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Original Article

Humulus lupulus L. Extract Prevents Ovariectomy-Induced Osteoporosis in Mice and Regulates Activities of Osteoblasts and Osteoclasts*

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ABSTRACT **Objective:** To systematically evaluate the protective effects of *Humulus lupulus* L. extract (HLE) on osteoporosis mice. **Methods:** *In vivo* experiment, a total of 35 12-week-old female ICR mice were equally divided into 5 groups: the sham control group (sham); the ovariectomy with vehicle group (OVX); the OVX with estradiol valerate [EV, 0.2 mg/(kg·d)]; the OVX with low- or high-dose HLE groups [HLE, 1 g/(kg·d) and 3 g/(kg·d)], 7 in each group. Treatment began 1 week after the ovariectomized surgery and lasted for 12 weeks. Bone mass and trabecular bone mircoarchitecture were evaluated by micro computed tomography, and bone turnover markers in serum were evaluated using enzyme-linked immunosorbent assay (ELISA) kits. *In vitro* experiment, osteoblasts and osteoclasts were treated with HLE at doses of 0, 4, 20 and 100 μg/mL. Biomarkers for bone formation in osteoblasts and bone resorption in osteoclasts were analyzed. **Results:** Compared with the OVX group, HLE exerted bone protective effects by the increase of estradiol ($P<0.05$), the improvement of cancellous bone structure, bone mineral density ($P<0.01$) and the reduction of serum alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP), bone gla-protein, c-terminal telopeptides of type Ⅰ collagen (CTX-Ⅰ) and deoxypyridinoline levels ($P<0.01$ for all). *In vitro* experiment, compared with the control group, HLE at 20 μg/mL promoted the cell proliferation ($P<0.01$), and increased the expression of bone morphogenetic protein-2 and osteopontin levels in osteoblasts (both $P<0.05$). HLE at 100 μg/mL increased the osteoblastic ALP activities, and HLE at all dose enhanced the extracellular matrix mineralization (both $P<0.01$). Furthermore, compared with the control group, HLE at 20 μg/mL and 100 μg/mL inhibited osteoclastic TRAP activity ($P<0.01$), and reduced the expression of matrix metalloproteinase-9 and cathepsin K (both $P<0.05$). **Conclusion:** HLE may protect against bone loss, and have potentials in the treatment of osteoporosis.

KEYWORDS *Humulus lupulus* L., osteoporosis, ovariectomy-induced mice, osteoblasts, osteoclasts

Osteoporosis, defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, affects millions of people worldwide.[1] In postmenopausal women, aging or hormonal deficiency induces the dysregulation of bone homeostasis, leads to a higher bone turnover, and hence, increases bone formation and even greater rates of bone resorption.[2] Postmenopausal hormone replacement therapy (HRT) can reduce the osteoporotic fractures and bone loss, but other risks such as mammary cancer, endometrial carcinoma and cardiovascular disease outweigh its bone protective benefits.[3] Therefore, it is urgent and necessary to develop suitable therapeutic alternatives for osteoporosis with less adverse effects.

Humulus lupulus L. also called hops, is an indispensable raw material in brewing industry all over the world, and has been traditionally used as a sleep aid and, more recently, by women for postmenopausal symptom relief.[4] Hops contains a varieties of chemical constituents, including bitter acids (e.g., α-acids, β-acids) and prenylflavonoids (e.g., xanthohumol, isoxanthohumol, 8-prenylnaringenin). Both bitter

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acids and prenylflavonoids from hops have been proved to possess many biological activities, such as preventing bone tissue injury, lowering body weight,[5,6] treating breast cancer,[7] and alleviating menopausal complaints. The prenylflavonoids from hops also show a significant activity on inhibiting osteoclastic bone resorption,[8] enhancing osteoblastic bone formation,[9] relieving vasomotor symptoms and other menopausal discomforts.[10] However, the antiosteoporotic activity of hops has not been scientifically evidenced.

Evidences have shown that chemical constituents in hops possess estrogen-like activities and regulatory effects on bone metabolism.[11] This led us to investigate whether hops could attenuate the bone loss induced by estrogen deficiency in mice, and exert regulatory effects on osteoblasts and osteoclasts, and provide more promising candidates for the prevention and treatment of osteoporosis.

METHODS

Drugs and Reagents

Humulus lupulus L. (PJH-01) was obtained from Xinjiang Autonomous Region, China, and identified by Prof. XIN Hai-liang, Department of Pharmacognosy, School of Phararmacy of the Second Military Medical University. Dried powders (70 g) of hops were reflux-extracted with 75% ethanol for 3 times. The filtrate was concentrated to dryness, and the extractum was suspended in ethanol and analyzed with high performance liquid chromatography (HPLC). The content of xanthohumol was up to 0.55% (Appendix 1).

Enzyme-linked immunosorbent assay (ELISA) kits for determination of bone gla-protein (BGP), c-terminal telopeptides of type Ⅰ collagen (CTX-Ⅰ) and deoxypyridinoline (DPD) were purchased from Xinyu Biological Engineering Co., Ltd., (China). Estradiol (E_2) assay kits were purchased from R&D Co., Ltd., (USA). Assay kits for determination of alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP) and calcium were purchased from Nanjing Jiancheng Bioengineering Institute (China). Estradiol valerate (EV) was purchased from Bayer Pharmaceutical Company (China). α-modified minimal essential medium (α-MEM) was purchased from Shanghai Jinuo Biological Technology Co., Ltd., (China). Fetal bovine serum (FBS) was purchased from Gibco (USA). Antibodies specific for osteopontin (OPN), bone morphogenetic protein-2 (BMP-2), matrix metalloproteinase-9 (MMP-9) and cathepsin K (CK) were purchased from Abcom (UK). Anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from CST (USA).

Animal Experimental Protocols

Totally 12-week-old female ICR mice of 19–22 g were purchased (Slacom, China) and maintained at the Experimental Animal Center of the Second Military Medical University. The mice were housed at 24±0.5 ℃ with a 12-h light and 12-h dark cycle and had free access to food and water. The acclimatized mice were anesthetized with 300 mg/kg chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., China) intraperitoneal injection and underwent either bilateral laparotomy (sham, *n*=7) or bilateral ovariectomized (OVX, *n*=28). One week after recovering from surgery, the OVX mice were randomly divided into 4 groups: OVX with vehicle (OVX); OVX with estradiol valerate [EV, 0.2 mg/(kg•d)]; OVX with ethanol extract of *Humulus lupulus* L. [HLE1, 1 g/(kg•d); HLE3, 3 g/(kg•d)]. The treatment started 1 week after the surgery and lasted for 12 weeks. All drugs were solved in saline and administrated orally. The body weights of mice were measured weekly during the experimental period. One day before the animals euthanized, urine samples were collected from mice that were housed individually without food for 24 h in a metabolic cage, and followed, the urine samples were centrifuged at 3,000×*g* for 10 min. After anesthetized with intraperitoneal injection of 300 mg/kg chloral hydrate, the blood samples of mice were obtained by plucking the eyeball. The serum was then prepared by centrifugation at 3,000×*g* for 10 min. Urine and serum were stored at –20 ℃ until biochemical assays. Uteruses were removed and immediately weighed. Right femurs were dissected and prepared for micro computed tomography (micro-CT) scanning. All studies were conducted according to the principles and procedures of the Care and Use of Laboratory Animals of the Second Military Medical University.

Micro-CT Analysis and Biochemical Markers Assay

The right femurs of mice were fixed in 4% paraformaldehyde for evaluating trabecular microarchitecture using a micro-CT system (eXplore Locus, GE Healthcare, USA) with MicroView ABA

analysis software version 2.1.2. The parameters setting were identical to literature.[12] The following bone indices were measured: bone mineral content (BMC), bone mineral density (BMD), tissue mineral content (TMC), tissue mineral density (TMD), bone volume fraction (BVF), bone volume to tissue volume (BV/TV) and trabecular number (TbN). The concentrations of BGP, CTX-Ⅰ, DPD and E_2 in serum were measured by enzyme immunoassays with ELISA kits according to the manufacturer's instructions. The concentrations of ALP, TRAP in serum and the calcium concentrations in urine were measured by commercial kits according to the manufacturer's instructions.

Cell Culture

Three days old Wistar rats were purchased from the Experimental Animal Center of the Second Military Medical University (Shanghai, China). Primary osteoblasts were prepared from the calvarias according to literature.[13] The cells were cultured in α-MEM with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The culture medium was changed every 3 days. Osteoclasts were induced with macrophage colony stimulation factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) from bone marrow cells, and the specific process was also referred to previous literature.[14]

Osteoblastic Proliferation and ALP Activity Assay

After attaching overnight, osteoblasts were treated with HLE (0, 4, 20, 100 μg/mL) and incubated for 48 h in 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and for 7 days in ALP activity assay. In MTT assay, 20 μL of 5 mg/mL MTT in phosphate buffered salt solution (pH 7.4) was added into plates and incubated for 4 h. The medium was discarded and the formazan crystals that formed in the cells were dissolved in 150 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm. For ALP activity assay, the cells were lysed and the protein concentration was determined by the kit. The ALP activity was measured according to the conversion of colorless p-nitrophenyl phosphate to colored p-nitrophenol after co-incubation for 30 min at 37 ℃.

Osteoblastic Mineralization Analysis by Alizarin Red Staining

Osteoblasts were plated in 24-well culture plates and allowed to attach overnight. When the density of the cells was nearly 80%, the cells were treated with osteogenic differentiation medium (α-MEM with 10% FBS, 50 μg/mL ascorbic acid, 10 mmol/L β-glycerophosphate). After 12 days culturing, cells were treated with HLE (0, 4, 20, 100 μg/mL) in osteogenic differentiation medium for another 6 days. In the end, cells were fixed in ice-cold 4% paraformaldehyde for 10 min and stained with 0.1% Alizarin Red staining solution (pH 8.3) for 30 min at 37 ℃. The stained cells were washed and further photographed. Alizarin Red was incubated with 5% (v/w) cetylpyridinium chloride at 37 ℃ for 30 min. The optical density was measured at 570 nm.

Osteoclastic Proliferation and TRAP Activity Assay

For MTT assay, after osteoclasts maturation, HLE was added to α-MEM cultures simultaneously with M-CSF (50 ng/mL), RANKL (100 ng/mL) and 10% FBS for 48 h. After that, 20 μL of 5 mg/mL MTT in phosphate buffered salt solution (pH 7.4) was added and incubated for 4 h. The medium was discarded and the formazan crystals that formed in the cells were dissolved in 150 μL of DMSO. The absorbance was measured at 570 nm. TRAP activity was also detected after 7 days culture. The cells added with 100 μL TRAP reaction solution of pH 3.5 and incubated at 37 ℃ for 30 min. Then, 0.4 g/mL NaOH were added to terminate the reaction. The absorbance was measured at 405 nm. The activity of TRAP was expressed as nanomoles p-nitrophenol per minute per 100 osteoclasts.

Western Blot Analysis

The osteoblasts and osteoclasts were treated with HLE in 2 mL α-MEM. Following treatment, the cells were lysed and centrifuged at 12,000 r/min for 10 min at 4 ℃. The protein concentration of the supernatants was determined by the bicinchoninic acid (BCA) kit. Each sample (20 μg protein) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubation with 5% bull serum albumin (BSA), the osteoblasts membrane was incubated with GAPDH, OPN and BMP-2 antibodies, and the osteoclasts membrane was incubated with GAPDH, MMP-9 and CK antibodies at 4 ℃ overnight. Each membrane was further incubated with anti-rabbit IgG antibody. The immunoreaction signals were detected with the enhanced electrochemiluminescence (ECL) reagent and exposed on Gel imaging system.

Statistical Analysis

All data were expressed as mean ± standard

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deviation ($\bar{x} \pm s$) and group differences were determined by one way analysis of variance (ANOVA) with the LSD test. The analyses were performed using a statistical software program (SPSS version 20.0, SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

RESULTS

HLE Increased E_2 Levels, Decreased Body Weight, but Did Not Affect Uterus Weight in OVX Mice

Ovariectomy causes a decrease of E_2 production, and then induces body weight gain. The levels of E_2 in serum of OVX mice were significantly decreased (P<0.01), while HLE could significantly increase the levels of E_2 in the serum of OVX mice (Figure 1A, P<0.05 or P<0.01). As shown in Figure 1B, there was no significant difference in the initial body weight among 5 groups at the beginning of the study. At the end of the study, the mean body weights of mice in the OVX group was significantly higher than that of the sham group (P<0.05). Treatment with HLE completely reversed the increase in body weights of OVX mice (P<0.05). As for the uterus weight, EV increased the uterus weight of OVX mice (P<0.05), but HLE did not produce any effect on uterus weight of OVX mice (P>0.05, Figure 1C).

HLE Reduced Levels of DPD, CTX and Excretion of Calcium in OVX Mice

As shown in Figures 2A and 2B, the levels of DPD and CTX-Ⅰ in serum of OVX mice were significantly increased compared with that of the sham group (both P<0.01). Treatment with HLE significantly decreased the DPD and CTX-Ⅰ levels in OVX mice (both P<0.01). As shown in Figure 2C, the urine calcium in OVX mice was significantly higher than that of the sham group (P<0.01), while HLE could reduce urine calcium levels of OVX mice (P<0.05).

HLE Reversed the Bone High Turnover in OVX Mice

As shown in Figures 3A and 3B, two indicators of bone formation, ALP and BGP were significantly increased in OVX mice (P<0.05 or P<0.01), while treatment with 3 g/(kg·d) HLE could suppress the activities of BGP and ALP in OVX mice (both P<0.01). The level of TRAP, an indicator of bone resorption, was significantly increased in serum of OVX mice compared with that of the sham group (P<0.05), while HLE significantly declined the TRAP activity in serum of OVX mice (Figure 3C, P<0.01).

HLE Improved Bone Microarchitecture in Distal Femoral Metaphysic of OVX Mice

Three-dimensional trabecular bone microarchitecture of mice was shown in Figure 4A. The OVX mice underwent a significant reduction in the trabecular area and trabecular number compared with that of the sham group. EV and HLE could prevent the bone loss caused by the ovariectomy, and improved the microarchitecture of trabecular bone after 12 weeks' treatment. As shown in Figures 4B and 4C, the OVX mice presented a significant decrease in femoral BMD and BMC after OVX (both P<0.01), while treatment with EV and HLE could increase the BMD and BMC in femurs of ovariectomized mice (both

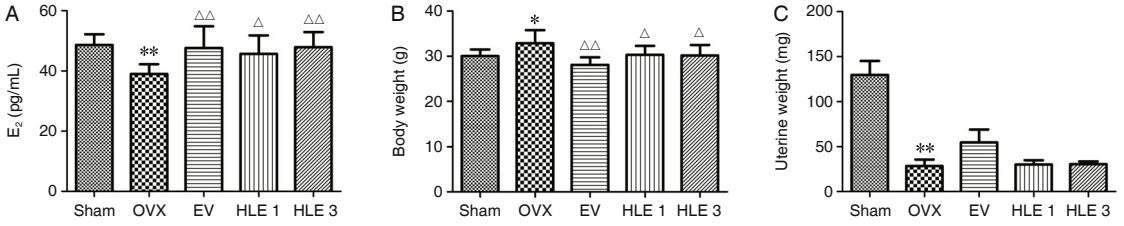

Figure 1. Effects of HLE on Serum E_2, Body Weight and Uterus Weight in Ovarietomized Mice (n=7, $\bar{x} \pm s$)

Notes: *P<0.05, **P<0.01, compared with the sham group; △P<0.05, △△P<0.01, compared with the OVX group; the same as in Figures 2–4

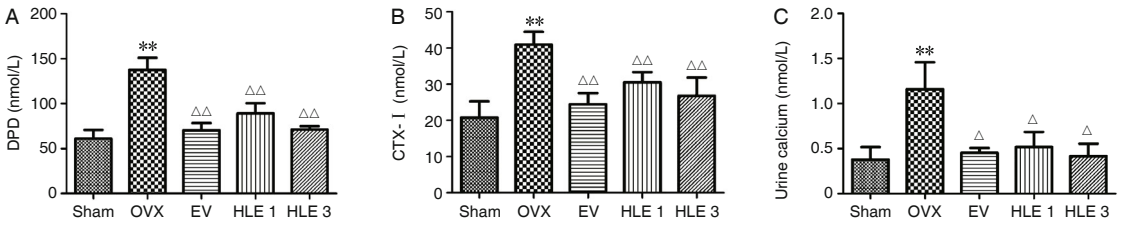

Figure 2. Effects of HLE on Serum DPD, CTX-Ⅰ and Urine Calcium in Ovarietomized Mice (n=7, $\bar{x} \pm s$)

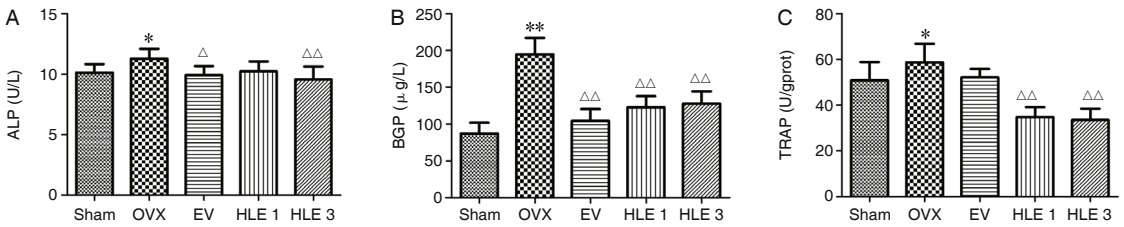

Figure 3. Effects of HLE on Biochemical Makers ALP, BGP and TRAP in Serum of Ovarietomized Mice (n=7, $\bar{x} \pm s$)

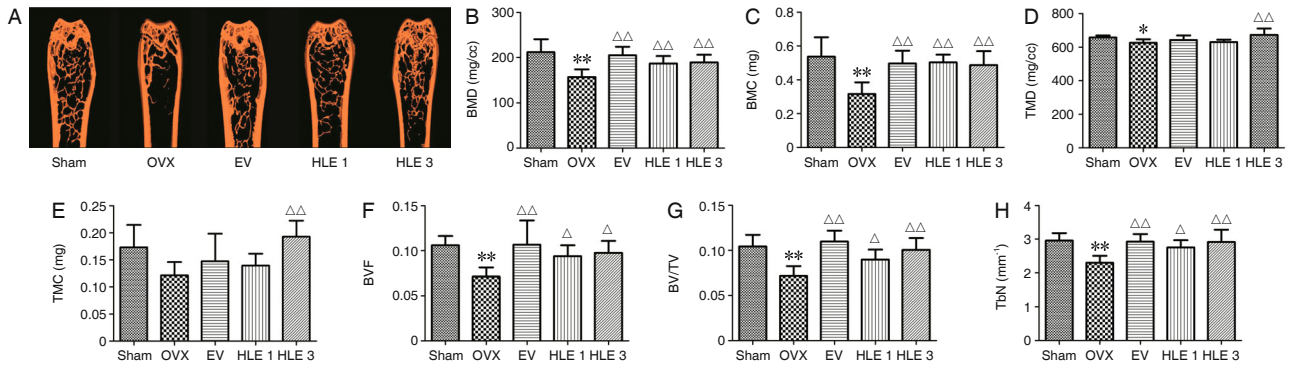

Figure 4. Effects of HLE on Bone Microarchitecture (A) and Femoral Trabecular Bone Morphologic Parameters (B–G) in Distal Femoral Metaphysic of Ovarietomized Mice (n=7, $\bar{x} \pm s$)

P<0.01). As shown in Figures 4D–4E, TMD and TMC also increased significantly after 12 weeks' treatment with 3 g/(kg•d) HLE (both P<0.01). Furthermore, the morphologic parameters, BVF, BV/TV and TbN were also significantly decreased in OVX mice compared with that of the sham group (all P<0.01). High dose [3 g/(kg•d)] of HLE showed excellent effects in reversing the alteration of bone morphologic parameters (P<0.05 or P<0.01, Figures 4F–4H).

HLE Enhanced Osteoblastic Bone Formation

As shown in Figure 5A, HLE significantly enhanced the cell proliferation at the concentrations of 4 μg/mL and 20 μg/mL after 48 h treatment (P<0.01). The activity of ALP in the presence of the HLE is shown in Figure 5B, and HLE significantly increased the ALP activity of primary osteoblasts at the concentration of 100 μg/mL (P<0.01). The extracellular matrix mineralization and the calcium deposition were determined by Alizarin red S staining. As shown in Figures 5C and 5D, after treatment with HLE for 18 days, the calcification nodules were significantly increased (P<0.01).

HLE Exerted No Toxic Effect and Inhibited TRAP Activity of Osteoclast

As shown in Figure 5E, there was no difference among 4 groups in MTT assay of osteoclasts (P>0.05). HLE at the concentration of 20 μg/mL and 100 μg/mL significantly inhibited osteoclastic TRAP activity (P<0.01, Figure 5F).

HLE Improved the Expression of OPN and BMP-2 Levels and Down-Regulated MMP-9 and CK Levels

As shown in Figures 6A–6C, HLE at the concentration of 4 and 20 μg/mL significantly improved the expression of BMP-2, and 20 μg/mL HLE also increased the OPN level (P<0.05 or P<0.01). In osteoclasts, treatment with HLE at the concentration of 20 μg/mL and 100 μg/mL both significantly suppressed the expressions of CK and MMP-9 (P<0.05 or P<0.01, Figures 6D–6F).

DISCUSSION

The present study found that HLE attenuated the bone loss, reversed bone high turnover in OVX mice. *In vitro* experiment, HLE increased the proliferation, differentiation and mineralization of bone matrix in osteoblasts, and also inhibited the bone resorption activity of osteoclasts. These suggested that hops exerted a concomitant effect on bone formation and bone resorption and possessed anti-osteoporosis effects.

As we all know that estrogens influence the shaping of the skeleton, and contribute to the maintenance of bone homeostasis and strength.

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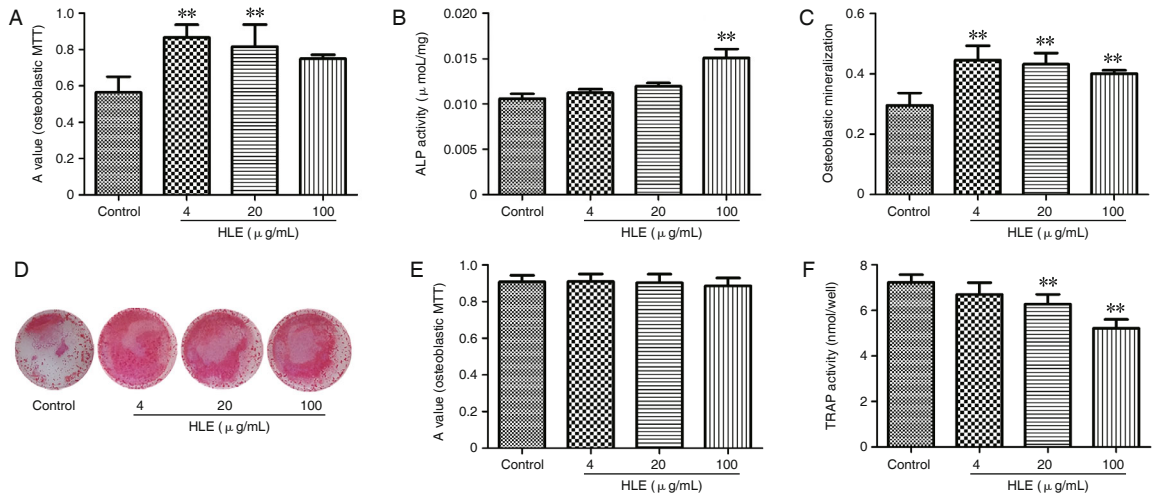

Figure 5. Effects of HLE on MTT, ALP Activity, Extracellular Matrix Mineralization, Bone Mineralization Nodule Stained with Alizarin Red in Osteoblast, and MTT, TRAP in Osteoclast (n=6, $\bar{x} \pm s$)

Notes: $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group; the same below

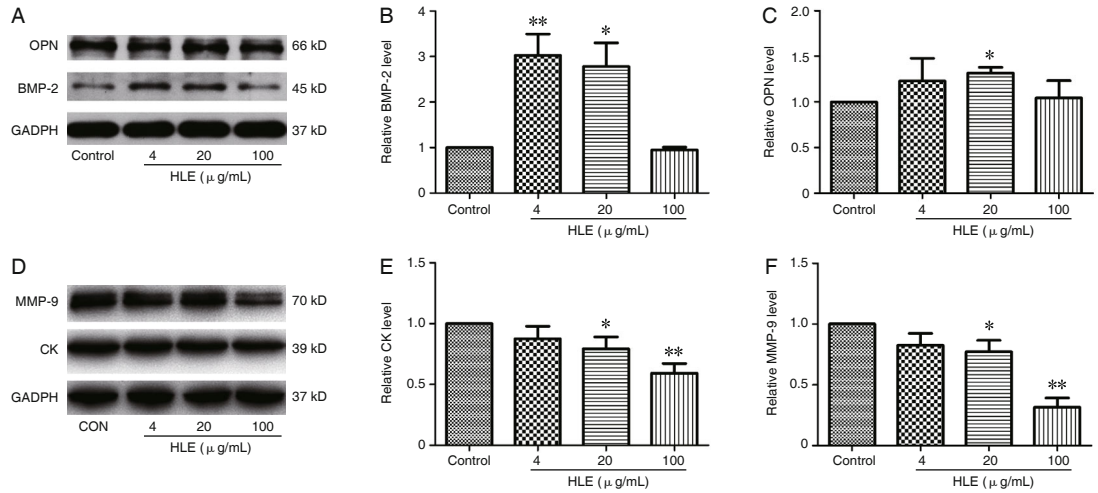

Figure 6. Effects of HLE on the Expression of BMP-2 and OPN in Osteoblast (A–C), and the Expression Level of MMP-9 and CK in Osteoclast (D–F) by Western Blot (n=3, $\bar{x} \pm s$)

The sharp decline of estrogen levels at menopause accelerates the age-dependent involution of the female skeleton and contributes to the loss of bone mass, architectural integrity, and strength.[15] The plant-derived substances, such as genistein and daidzein, possess estrogen-like activities, and can be used to prevent and treat relevant diseases, such as osteoporosis and menopausal syndrome.[16] The present study found that hop extracts could increase the estrogen levels in serum of ovariectomized mice, showing a potential estrogen-like activity. In addition, ovariectomy causes a decline of levels of estrogen, and then indeces an increase of body weight and a decrease of uterine weight in mice.[12] Estrogen treatment can counteract the effects of ovariectomy, reduce body weight and enhance uterine weight in animal and human. This may be beneficial to the control of body weight, but increase the risk of uterus suffering from endometrial hyperplasia.[17] Our study exhibited that HLE inhibited the increase of body weight, but did not produce any effect on uterine weight in OVX mice, showing that hops did not have adverse effects on uterus. Previous study has noted that hops extracts did not have an effect on the rat uterus weight, while 8-PN from hops at equivalent doses to this plant extracts used in humans did have a stimulating effect.[18] The deep reason needs to be further investigated.

OVX induces postmenopausal osteoporosis, which is characterized by bone loss and bone high turnover. The bone turnover markers, which indicate either osteoclastic bone resorption (TRAP and CK) or osteoblast function (ALP and BGP), were elevated

in serum of OVX animals.[19] Among them, ALP and BGP are essential in osteoblastic bone formation, bone matrix mineralization, controlling the formation of abnormal hydroxylapatite, crystallization and cartilage mineralization, and are extensively used as indicators of bone formation.[20] TRAP is correlated with osteoclastic boneresorptive activity, and involved in the degradation of bone mineral substances.[21] In this study, BGP and ALP were significantly increased in OVX rats, leading to a high bone turnover with an excess of bone resorption over bone formation. HLE could not only inhibit the activities of BGP and ALP, but also notably decrease the activity of TRAP in serum of OVX mice, suggesting that HLE ameliorated the OVX-induced bone high turnover, and inhibited bone loss.

Rapid postmenopausal bone loss, which occurs in female rats following ovariectomy, is characterized by a decrease in trabecular bone density and a deterioration of the bone architecture, with a particular diminution in the total number of bone trabecula and an increase in the number of their perforations. In this study, HLE could increase the BMD, improve bone micro-architecture, and reduce the excretion of calcium from urine, indicating that HLE could effectively prevent bone loss in ovariectomized mice. Actually, it has been reported in Europe that treatment with a combination of hops rho iso-alpha acids, berberine sulfate trihydrate, vitamin D3 and vitamin K1 could produce a more favorable bone biomarker profile that supports a healthy bone metabolism.[22] It was further confirmed that hops could be used to prevent and treat osteoporosis.

Bone remodeling is a dynamic process which is maintained by a balance between osteoblastic bone formation and osteoclastic bone resorption. Bone formation is likely to be affected by reductions in osteoblast proliferation, differentiation and mineralization.[23] During the differentiation of osteoblasts, it can produce ALP to regulate the mineralization of bone matrix. Mineralized nodule formation is a symbol of differentiation and formation of bone matrix.[24] In this study, HLE could not only enhance the osteoblastic proliferation and ALP activity, but also increase the formation of mineralized nodules, suggesting that HLE promoted osteoblastic bone formation. OPN and BMP-2 are key indicators of bone formation in osteoblasts, both of which are involved in bone mineral deposition and regulating osteoblastic activity.[25] Our study discovered HLE could increase the expressions of BMP-2 and OPN, suggesting its auxo-action on osteoblastic bone formation. In osteoclast, TRAP is a crucial enzyme characterized the capacity of bone resorption.[21] Cathepsin K is a cysteine protease that is highly expressed by activated osteoclasts, where it degrades type Ⅰ collagen.[26] MMP-9 is another key enzyme reflecting bone resorption.[27] In this study, HLE could not only inhibit osteoclastic TRAP activity, but also suppress the CK and MMP-9 expressions through Western blot analysis, indicating hops also ameliorated bone loss by inhibiting osteoclastic activity.

Taken together, this report demonstrates that the HLE administration has potential to prevent the reduction of bone mass, improve the cancellous bone structure and biochemical properties in an OVX mice model of osteoporosis. Furthermore, our *in vitro* experiments proves that HLE regulates the activities of both osteoblasts and osteoclasts. Therefore, *Humulus lupulus* L. may serve as a new candidate for the development of anti-osteoporosis drugs. However, the target molecule of hops is not clear and the mechanism of action is also unknown. Further detailed studies with hops and its chemical constituents will be needed.

Conflict of Interest

The authors report no conflicts of interest in this work.

Author Contributions

Xin HL and Qin LP designed the study protocol; Xia TS, Lin LY, Li CH and Liu XY performed the experiments; Lin LY and Jiang YP performed the data analysis; Xia TS and Lin LY wrote the paper; Zhang QY and Xia TS edited and revised the manuscript. All authors read and approved the final manuscript.

Electronic Supplementary Material: Supplementary materials (Appendixes) are available in the online version of this article at https://doi.org/10.1007/s11655-019-2700-z

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