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Original Article

Improvement of osteoporosis by *Lycium chinense* administration in ovariectomized mice

Mi Hye Kim, Ji Eun Lee, Jae Sung Lee, Woong Mo Yang*

Department of Convergence Korean Medical Science, Graduate school, Kyung Hee University, Seoul, Republic of Korea

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Abstract

Background: The fruit of *Lycium chinense* (LC), one of the most popular natural materials, has been used to counter the effects of musculoskeletal diseases in oriental medicine. In particular, this fruit is known to have antiaging effects, which is of significant interest to the research community. The purpose of our investigation was to observe and confirm the ameliorative effect of LC on osteoporosis, which was verified in this *in vivo* study.

Methods: Female ICR mice aged 5 weeks were surgically ovariectomized (OVX) to induce osteoporosis. After 7 weeks of induction, oral administration with 1 mg/kg and 100 mg/kg LC was continued for 6 weeks. At the end of the experiment, the levels of bone mineral content (BMC) and bone mineral density (BMD) were estimated by dual-energy X-ray absorptiometry. Hematoxylin and eosin staining was performed; additionally, serum osteocalcin and calcium concentrations were analyzed.

Results: LC treatment significantly reduced the deterioration of BMC and BMD compared with OVX mice. Histological analysis showed that LC ameliorated osteoporosis including the hypertrophy of the epiphyseal plate. Additionally, decreased serum osteocalcin as well as the calcium levels recovered following the administration of LC.

Conclusion: These results demonstrated that LC ameliorates osteoporosis in OVX mice by improving several important parameters including BMC, BMD, and bone-turnover markers such as osteocalcin and calcium levels in serum.

Keywords: bone density; functional food; osteoporosis

1. Introduction

Osteoporosis is a common public health problem affecting populations around the world. The typical characteristics of osteoporosis, such as low bone mineral density (BMD) and defects in the microarchitecture of bone tissue, result in an increase of bone fracture risk.[1] The prevalence rate of osteoporosis increases as patients get older.[2] In particular, there are many earlier studies demonstrating that the vital factor associated with an increased occurrence of osteoporosis in women aged 50 years is the extent of estrogen deficiency after menopause.[3]

Since estrogen regulates the balance between bone resorption and bone formation, hormone replacement therapy is believed to effectively treat postmenopausal osteoporosis. However, prolonged exposure to estrogen is associated with breast cancer, dysfunction of the vascular system, and serum lipid abnormality.[4] While administration of the parathyroid hormone to osteoporotic patients induces bone formation by binding osteoblast lineage, its continuous treatment finally leads to bone loss due to downregulation of bone forming genes.[5] In addition, the widely used antiresorptive drugs bisphosphonate and denosumab are reported to have serious

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Prof. Woong Mo Yang, Department of Convergence Korean Medical Science, College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemoon-gu, Seoul 02447, Republic of Korea.

E-mail address: wmyang@khu.ac.kr (W.M. Yang).

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side effects.[4] For those reasons, it is important to develop a treatment for osteoporosis with appropriate efficacy without undesirable adverse effects.

Lycii fructus, a dried mature fruit of *Lycium chinense* Mill (Solanaceae), has been traditionally used to treat musculoskeletal disorders based on classic Korean literature.[6] In a previous report, *Lycii radicis* cortex inhibited osteoclast differentiation in nuclear factor kappa-B ligand-induced RAW 264.7 cells.[7] In addition, a herbal formula containing *L. fructus* exerted estrogenic activity in ovariectomized (OVX) rats.[8] However, the ameliorative effect of *L. fructus* on osteoporosis has not yet been elucidated. In the present study, we aimed to analyze the bone mineral content (BMC) and BMD following *L. fructus* treatment in OVX-induced osteoporotic mice. In addition, histologic structure changes of bone were monitored along with serum osteocalcin and calcium concentrations.

2. Methods

2.1. Sample preparation

The dried fruit of *L. chinense* Mill. (Family, Solanaceae) was obtained from Jung-do Herb (Seoul, Korea). The fruit was extracted in 30% ethanol for 24 hours at room temperature. The extract was filtered, evaporated in a rotary vacuum evaporator, and lyophilized (called LC) by means of a freezing dryer. The obtained powder (yield, 26.8%) was kept at −20°C until use. A voucher specimen (LC-E30) was deposited at our laboratory.

The betaine concentration in LC was determined by high-performance liquid chromatography-evaporative light scattering detector (Agilent 1100 series (Agilent Technologies, Waldbronn, Germany)). First, 50-mg LC was dissolved in 2-mL 100% methanol and filtered through a 0.45-μm filter membrane. Then, chromatographic separation was achieved using the Atlantis HILIC silica (Waters Corporation, Milford, MA, USA) (4.6 mm × 150 mm, 5 μm, 100 Å) with 1.0 mL/min flow rate at 25°C. As shown in Fig. 1, the concentration of betaine in LC was 360.32 μg/mL (1.441%).

2.2. Ovariectomy-induced osteoporotic animals and LC treatment

ICR mice (6-week-old females, 20 ± 2 g) were purchased from RAONBIO Inc. (Yongin, Korea). All mice were maintained in an air-conditioned room (20 ± 2°C temperature and 50 ± 5% humidity) using a 12-hour light/dark cycle. Standard chow diet and tap water were freely available to all mice. All experiments were approved by the Committee on Care and Use of Laboratory Animals of Kyung Hee University [KHUASP(SE)-15-079; Seoul, Korea].

After 1 week of acclimatization, the mice were randomly placed into five groups ($n = 6$): (1) SHAM, sham-operated and nontreated as normal control; (2) OVX, OVX and vehicle-treated as negative control; (3) 17β-estradiol (E2), OVX and E2-treated as positive control; (4) LC1, OVX, and 1 mg/kg LC-treated; and (5) LC100, OVX, and 100 mg/kg LC-treated. Thereafter, osteoporosis induction was allowed to continue in the mice for a period of 9 weeks. The SHAM, OVX, and LC-treated groups were orally administered, while the E2 group was injected intraperitoneally10-μg/kg E2. All procedures for treatment continued five times/wk for 3 weeks. At the end of treatment, both sides of the femur without connective tissues and blood sample were collected by cardiac puncture.

2.3. Determination of BMC and BMD

The levels of BMC and BMD were immediately determined after collecting the left femurs using dual-energy X-ray absorptiometry with a PIXImus instrument (Lunar, Madison, WI, USA).

2.4. Histological analysis of bone

Fixed right femurs in 10% neutralized formalin for 18 hours were demineralized with 0.1M ethylene diamine tetraacetic acid aqueous solution for 1 month for histological analysis. The bone tissues were dehydrated with ethanol and xylene, and paraffin-embedded samples were cut into 7 μm

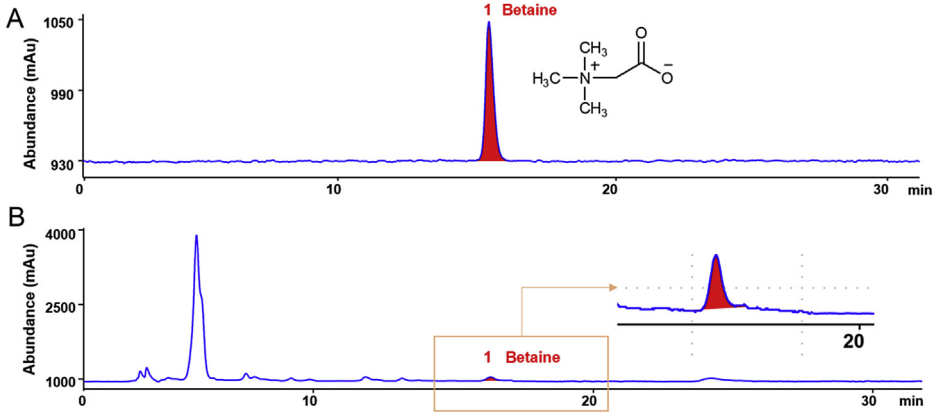

Fig. 1. Standardization of (A) *Lycium chinense* extract (LC) and (B) standard compound using high-performance liquid chromatography (HPLC) systems. Betaine, which is main component of *L. chinense,* were detected in *L. chinense* extract.

thickness by sagittal sections. The tissue samples were stained with hematoxylin and eosin. The histological changes of the capital femoral epiphysis were monitored using digital microscope (Leica Microsystems, Buffalo Grove, IL, USA).

2.5. Measurement of serum osteocalcin and calcium concentrations

The collected blood samples were centrifuged at 12,000*g* for 30 minutes to obtain serum. Serum osteocalcin and calcium concentrations were estimated with the Mouse Gla-osteocalcin High Sensitive EIA kit (Takara, Otsu, Japan) and calcium enzyme-linked immunosorbent assay kit (Adipogen, Shizuoka, Japan), respectively, according to the manufacturer's instructions. All samples were analyzed in triplicate.

2.6. Statistical analysis

Significance was determined by one-way analysis of variance and Dunnett's multiple comparison tests. In all analyses, $p < 0.05$ was designated to indicate statistical significance.

3. Results

3.1. LC increased low femoral BMC and BMD levels

The femoral BMC level in the OVX group (0.034 ± 0.004 g) was significantly lower than in the SHAM group (0.044 ± 0.002 g). BMC was significantly increased in the E2-injected group (0.038 ± 0.002 g), and about 12.6% compared with the OVX group. Treatment with 1 mg/kg and 100 mg/kg LC restored low BMC levels to about 12.6% and 18.5%, respectively, in comparison with the BMC level of OVX mice (LC1 = 0.038 ± 0.001 g and LC100 = 0.04 ± 18.5 g).

Similar to BMC, BMD was significantly decreased by ovariectomy surgery. The levels of femoral BMD were 0.082 ± 0.006 g/cm^2 in the SHAM group. Compared with the OVX group (0.073 ± 0.003 g/cm^2), E2 treatment significantly upregulated the decreased BMD (0.080 ± 0.003 g/cm^2). Also, 1 mg/kg and 100 mg/kg LC administration increased BMD levels (LC1 = 0.076 ± 0.004 g/cm^2; LC100 = 0.077 ± 0.003 g/cm^2). Taken together, LC treatment increased both the femoral BMC and BMD levels (Fig. 2).

3.2. LC recovered the bone structure

The histological changes of capital femoral epiphysis were showed by hematoxylin and eosin staining. There was an appreciable increase of epiphyseal plate thickness in OVX-induced osteoporotic mice compared with the SHAM group. As a positive control, E2 injection reduced the thickness of the growth plate compared with the OVX group. Similarly, the thicknesses of the growth plate were markedly thinner than OVX mice in 1 mg/kg and 100 mg/kg LC-treated groups (Fig. 3). However, administration of LC ameliorated the growth plate hypertrophy.

3.3. LC increased osteocalcin and calcium concentrations in serum

The reduction rate of serum osteocalcin concentration by OVX was 26.1% compared with the SHAM group (SHAM = 54.01 ± 3.00 pg/mL; OVX = 39.91 ± 2.69 pg/mL). E2 treatment showed an approximate 26.5% increase of osteocalcin level (50.47 ± 0.94 pg/mL). In the 100 mg/kg LC-treated group, the serum osteocalcin concentration was significantly upregulated about 13.5%, while that in the 1 mg/kg LC-treated group was unchanged compared with OVX mice (LC1 = 40.46 ± 1.96 pg/mL; LC100 = 45.30 ± 1.70 pg/mL) (Fig. 4).

In addition, serum calcium level in the OVX group (8.997 ± 0.103 mg/dL) was significantly decreased compared to SHAM group (9.838 ± 0.861 mg/dL). By E2 injection, serum calcium level showed a 4.6% recovery (9.532 ± 0.391 mg/dL). Lowed calcium level in OVX mice was significantly restored by the administration of 100 mg/kg LC (LC1 = 9.410 ± 0.033 mg/dL; LC100 = 9.628 ± 0.483 mg/dL).

4. Discussion

Finding effective treatment for osteoporosis is considered important because the fracture risk and difficulty of movement increases as bone strength weakens.[1] Bone microstructure disruption and mechanical strength deterioration, result from a reduction in bone mass and BMD, and are hallmarks of osteoporosis.[9] In particular, it is well-established that the assessment of BMC and BMD by dual-energy X-ray absorptiometry or microcomputed tomography are diagnostic criteria for osteoporosis.[10] In this study, ovariectomy decreased the femoral BMC as well as BMD. The BMD of the distal femur in the LC-treated groups at 1 mg/kg and 100 mg/kg was much higher than that of the OVX group. The oral administration of LC also restored the femoral BMC levels. In conjunction with BMC and BMD, the histological change of bone structure was also observed. The hypertrophy of epiphyseal plate was markedly attenuated by LC treatment.

We further evaluated the serum osteocalcin concentrations in each group to investigate the effects of LC on bone-turnover marker. Controlled remodeling between bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) mainly occurs to maintain bone homeostasis.[11] When osteoblasts form bone matrix consisting of collagen type I, osteopontin, and alkaline phosphatase, the process of bone growth or maturation of mineral phase is continued by secreting bone-turnover factors including osteocalcin and calcium. Therefore, osteocalcin and calcium are classic markers of osteoblastic maturation.[12] A previous report indicates that serum osteocalcin is significantly decreased in OVX-induced osteoporotic mice.[13] The treatment of LC increased the serum osteocalcin concentration. Additionally, the reduction of serum calcium in OVX mice was ameliorated by LC administration. Taken together, LC treatment increased not only serum osteocalcin, but also calcium concentration.

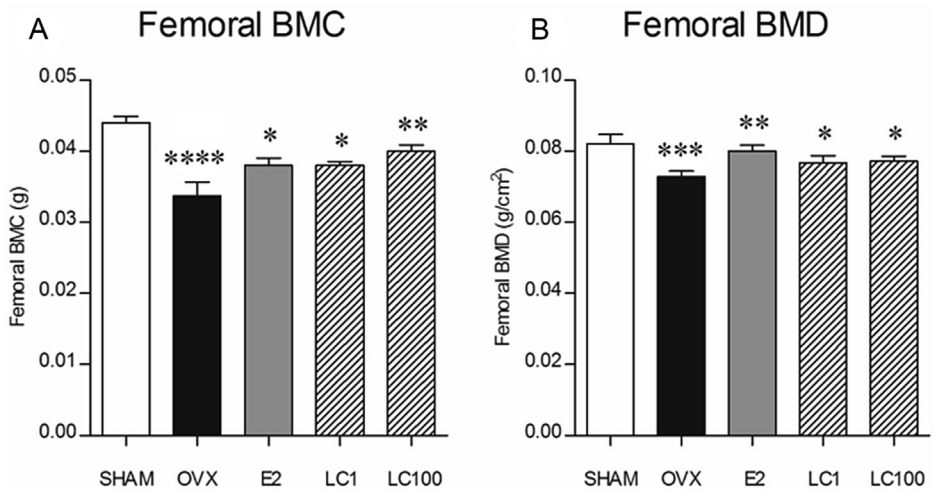

Fig. 2. *Lycium chinense* ameliorated the (A) femoral bone mineral content (BMC) and (B) bone mineral density (BMD). * $p < 0.05$ compared with OVX group. ** $p < 0.01$ compared with OVX group. *** $p < 0.01$ compared with SHAM group. **** $p < 0.001$ compared with SHAM group. E2 = 17β-estradiol; LC = *Lycium chinense* extract; OVX = ovariectomized.

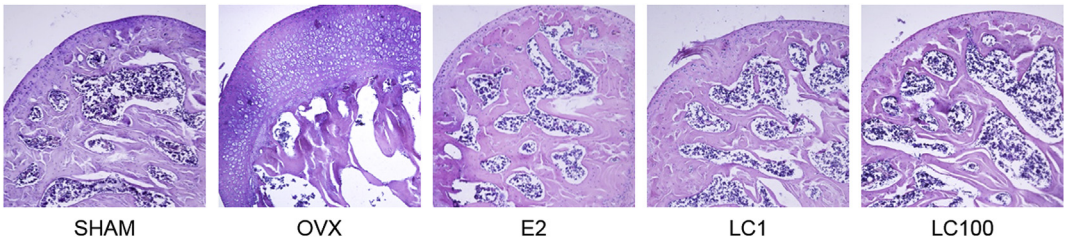

Fig. 3. *Lycium chinense* recovered the bone integrity. The growth plate thickness of the capital femoral epiphysis was shown by hematoxylin and eosin staining. Magnification, ×100. E2 = 17β-estradiol; LC = *Lycium chinense* extract; OVX = ovariectomized.

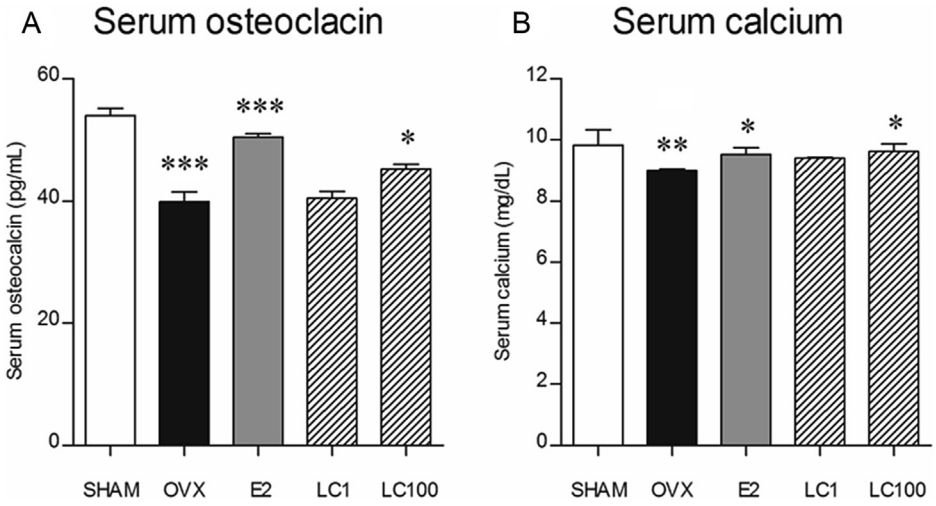

Fig. 4. *Lycium chinense* increased the (A) serum osteocalcin and (B) calcium concentrations. * $p < 0.05$ compared with OVX group. ** $p < 0.05$ compared with SHAM group. *** $p < 0.001$ compared with SHAM group.

In conclusion, the treatment with LC improves osteoporosis in OVX mice. The destruction of bone structure, and decreases of BMC and BMD were recovered by LC treatment along with bone-turnover markers. One possible mechanism might be associated with the maturation of osteoblasts. However, further in-depth study with detailed mechanisms should be undertaken.

Acknowledgments

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