

Bone regenerative effect of aqueous *Cynanchum wilfordii* extract in receptor activator of nuclear factor- κ B ligand-induced osteoclast differentiation and estrogen deficiency-induced osteoporosis

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Abstract

Osteoporosis increases with age, most frequently in postmenopausal women because of reduced ovarian hormone levels. Furthermore, estrogen deficiency impairs trabecular metaphyseal bone. Although efficacious, long-term hormone replacement therapy (HRT) has estrogen-like side effects including breast and endometrial cancers, and non-hormonal or herbal therapies maybe safer alternatives. Therefore, the aim of this study was to investigate the effects of aqueous extracts of *Cynanchum wilfordii* (CWW) on receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL)-induced osteoclast differentiation *in vitro* and ovariectomy-mediated osteoporosis *in vivo*. CWW inhibited RANKL-induced osteoclast formation and tartrate-resistant acid phosphatase (TRAP) activity in primary mouse bone marrow-derived cells. We investigated the osteoprotective effect of CWW in an ovariectomized (OVX) Sprague-Dawley rat model treated with vehicle (OVX/vehicle), 17 β -estradiol (OVX/E2), or three CWW doses (100, 200, and 400 mg/kg). After a 24-week treatment, the body and uterus weights were not affected except in the OVX/E2 group. Additionally, bone mineral density (BMD) and histological analyses showed that the BMD of the femurs of CWW400-treated rats was significantly higher than that of the OVX/vehicle rats, and comparable to that of the OVX/E2 group rats. Serum levels of bone turnover markers alkaline phosphatase (ALP), osteocalcin, collagen type I C-telopeptide, and TRAP significantly decreased in the CWW400 group. Our results show that compared to the vehicle, CWW had a significant anti-osteoporotic effect in the OVX model. Taken together, CWW exhibited inhibitory effects on osteoclastogenesis *in vitro*, and we confirmed its *in vivo* efficacy in the prevention of osteoporosis.

Keywords : Bone mineral density, *Cynanchum wilfordii* aqueous extract, estrogen deficiency-induced osteoporosis, osteoclast differentiation

Introduction

Osteoporosis is an age-dependent multifunctional skeletal disease characterized by decreased bone mineral density (BMD), deterioration of bone microarchitecture, and increased risk of fragility fractures [1]. Bone mass is balanced by bone remodeling through bone-forming osteoblasts and bone-resorbing osteoclasts [2]. Consequently, bone homeostasis maintains continuous remodeling of osteoblasts and osteoclasts, which is ultimately determined by the proliferation and differentiation of precursors of these two bone-associated cells. Especially, osteoclasts, which are bone-resorbing multinucleated giant cells, are differentiated from hemopoietic progenitors of monocyte-macrophage lineage precursor cells following their activation by crucial cytokines:

macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) [3]. M-CSF plays a crucial role in the proliferation and survival of osteoclast precursors and the constitutive expression of RANK [4]. Osteoclasts secrete tartrate-resistant acid phosphatase (TRAP), a biomarker of osteoclast differentiation, during bone resorption and the secretion is found to correlate positively with resorptive behavior [5]. Excessive osteoclastogenesis is associated with the pathogenesis of osteoporosis [6].

In preclinical and clinical research, bone turnover biomarkers are categorized into bone formation and resorption markers [7]. Bone formation markers are osteoblastic enzymes or by-products of active osteoblasts produced during the various phases of bone development. Most bone resorption markers are degradation products such as collagen type I, non collagenous bone matrix

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proteins, and osteoclastic enzymes. In addition, several regulators of bone cell activity and, therefore, bone turnover may also be used as biomarkers. Bone turnover biomarkers used in preclinical and clinical research of bone metastasis are serum bone-specific alkaline phosphatase (BALP), osteocalcin (OCN), and procollagen type I N propeptide (P1NP), as well as pyridinoline (PYD), deoxypyridinoline (DPD), aminoterminal crosslinked telopeptide of type I collagen (NTX-I), and carboxy-terminal crosslinked telopeptides of collagen type I (CTX-I and ICTP) [8].

A menopause-triggered decrease in BMD and high turnover bone metabolism is associated with the occurrence of post-menopausal osteoporosis [9]. Estrogens are also the main factors regulating bone mass in postmenopausal women by maintaining the balance of osteoblastic bone formation and osteoclastic bone resorption [10]. Ovariectomy-induced estrogen deficiency triggers similar bone imbalances in experimental animals while estrogen supplementation was shown to recover the normal bone mass and turnover in post-menopausal women and ovariectomized animals [11].

The occurrence of osteoporosis increases with age, and most frequently in postmenopausal women owing to the menopausal decline in ovarian hormones [12]. In addition, estrogen deficiency impairs trabecular metaphyseal bone, which reduces bone mass in humans and animals [13]. Hormone replacement therapy (HRT) has proven efficacious in preventing osteoporosis and reducing the incidence of bone fractures in postmenopausal women [14]. However, long-term HRT has estrogen-like side effects such as breast and endometrial cancers [15]. Therefore, non-hormonal or herbal medicine therapies have gained attention as alternative therapies from natural sources [16].

Cynanchum wilfordii components have been reported to show anti-inflammatory, hepatoprotective, neuroprotective, and gastroprotective effects [17]. In the treatment of menopausal symptoms, herbal mixtures containing *C. wilfordii* have been used to improve symptoms including hot flashes, vaginal dryness, night sweats, and insomnia [18]. However, the anti-osteoporotic effect of *C. wilfordii* in an ovariectomized (OVX)-induced rat model and on osteoclastic bone resorption has not been reported. Therefore, in the present study, we investigated the anti-osteoporotic effect of *C. wilfordii* on RANKL-induced osteoclastogenesis *in vitro* and ovariectomy-induced bone loss *in vivo*.

Material and Methods

Chemicals

α -Modified minimal essential medium (α -MEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic-antimycotic solution, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen Co., (Grand Island, NY, USA). RANKL and M-CSF were purchased from Peprotech (Rocky Hill, NJ, USA).

The rat Gla-OCN high sensitive enzyme immunosorbent assay (EIA), TRACP and ALP assay (Takara), TRAP staining (Cosmotic), and rat CTx enzyme-linked immunosorbent assay (ELISA, MyBiosource) kits, as well as eosin Y solution, hematoxylin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Preparation of aqueous extract of *C. wilfordii* (CWW)

C. wilfordii root (2 kg) was extracted with distilled water at 100°C for 4 h at 0.7–0.75 kgf/cm², filtered through Whatman No. 4 filter paper (Maidstone, UK), and then concentrated using a rotary evaporator (R-210, Buch, Flawil, Switzerland). The concentrated extract was then lyophilized using a freeze-dryer and stored at -20°C until it was used. The aqueous extract of *C. wilfordii* was designated as CWW, which was used as the test sample.

Murine bone marrow-derived osteoclasts

Bone marrow cells were isolated from 5-week-old ICR mouse as previously described [19]. In brief, bone marrow from the tibia and femur of each mouse was flushed with Hank's balanced salt solution (HBSS) and the cells were plated in 24-well plates in 30 ng/mL M-CSF for 24 h. Next, the cells were treated with the indicated concentrations of CWW in the osteoclastogenic medium (30 and 200 ng/mL M-CSF and RANKL, respectively) for 5 days. The medium was replaced with a 50% volume with and without CWW every 2 days.

TRAP staining

Osteoclast differentiation was assessed by analyzing the TRAP activity. Five days after stimulating the cells with M-CSF and RANKL (30 and 200 ng/mL, respectively), they were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 5 min. Then, the cells were rinsed in deionized water, incubated in tartrate-staining solution in the dark for 1 h at 37°C, rinsed in deionized water, and allowed to air dry. Images of the TRAP-positive multinucleated cells were captured using a microscope (Foculus, Leica, Germany). To measure the TRAP activity, the reaction mixture was transferred to new plates containing an equal volume of 0.1M sodium hydroxide (NaOH), the absorbance was measured at 410nm, and TRAP was expressed as a percentage (%) of the control.

Animals

Female Sprague-Dawley rats (230–250 g, 10-week-old) were purchased from Samtako (Osan-si, Gyeonggi-do, Korea). The animals were acclimated to standard laboratory conditions with free

access to food and water for 1 week before the experiment. The temperature was thermostatically regulated to $23 \pm 2^\circ\text{C}$ and a 12-h light-dark schedule was maintained. Prior to their use, the rats were allowed a 1-week acclimatization to the experimental environment, and all the animal experimental protocols were approved by the Animal Ethical Committee of Jeollanamdo Institute for Natural Resources Research (JINR1422). At 11 weeks old, 50 and 10 Sprague-Dawley rats were bilaterally OVX, and sham-operated (Sham/vehicle), respectively.

After a 1-week recovery from the surgery, the OVX rats were randomly divided into the following five groups of 10 rats each and treated as indicated: OVX administered vehicle (OVX/vehicle); OVX administered 17β -estradiol $91 \mu\text{g}/\text{kg}$ (OVX/E2); and OVX administered CWW100, 200, or 400 mg/kg (OVX/CWW100, 200, or 400, respectively). CWW was orally administered in distilled water (0.3 mL) for 24 weeks, and the Sham/vehicle and OVX/vehicle groups were administered the same volume of distilled water. After 24 weeks of treatment, the animals were euthanized, and blood samples were collected for serum isolation. The femur bones were dissected, and the soft tissue removed to enable the analysis of the trabecular microarchitecture.

Dual-energy X-ray absorptiometry (DEXA) measurement

All dual-energy X-ray absorptiometry (DEXA) measurements were performed using the Norlandp DEXA Sabre (Fort Atkinson, WI, USA) equipped with the Sabre Research software (v3.6). The inter-assay coefficient of variation (CV) for the BMD and BMC was 1.7%. The scanner was calibrated daily to a dual-material standard according to the manufacturer's recommendations, and the scanner performance was controlled using a quality assurance protocol from our laboratory. The rats were scanned using DEXA to determine the BMC and BMD and the DEXA measurements were collected using a special collimator (0.8-mm diameter). The scan length, width, and speed were 5 cm, 2 cm, and 10 mm/s, respectively at a resolution of 0.2 mm \times 0.2 mm [20]. The deltoid tuberosity was positioned upward to avoid an irregular projecting shape. The scan started above the distal condyle of the femur and ended proximal to the femoral end so that the scanner arm moved along the long axis of the femoral shaft, which allowed us to evaluate the femur length. The baseline point was on the cotton [21], and the BMD was calculated using the BMC of the measured area.

Preparation of histological specimens

One day following the final treatment, the animals were euthanized by exsanguination, the uteri were dissected out, and then weighed

immediately [22]. The right femurs were fixed in 4% paraformaldehyde for 24 h and then decalcified in 10% EDTA at 4°C for 4 weeks. Then, the decalcified samples were dehydrated in an ethanol gradient of 80%, 90%, and 100% for 2 days at each step, defatted in xylene for 2 days, and embedded in plastic polymer. Then decalcified sections (5- μm) were cut using a microtome (Reichert-Jung 2040, Leica, Germany), and stained with hematoxylin and eosin (H&E).

Analysis of serum bone turnover biomarkers

The serum concentrations of OCN and ALP activity were assayed using an ELISA kit (Biomedical Technologies Inc., Stoughton, MA, USA) and QuantiChrome ALP assay kit (DALP-250, BioAssay Systems, CA, USA), respectively according to the manufacturers' instructions. The serum levels of CTx bone resorption biomarkers that are indicative of osteoclastic activity were also analyzed using commercial ELISA kits (Serum Cross Laps, Nordic Bioscience, Herlev, Denmark). The TRAP concentration was determined using a rat TRAP assay kit (Suomen Bioanalytikka Oy, Turku, Finland).

Statistical analysis

The results are expressed as mean \pm standard error of the mean (SEM) and the group data were compared using an analysis of variance (ANOVA), followed by Dunnett's post hoc test. All statistical analyses were performed using the Graph Pad Prism 5 for Windows (Graph Pad Software, San Diego, CA, USA) and a p -value < 0.05 was considered statistically significant.

Results

Effect of CWW on osteoclast differentiation

We investigate the effects of CWW on osteoclast differentiation using TRAP staining and activity assays in BMCs cultured for 3 days in RANKL (200 ng/mL) with or without CWW. We found that CWW or E2 significantly decreased TRAP activity. Furthermore, TRAP staining was performed to evaluate the effect of CWW on RANKL-induced osteoclast differentiation. Figure 1 shows that no cytotoxicity was observed against BMCs at concentrations up to 600 $\mu\text{g}/\text{mL}$ CWW as determined using the MTT assay ($>85\%$ cell survival). BMCs were differentiated into osteoclasts in the presence of RANKL and M-CSF for 5 days. CWW inhibited TRAP-positive cell formation during RANKL-induced osteoclast differentiation in a concentration-dependent manner (Figure 1).

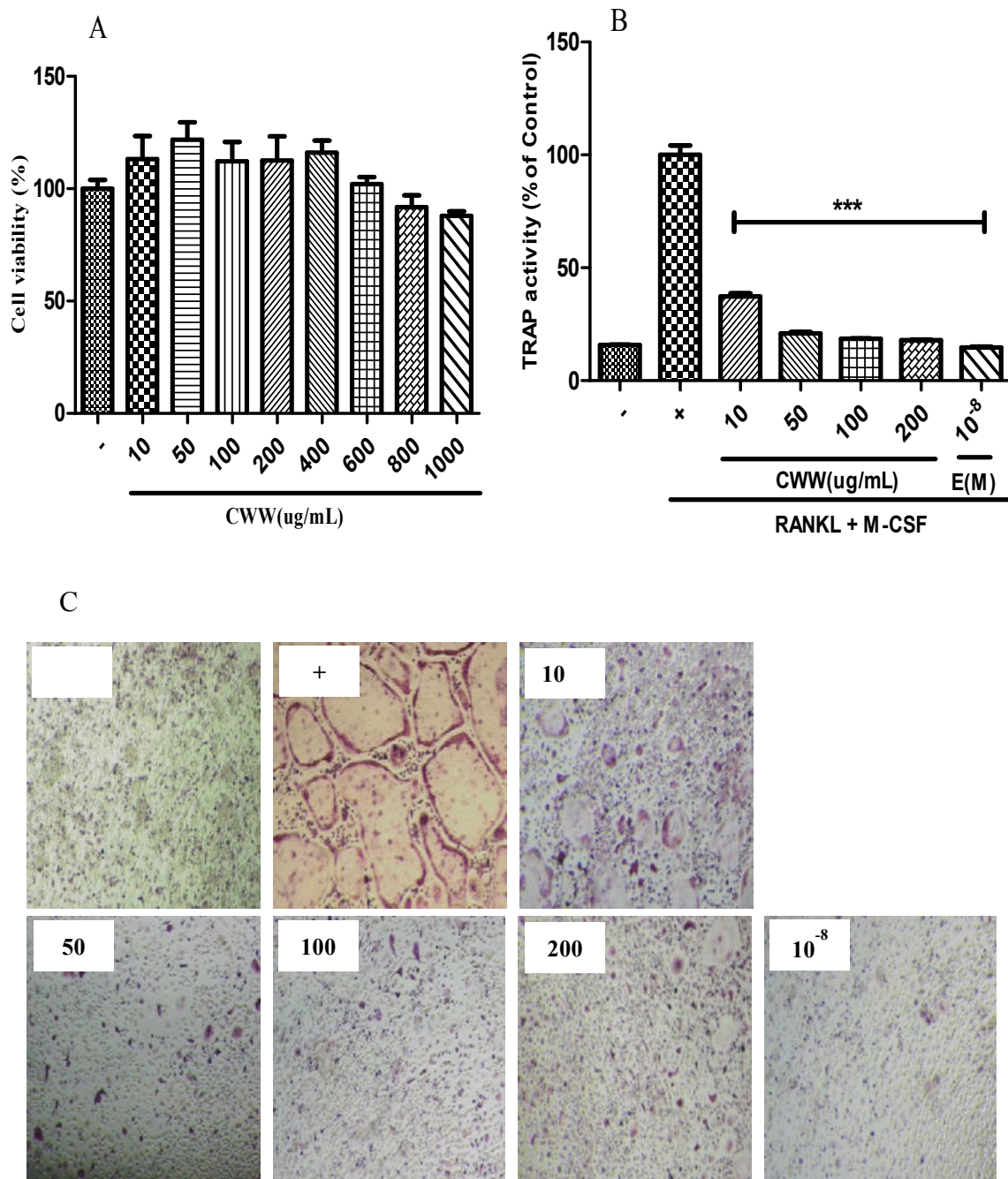


Figure 1. Effect of aqueous extract of *Cynanchum wilfordii* (CWW) on osteoclast differentiation. (A) Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Bone marrow cells (BMC, 1×10^4 cells/mL) were exposed receptor activator of nuclear factor kappa-B ligand (RANKL, 200ng/mL) and macrophage-colony stimulating factor (M-CSF, 30 ng/mL) for 5 days with or without different CWW concentrations. Tartrate-resistant acid phosphatase (TRAP) activity was measured at 410 nm. (C) Cells were fixed and stained for TRAP. 40. Data are expressed as a percentage of TRAP activity of untreated cells. Mean \pm standard deviation (SD, n = 4); *** p < 0.005 vs. control group.

Body and uterine weights

The effects of CWW on the body and uterine weight are presented in Table 1, which shows that the initial body weights were similar among groups. After 24-week treatments, the body weight of the OVX/vehicle group was significantly higher than that of the Sham/vehicle was. The OVX-induced body weight increase was

significantly inhibited in the OVX/E2 group, but CWW did not significantly affect the body weight at all three doses. Additionally, ovariectomy caused atrophy, which was mediated by estrogen deficiency in the OVX/vehicle rats. E2 administration increased the uterus index significantly compared to that of the OVX/vehicle group, but the uterine weight was still lower than that of the Sham/vehicle group while CWW showed nouterotrophic effects.

Table 1. Comparison of body weight and uterine index before and after 24-week treatment

Group	Body weight (g)		Uterine index (g/100 g BW)
	Initial	Final	
Sham/vehicle	229.2 ± 4.72	319.4 ± 14.77	0.37 ± 0.07
OVX/vehicle	231.0 ± 4.61	414.5 ± 8.83###	0.05 ± 0.01###
OVX/E2	230.5 ± 4.14	348.9 ± 6.93**	0.12 ± 0.01***
OVX/CWW100	231.6 ± 3.92	365.4 ± 20.09	0.07 ± 0.01
OVX/CWW200	228.9 ± 3.63	385.9 ± 18.20	0.04 ± 0.01
OVX/CWW400	229.1 ± 3.49	377.1 ± 9.76	0.05 ± 0.14

Values are mean ± standard error of the mean (SEM), n = 8 rats. In columns, superscripts show significant differences; ### $p < 0.005$ vs Sham/vehicle, ** $p < 0.01$ and *** $p < 0.001$ vs.OVX/vehicle. Groups and treatments: Sham/vehicle, sham-operated, vehicle; OVX/vehicle. Ovariectomized, vehicle; OVX/E2 ovariectomized, 17β-estradiol 91 μg/kg; and OVX/CWW100, 200, or 400, ovariectomized, CWW100, 200, or 400 mg/kg, respectively.CWW, aqueous extract of *Cynanchum wilfordii*.

Effects of CWW on BMD, BMC, and projected bone area

To investigate the anti-osteoporotic effects of CWW, the total femur BMD, BMC, and projected bone area (AREA) were measured using DEXA. Figure 2 shows that no differences occurred in the total

femur BMC and AREA between the OVX groups by the end of the treatment. However, ovariectomy significantly decreased the total femur BMD compared with that of the Sham/vehicle ($p < 0.05$).After 24-week treatments, the final femur BMD of the OVX/E2 and OVX/CWW400 groups increased more significantly than that of the OVX/vehicle group did.

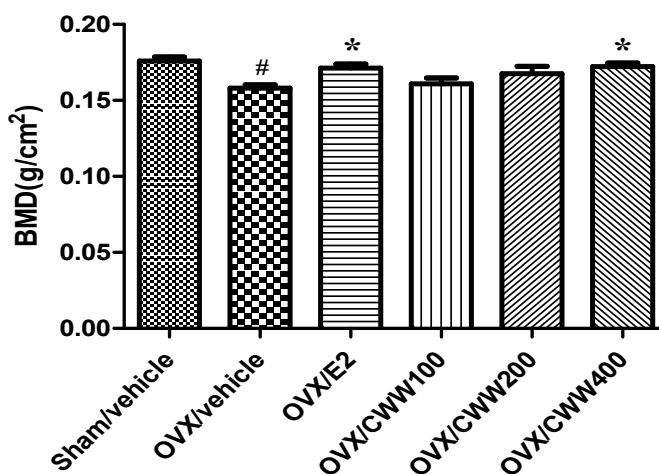


Figure 2.Anti-osteoporotic effect of aqueous extract of *Cynanchum wilfordii* (CWW).Effects of CWW on bone mineral density (BMD)of femurs measured using dual-energy X-ray absorptiometry (DEXA);# $p < 0.05$ vs Sham/vehicle, * $p < 0.05$ vs. OVX/vehicle group. Groups and treatments: Sham/vehicle, sham-operated, vehicle; OVX/vehicle, ovariectomized, vehicle; OVX/E2 ovariectomized, 17β-estradiol 91 μg/kg; and OVX/CWW100, 200, or 400,ovariectomized, CWW 100, 200, or 400 mg/kg, respectively.

Effect of CWW on histological morphology of femur bone

Bone turnover is a life-long process involving two remodeling processes (bone resorption and formation). In this study, the histomorphology of the femur bone region of OVX rats was investigated using H&E staining. The femur region of the OVX group was small, thin, and sparse compared with that of the sham group. Furthermore, compared with the OVX group, the CWW

group showed less trabecular loss in the femurs. Figure 3 shows a representative histological section of the femur bone region of the Sham/vehicle and OVX/vehicle rats and those treated with E2 and CWW. Sections from sham-operated rats showed thick trabecular bone with relatively scant marrow space (Figure 3A). Ovariectomy markedly reduced the trabecular bone volume and increased the marrow space (Figure 3B) while 24-week treatment with E2 and CWW markedly increased trabecular bone volume (Figure 3E and F), although E2 was more potent than CWW was (Figure 3C).

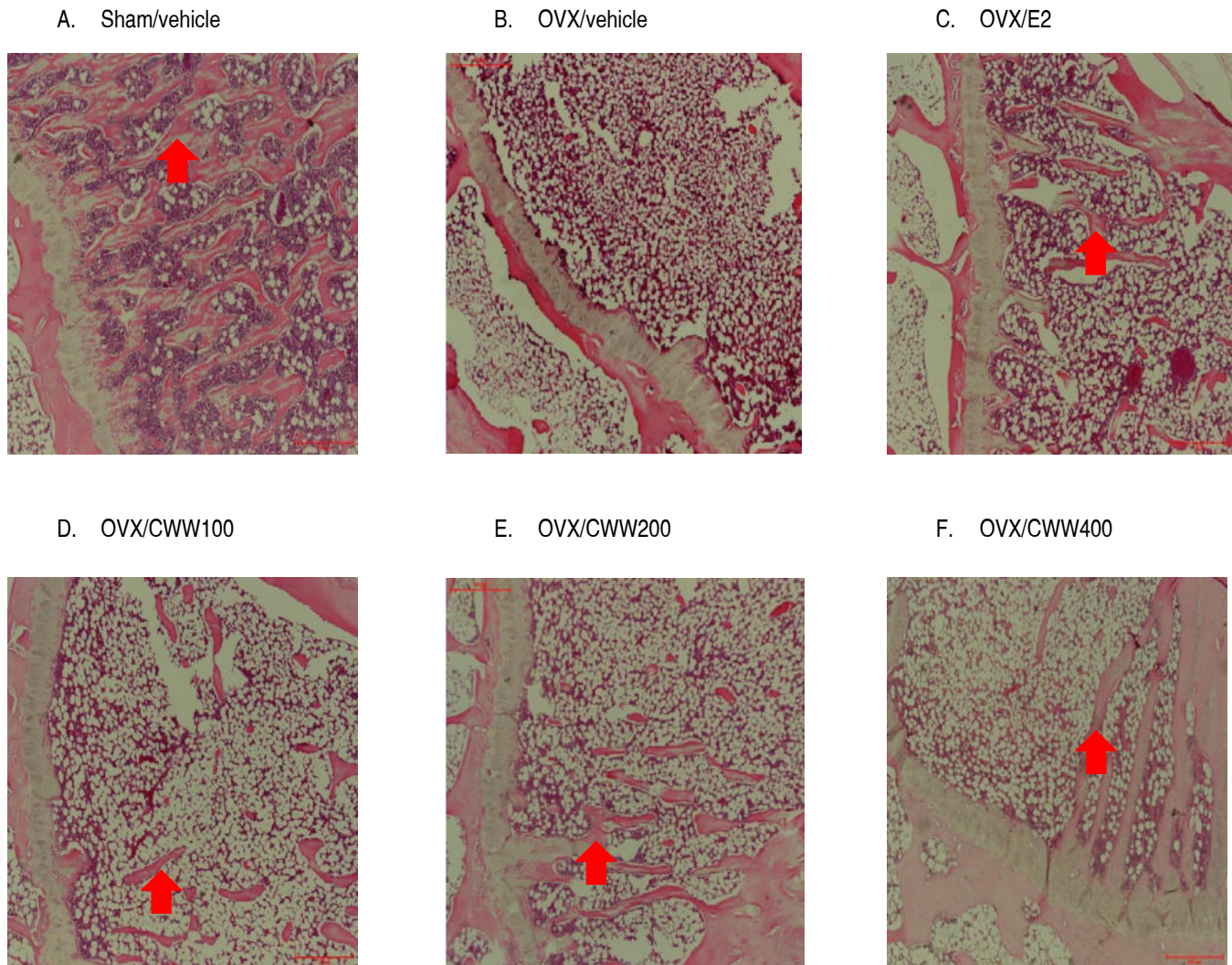


Figure 3. Histology of trabecular bone region of femur in rats. Sections were hematoxylin and eosin (H&E)-stained; scale bar = 100 μ m; original magnification, 40. Red arrow indicates trabecular bone. Groups and treatments: Sham/vehicle, sham-operated, vehicle; OVX/vehicle, ovariectomized, vehicle; OVX/E2 ovariectomized, 17 β -estradiol 91 μ g/kg; and OVX/CWW100, 200, or 400, ovariectomized, aqueous extract of *Cynanchum wilfordii* 100, 200, or 400 mg/kg, respectively.

Effect of CWW on serum biochemical markers in OVX rats

The effects of CWW on bone metabolic biomarkers are presented in Figures 4 and 5. The OVX-induced bone loss was characterized

by high bone turnover in the rats, as evidenced by high serum ALP, OCN, CTx, and TRAP levels. E2 treatment reversed the OVX-induced increase in ALP concentration. OCN markedly increased in OVX rats, but CWW and E2 treatment inhibited this effect. The serum levels of bone formation markers (ALP and OCN) significantly increased in the OVX/vehicle group compared to levels in the Sham/vehicle group. However, E2 and CWW decreased the

elevated serum ALP and OCN levels in the OVX rats ($p < 0.05$). Additionally, the serum TRAP level, which is responsible for enhanced osteoclastogenesis and activation of mature osteoclasts for bone resorption, was increased in the OVX/vehicle group, but CWW significantly reduced TRAP activity ($p < 0.05$). The increase in another bone resorption marker, CTx, in the OVX/vehicle group was also inhibited by CWW ($p < 0.05$).

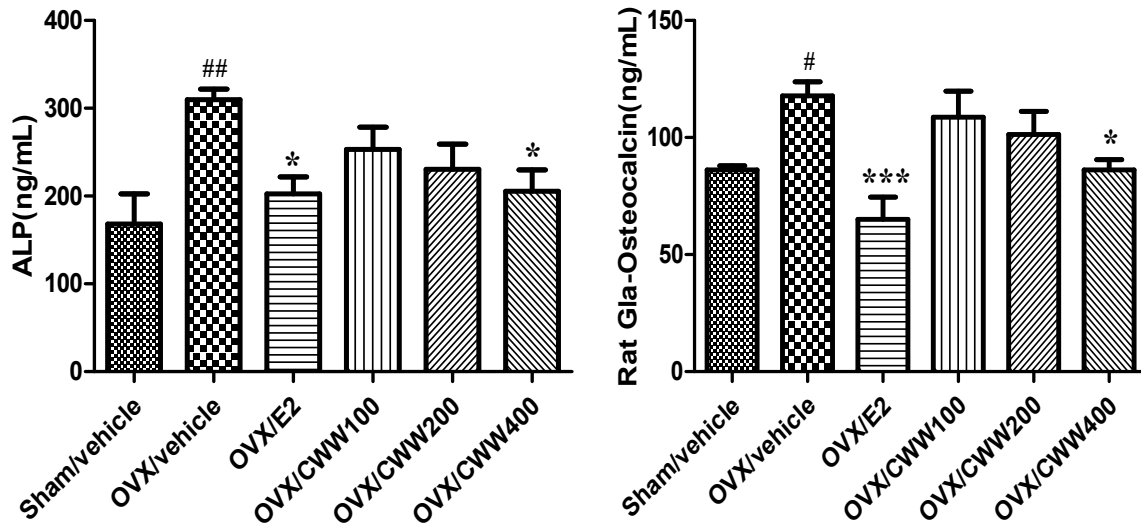


Figure 4. Effect of aqueous *Cynanchum wilfordii* extract (CWW) on serum alkaline (ALP) and osteocalcin (OCN). Rats were treated for 24 weeks; # $p < 0.05$ and ## $p < 0.01$ vs Sham/vehicle, * $p < 0.05$ and *** $p < 0.005$ vs. OVX/vehicle group. Groups and treatments: Sham/vehicle, sham-operated, vehicle; OVX/vehicle, ovariectomized, vehicle; OVX/E2 ovariectomized, 17 β -estradiol 91 μ g/kg; and OVX/CWW100, 200, or 400, ovariectomized, CWW 100, 200, or 400 mg/kg, respectively.

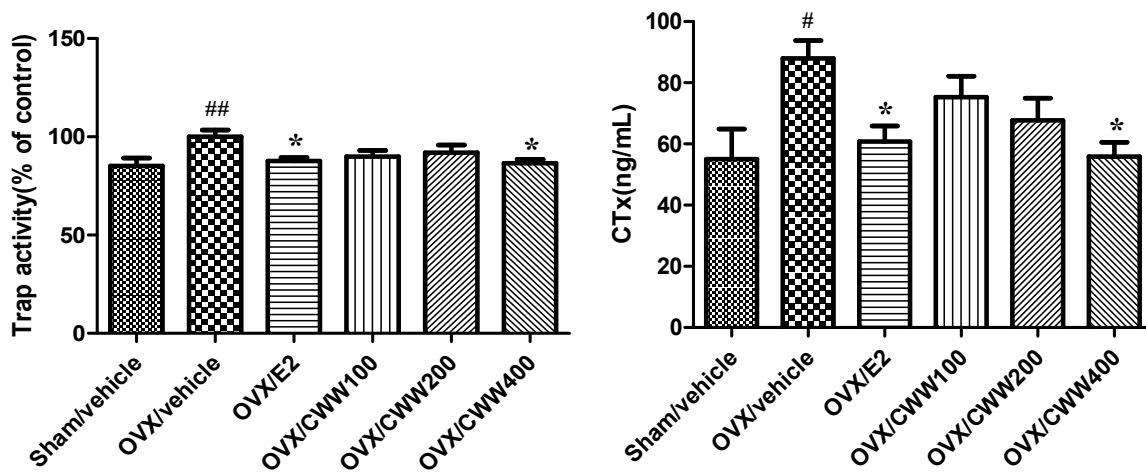


Figure-5. Effect of aqueous extract of *Cynanchum wilfordii*(CWW) on bone metabolic markers. Effects of CWW on C-terminal cross-linked telopeptides of type I collagen (CTx) and tartrate-resistant acid phosphatase (TRAP) in serum after 24-week treatment; # $p < 0.05$ and ## $p < 0.01$ vs Sham/vehicle, * $p < 0.05$ vs. OVX/vehicle group. Groups and treatments: Sham/vehicle, sham-operated, vehicle; OVX/vehicle, ovariectomized, vehicle; OVX/E2, ovariectomized, 17 β -estradiol 91 μ g/kg; and OVX/CWW100, 200, or 400, ovariectomized, CWW 100, 200, or 400 mg/kg, respectively.

Discussion

Bone remodeling is regulated by a balance between the formation of new bone by osteoblasts and resorption of old bone by osteoclasts, and an imbalance in osteoclastogenesis causes the bone loss in osteoporosis [23]. M-CSF and RANKL are important cytokines that induce the differentiation of osteoclast precursors into activated osteoclasts [24]. Using *in vitro* and *in vivo* studies, we demonstrated the anti-osteoclastogenic activity of CWW, which exhibited anti-osteoporotic effects. *In vitro*, CWW effectively inhibited RANKL-induced osteoclast differentiation. Osteoporosis is a disease characterized by bone loss and deterioration of bone tissue, and patients exhibit lower bone density and bone mass than healthy individuals do [25]. Bone loss triggered by estrogen decline in experimental animals and humans is due to an increase in osteoclastic bone resorption [26]. OVX rats, which exhibit most of the characteristics of human postmenopausal osteoporosis [27], are widely used to evaluate potential osteoporosis treatments [28]. Although the exact mechanisms by which OVX increases body weight are not clear, a recent study reported that an estrogen-deficient state induced body fat accumulation and increased body weight [29]. OVX-rats show increased body weight compared to sham-operated rats while E2 treatment prevents body weight gain [30]. Estrogen deficiency induces body fat accumulation and subsequently increases body weight [29]. Estrogen acts by binding to different ERs (ER and ER β). ER β is more abundant than ER is in bone tissue, while ER, which, is the dominant receptor mediating the effects of E2 on the breast and uterus [31].

Selective estrogen receptor modulators (SERMs) are synthetic non-steroidal agents that exhibit ER agonistic or antagonistic activities depending on the target tissue. A novel approach to postmenopausal therapy is tissue-selective estrogen complex (TSEC) treatment, which pairs a SERM with one or more estrogens. The goals of TSEC are to provide the benefits of estrogens such as reduced hot flashes and vulvar-vaginal atrophy, thereby preventing menopausal osteoporosis events, and protecting the endometrium and breast tissue from estrogen stimulation [32]. In our experiments, oral administration of CWW did not affect body weight gain and uterotrophic activity in OVX rats. These results suggest that CWW might have anti-osteoporotic effects in OVX rats without the hormonal effects of estrogen. These results are in accordance with previous studies [33]. Therefore, it would be useful to study the interaction between *C. wilfordii* and ERs.

Bone loss is a major factor jeopardizing bone integrity, resulting in reduced bone strength and increased susceptibility to fracturing [34]. BMD is the gold standard for evaluating individuals who are at risk for osteoporosis because it best predicts the fracture risk in people without previous fractures [35]. Following ovariectomy, BMD markedly decreases owing to increased bone turnover in OVX rats

compared to the Sham rats. The current results reveal that OVX significantly decreased the total femur BMD compared with that of the sham group. CWW prevented OVX-induced bone loss of the total femur.

Bone turnover biochemical markers have been determined previously, and allow evaluation of the bone remodeling status [36]. ALP is a ubiquitous ectoenzyme, which catalyzes the hydrolysis of monophosphate ester groups. Four genes encode the isoenzymes, which are the intestinal, placental, germ cell, and tissue non-specific (bone/osteoblast, liver, and kidney isoforms) [37]. OCN, a critical non collagenous protein synthesized by osteoblasts, is primarily deposited in the bone extracellular matrix and considered a specific marker of osteoblast function [38]. The crosslinked telopeptides are released from type I collagen degradation by proteases during bone resorption generate neoepitopes (CTX-I, ICTP, NTX-I). Serum ALP and OCN are phenotypic biomarkers of osteoblastic activity, and CTx and TRAP are widely accepted biomarkers of bone resorption. Serum ALP, OCN, CTx, and TRAP concentrations in OVX rat were significantly higher than those in the Sham group were while 24-week E2 or CWW treatments lowered the increased bone turnover. This was evidenced by the significant decreases in ALP, OCN, CTx and TRAP. In this study, we found that CWW prevented bone loss, suggesting it most likely prevented bone loss by decreasing bone turnover. Finally, we showed that CWW inhibited osteoclast differentiation, prevented bone mass reduction, and improve trabecular bone structure and biochemical markers in OVX-induced osteoporosis.

Conclusion

In conclusion, *C. wilfordii* inhibited osteoclast differentiation, prevented bone mass reduction, and improved trabecular bone structure and biochemical markers in OVX-induced osteoporosis. These results indicate that *C. wilfordii* has ameliorative effects on osteoporosis. In addition further studies are needed to clarify the molecular mechanisms.

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Conflict of interest

All the authors state that there are no conflicts of interest within this article.

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