2 cells were plated in 6-well culture plates at $0.8x10^5$cells/well. CW (1, 10 and 100 μg/ml) in FBS-free DMEM medium was administered for 24 h.

Western blot analysis. RIPA buffer (50 mM Tris-HCl; pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) was used for uterus and Saos-2 cell protein extraction. The lysate (30 μg) was denatured with 2X loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and separated on a 10% sodium dodecyl sulfate (SDS) -polyacrylamide gel, and then electrotransferred onto a PVDF membrane (Bio-Rad Laboratories, Inc.). Primary antibodies targeting osterix (cat. no. Ab94744; Abcam, Cambridge, UK), osteoprotegerin (cat. no. sc-11383; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) in TBS-T (1:1,000 dilution) were incubated overnight at 4˚C and secondary antibody anti-mouse IgG (1:2,000 dilution; Cell Signaling Technology, Inc.) in TBS-T was incubated for 1 h at RT. The proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Uppsala, Sweden). Visualized bands were quantified using a computerized densitometry system

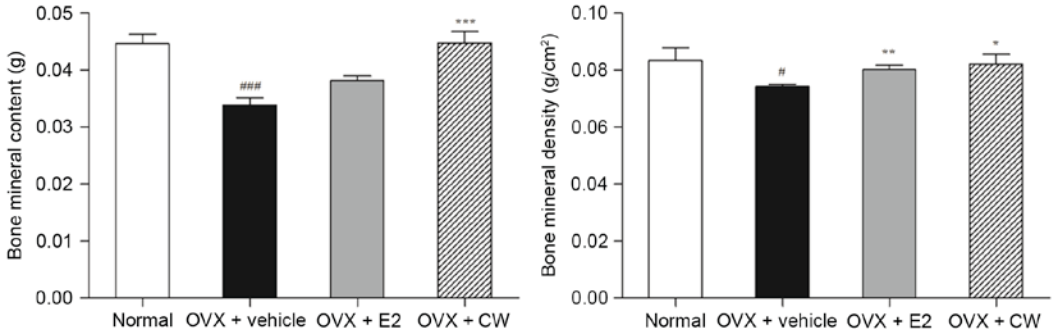

Figure 2. The effects of CW on bone mineral content and bone mineral density of femurs. Results are presented as the mean ± standard error. #P<0.05 and ###P<0.001, normal group vs. OVX + vehicle group. *P<0.05, **P<0.01 and ***P<0.001, OVX + CW or E2 group vs. OVX + vehicle group. CW, *C. wilfordii* water extract; OVX, ovariectomized group; E2, 17β-estradiol 10 μg/kg/day group.

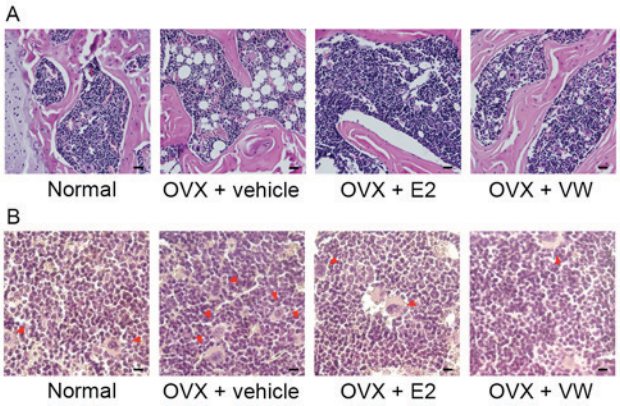

Figure 3. The effect of CW on pores within (A) interstitial cells filling the lateral and medial epicondyles and (B) osteoclast population. Sections in panel A were stained with hematoxylin and eosin (magnification, x200), and in panel B with tartrate resistant acid phosphatase (magnification, x400). Red arrows indicate multi-nuclei osteoclasts. CW, *C. wilfordii* water extract.

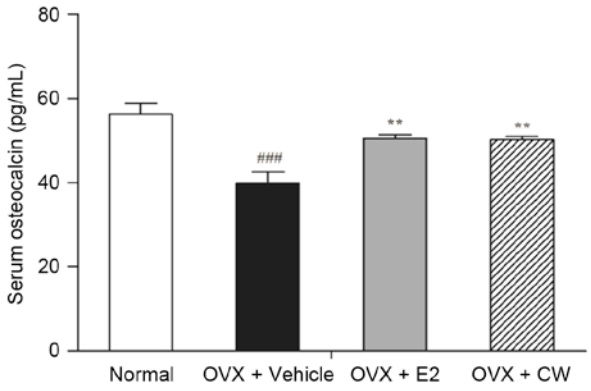

Figure 4. The effects of CW on serum osteocalcin concentrations. Results are presented as the mean ± standard error. ###P<0.001, normal group vs. OVX + vehicle group. **P<0.01, OVX + CW or E2 group vs. OVX + vehicle group. CW, *C. wilfordii* water extract; OVX, ovariectomized group; E2, 17β-estradiol 10 μg/kg/day group.

ImageJ (version 1.38e; National Institutes of Health, Bethesda, MD, USA). All samples were analyzed in triplicate.

Statistical analysis. Significance was determined by one-way analysis of variance and Dunnett's multiple comparison tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of CW on BMC and BMD. The BMC level in the OVX + vehicle group was significantly decreased to 0.034±0.003 g compared with the normal group (0.045±0.004 g). CW treatment significantly increased the level of BMC ~32% (0.045±0.004 g; Fig. 2). In addition, the BMD level of the OVX + vehicle group (0.074±0.001 g/cm^2) was significantly lowered ~10.8% compared with the normal group (0.083±0.008 g/cm^2). There was a 10.5% increase in BMD level following treatment of CW (0.082±0.006 g/cm^2; Fig. 2). Taken together, CW treatment demonstrated recoveries of BMC in addition to BMD levels.

Effect of CW on histological changes of epicondyles. In the OVX + vehicle group, the pores within interstitial cells filling the lateral and medial epicondyles were markedly increased in comparison with the normal group. E2 injection as a positive control drug ameliorated histopathological changes of epicondyles. As recoveries in the E2 injected group, CW-treated mice demonstrated dense and well-formed bone marrow cells. Additionally, CW treatment reduced the bone marrow pores in the lateral and medial epicondyles (Fig. 3A).

Effect of CW on TRAP-positive osteoclasts in epicondyles. The OVX + vehicle group demonstrated substantial increases of TRAP-stained multinucleated osteoclasts compared with normal mice. In lateral and medial epicondyles of the CW-administrated group, stained TRAP-positive cells were fewer compared with the OVX + E2 group (Fig. 3B).

Effect of CW on serum osteocalcin concentration. The value of serum osteocalcin concentration was significantly lower in the OVX + vehicle group compared with the normal group. While the serum concentration of osteocalcin in normal group was 56.26±2.6 pg/ml, that in the OVX + vehicle group was 39.91±2.69 pg/ml; a significant ~29.06% decrease. Following treatment by CW, serum osteocalcin level demonstrated a 25.93% recovery (50.26±0.7 pg/ml; Fig. 4).

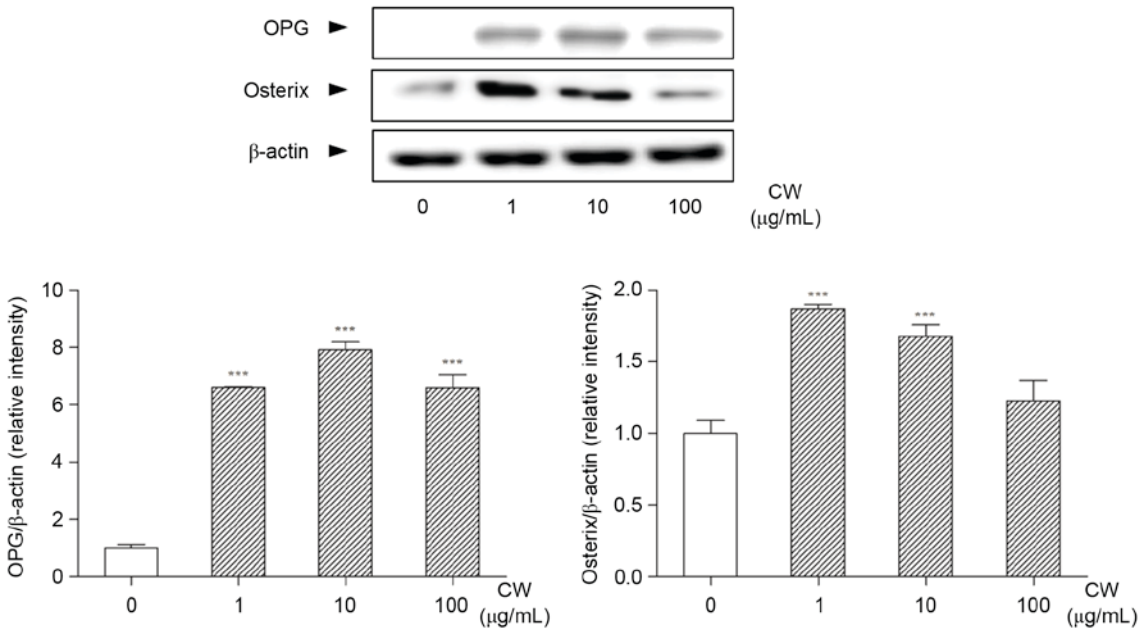

Figure 5. The effects of CW on the expression levels of OPG and osterix in Saos-2 cells. Results are presented as the mean ± standard error. ***P<0.001, non-treated vs. CW-treated cells. CW, *C. wilfordii* water extract; OPG, osteoprotegerin.

Effect of CW on bone differentiation-related markers in Saos-2 osteoblast cells. When the cells were treated with various concentrations of CW, the expression of OPG was significantly increased (Fig. 5). In addition, the expression of osterix in Saos-2 cells was increased by CW treatment compared with non-treated cells (Fig. 5).

Discussion

Osteoporosis is a skeletal disorder characterized by low BMD level and microarchitectural deterioration of bone tissue (19). Several prospective studies have demonstrated that decline in BMC or BMD is strongly associated with an increased risk of fragility fractures (20-22). The present study demonstrated that treatment with CW significantly decreased the loss of BMC and BMD in the femur. In addition, the improvement in bone mass was accompanied by complete normalization of bone structure in CW-treated mice. Histological analysis demonstrated that the bone marrow cells were restored to dense and well-formed tissue with reduced bone marrow pores by CW treatment, indicating that CW is efficacious in the maintenance of bone integrity in osteoporosis and with the enhancement of BMD.

Osteoporotic bone results from a homeostatic imbalance between bone resorption and bone formation (23). Continuous and well-balanced bone remodeling by bone-resorbing osteoclasts and bone-forming osteoblasts is essential to retain bone homeostasis, which is fundamentally controlled by proliferation and maturation of precursors of these two cells (24,25). Accordingly, bone metabolism and activities of osteoclast and osteoblast were examined to determine the improvement of osteoporosis by CW treatment *in vivo* and *in vitro*.

Osteoclasts secrete TRAP, a biomarker of osteoclast differentiation, during bone resorption and its secretion is identified to correlate positively with resorptive behavior (26,27). Histopathological examination demonstrated that OVX increased the number of TRAP-positive multinucleated osteoclasts, as expected. There was a significant decrease in the number of mature osteoclasts following CW administration in OVX-induced osteoporotic mice. Osteoblastic lineage cells are responsible for production of OPG, a secreted member of the tumor-necrosis factor receptor family, which is a crucial inhibitor of osteoclastogenesis (28). To elucidate the further mechanism of CW on osteoclasts formation, the possible effects of CW on OPG production in Saos-2 osteoblast cells was evaluated. In the present study, the expression of OPG was notably increased by CW treatment *in vitro,* which meant that the development, function and survival rate of osteoclasts can be inhibited by CW. Together, these results appear to suggest that CW treatment can inhibit bone resorption by exerting an anti-osteoclastic effect due to the increase of OPG.

Osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein, is the most abundant non-collagenous extracellular matrix protein (29). Serum osteocalcin concentration is regarded as a bone-turnover marker closely associated with bone formation (30). In particular, osteocalcin is expressed in high amounts by osteoblasts during bone matrix mineralization (31). In the present study, serum osteocalcin was decreased by OVX and partially recovered by CW treatment *in vivo*. To confirm the effect of CW on osteoblast differentiation, the expression of osterix was evaluated in Saos-2 cells. Osterix, an indispensable transcription factor that regulates osteoblastogenesis and osteogenesis, serves a critical role in the commitment and differentiation of osteoblast precursor cells to osteoblasts (32). The expression of osterix in Saos-2 cells demonstrated a noticeable increase following CW treatment, which provides evidence to support that CW promoted bone formation by upregulating the osteoblast differentiation. These results clearly indicate that CW treatment can expedite recovery from bone loss by inducing the development of mature osteoblasts and the formation of bone tissue.

Taken together, treatment of CW maintained the bone integrity, inhibited the osteoclast formation and induced the osteoblast differentiation. These results suggest that CW has ameliorative effects on osteoporosis and could be used as a treatment for osteoporosis. Further studies are required to clarify the molecular mechanisms through which CW ameliorates osteoporosis.

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