Original Research Article

Caulis Lonicerae Japonicae extract shows protective effect on osteoporosis in rats

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Abstract

Purpose: *To investigate the effect of Caulis lonicerae Japonicae extract (CLJE) on ovariectomy-induced osteoporosis in rats.*
Methods: *Female Sprague-Dawley rats were randomly assigned to a control group (normal rats) and five ovariectomy (OVX) subgroups: OVX with vehicle (OVX), OVX with 17ß-estradiol (E2, 25µg/kg/day), and OVX with CLJE doses (150, 300, and 600 mg/kg/day). Daily oral administration of E2 or CLJE started 4 weeks after OVX and lasted for 16 weeks. The bone mineral density (BMD) of L4 vertebrae and right femurs was determined. The length of each femur was measured with a micrometer, and the center of the diaphysis was determined. Three representative L4 vertebrae were selected to evaluate trabecular microarchitecture. Serum alkaline phosphatase (ALP), urinary calcium (U-Ca), urinary phosphorus (U-P), urinary creatinine (Cr) and osteocalcin (OC) levels were measured using a diagnostic reagent kit.*
Result: *The results show that a high-dose of CLJE (600 mg/kg) significantly inhibited bone mineral density (BMD) reduction of L4 vertebrae (0.24 ± 0.02, $p < 0.05$) and femur (0.24 ± 0.03, $p < 0.05$) caused by OVX, and prevented the deterioration of trabecular microarchitecture ($p < 0.05$). High-dose CLJE also improved morphometric parameters, viz, trabecular number (Tb-N) (4.6 ± 0.3, $p < 0.05$), trabecular thickness (Tb-Th, 0.084 ± 0.012, $p < 0.05$) and trabecular separation (Tb-Sp, 0.14 ± 0.02, $p < 0.05$) in L4 vertebrae significantly.*
Conclusion: *The results indicate that CLJE prevents OVX-induced osteoporosis in rats. Thus, CLJE is a potential natural alternative for the treatment of postmenopausal osteoporosis in future.*

Keywords: *Caulis Lonicerae Japonicae, Post-menopausal osteoporosis, Ovariectomy, Bone mineral density, Trabecular microarchitecture, Diaphysis*

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by reduced bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fractures [1]. According to data released by the World Health Organization (WHO), osteoporosis affects approximately a million people throughout Europe, the USA, and Japan [2]. The incidence of osteoporosis increases dramatically with life expectancy. Accordingly, the risk of osteoporotic fractures and their associated costs is rising rapidly due to aging population [3]. In the elderly, hip fractures are closely associated with mortality [4].

Hormone deficiency is known to impair cancellous metaphyseal bone and reduce BMD in humans and animals. Therefore, estrogen

deficiency in post-menopausal women has been regarded as a critical cause of this population's susceptibility to osteoporosis [5]. Osteoporosis is twice as common in women as in men [6], and approximately one in three women over 50 years old experiences an osteoporotic fracture in her lifetime [7].

Clinically, hormone replacement therapy (HRT) has been a popular therapeutic strategy designed for postmenopausal osteoporosis [8,9]. However, the long-term application of HRT has potential malignant effects on reproductive tissues [10-13]. Other medicines that stimulate bone formation (e.g. growth hormone, sodium fluoride, and parathyroid hormone) or inhibit bone resorption (e.g. bisphosphonates and calcitonin) may prevent bone loss progression in established osteoporosis. However, these medications are not effective for a large proportion of the world population, especially in developing countries, and these medicine have side effects, such as gastrointestinal reactions, cancers, osteonecrosis of the jaw, and reduced skeletal strength [14,15]. Consequently, to substitute or reduce the medicines used currently, there are efforts to study new drugs with improved therapeutic efficacy, fewer undesirable side effects, and lower price.

Caulis Ionicerae has been widely used as a kidney-tonifying and anti-osteoporosis herb for the treatment of nephrasthenia syndrome [16], osteoporosis [17] and bone fracture [18] for thousands of years in China. The aim of the present study was to systematically evaluate the effect of CLJE on osteoporosis induced by OVX in rats.

EXPERIMENTAL

Preparation of *Caulis Ionicerae* extract

The herbal samples of *Caulis Ionicerae Japonicae* were collected from Zhangjiajie City, Hunan Province in China in May 2016. Taxonomic identification of the plant was performed by Professor WeiWang of Shandong University of Traditional Chinese Medicine, in China. A voucher specimen (no. CLJE 201605023) was assigned and the specimen deposited in the herbarium of Shandong University of Traditional Chinese Medicine, China for future reference.

One batch (100 g) of the herbal material *Caulis Ionicerae* was dried in an oven. Aqueous extract of CLJE was obtained by steeping the dried *Caulis Ionicerae* in water at 60 ℃ three times for 1 h each. The liquid *extract* was dried in an oven

and freeze-dried to obtain CLJE [19]. The yield was 58.82 %.

Animals and treatments

Healthy three-month-old female Sprague-Dawley rats (weight, 220 ± 10 g) provided by the Experimental Animal Center of Shandong Province (Certificate no. SYXK 2004 - 0003). The animals had free access to feed and water, and were allowed to acclimatize for at least one week before use. The rat experiment was approved by the Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine (approval ref no. 20130709) and was carried out in compliance with the Directive 2010/63/EU on the handling of animals used for scientific purposes [20,21].

Sixty rats were randomly divided into six groups of ten individuals: a sham-operated group (control) and five ovariectomy (OVX) subgroups, that is, OVX with vehicle (OVX), OVX with 17ß-estradiol (E_2, 25 mg/kg/day), and OVX with CLJE doses (150, 300 and 600 mg/kg/day). Daily oral administration of E_2 or CLJE started 4 weeks after OVX and lasted for 16 weeks.

Bone mineral density measurement

The BMD of the L4 vertebrae and right femurs was estimated using dual-energy X-ray absorptiometry scanning (DEXA, GE Healthcare, USA) with small animal measurement. The measurements were expressed as grams of mineral contents per cm^2 of surface area. Scans were performed by the same blinded technician.

Three-point bending test

Before mechanical testing, the rats were sacrificed by cervical spondylosis. Then the left femurs were slowly thawed at room temperature. The length of each femur (distance from the intermalleolar to the intercondylar region) was measured with a micrometer, and the center of the diaphysis was determined.

Micro-CT analysis

Based on the BMD, three representatives L4 vertebrae from each group were selected to evaluate the trabecular microarchitecture using eXplore Locus SP preclinical specimen microcomputed tomography (MicroCT, GE Healthcare, USA). Before the scans, the bones were positioned with gauze in the sample holder and allowed to reach room temperature. The L4 vertebrae were scanned from the anterior endplate in the posterior endplate direction (22

um/slice). The isotropic voxel resolution of bone was 22 um^3. The volume of interest (VOI) was selected as a region 25 slices away from the anterior endplate to the posterior endplate, ranging to 125 slices. The three-dimensional images were reconstructed with the purpose of visualization and display. After analyzing the VOI, morphometric bone parameters, including trabecular number (Tb-N), trabecular separation (Tb-Sp), trabecular thickness (Tb-Th) were obtained. The VOI analysis was performed blindly by the same operator.

Biochemical analysis of serum and urine specimens

The serum of rats were collected by picking eyeball, and the urine of rats were collected by stimulating back. The levels of serum alkaline phosphatase (ALP), urinary calcium (U-Ca), urinary phosphorus (U-P), and urinary creatinine (Cr) were measured on an automatic analyzer (Ciba-Corning 550, USA) using a diagnostic reagent kit. Serum osteocalcin (OC) concentration was determined using a rat OC ELISA kit (San Clemente, CA, USA).

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA combined with Bonferroni's multiple comparison test using SPSS 16.0. Differences were considered statistically significant at $p < 0.05$.

RESULTS

BMD of L4 vertebrae and femurs

The BMD of the L4 vertebrae and femurs presented in Table 1. These results demonstrate that OVX significantly decreased the BMD in the L4 vertebrae and femurs compared to the sham group ($p < 0.05$). Compared to the OVX group, CLJE treatment significantly prevented the BMD decrease in OVX-induced L4 vertebrae and femurs (all $p < 0.05$) in a dose-dependent manner. E_2 also significantly increased the BMD of the L4 vertebrae and femurs (both $p < 0.05$), which was similar to that observed in the H-CLJE group ($p > 0.05$).

Mechanical characteristics of femur

The results of the mechanical testing of femur are presented in Table 2. Compared with the sham group, 16 weeks of estrogen deficiency significantly decreased the maximum load and maximum stress (both $p < 0.05$). Meanwhile, higher dosage of CLJE treatments (300 or 600 mg/kg/day) markedly prevented the OVX-induced tendency to decrease these parameters ($p < 0.05$). E_2 also increased these biomechanical parameters, which were significantly higher than those of the OVX group ($p < 0.05$). It is worth noting that the increase in maximum load observed for the H-CLJE group was similar to that of E_2 ($p > 0.05$).

Table 1: Effect of CLJE on BMD of L4 vertebrae and femur (n = 10)

Group	Dosage(mg/kg)	BMD of vertebra(g/cm^2)	BMD of femur(g/cm^2)
Control	-	0.28±0.03[*]	0.27±0.03[*]
OVX	-	0.13±0.02	0.13±0.03
E_2	0.025	0.24±0.03[*]	0.17±0.04[*]
L-CLJE	150	0.15±0.02[*]	0.15±0.03[*]
M-CLJE	300	0.19±0.02[*]	0.17±0.02[*]
H-CLJE	600	0.24±0.02[*]	0.24±0.03[*]

[*]$P < 0.05$ and [**]$p < 0.01$ versus OVX group. L-CLJE: low dose CLJE, M-CLJE: medium dose CLJE, H-CLJE: high dose CLJE

Table 2: Effect of CLJE on mechanical characteristics of femur (n = 10)

Group	Dose (mg/kg)	Maximum load(N)	Maximum stress(MPa)
Control	-	132.5±5.3[*]	203.7±6.1[*]
OVX	-	95.6±4.3	148.3±5.6
E_2	0.025	121.4±5.0[*]	181.4±5.4[*]
L-CLJE	150	92.3±4.5	149.3±4.6
M-CLJE	300	102.4±5.2[*]	170.5±5.1[*]
H-CLJE	600	117.1±4.7[*]	176.3±5.3[*]

[*]$P < 0.05$ and [**]$p < 0.01$ versus OVX group. L-CLJE: low dose CLJE, M-CLJE: medium dose CLJE, H-CLJE: high dose CLJE

Table 3: Effect of CLJE on morphometric parameters of L4 vertebrae (n = 10)

Group	Dose (mg/kg)	Tb-N(1/mm)	Tb-Th(mm)	Tb-Sp(mm)
Control	-	5.8±0.3[*]	0.098±0.013[*]	0.11±0.02[*]
OVX	-	2.4±0.2	0.068±0.014	0.46±0.03
E$_2$	0.025	4.3±0.3[*]	0.093±0.011[*]	0.24±0.02[*]
L-CLJE	150	2.7±0.3	0.053±0.015	0.38±0.03
M-CLJE	300	3.5±0.3[*]	0.085±0.014[*]	0.26±0.03[*]
H-CLJE	600	4.6±0.3[*]	0.084±0.012[*]	0.14±0.02[*]

[*]$P < 0.05$ and [**]$p < 0.01$ versus OVX group. L-CLJE: low dose CLJE, M-CLJE: medium dose CLJE, H-CLJE: high dose CLJE

Table 4: Effect of CLJE on biochemical profile of rat serum and urine (n = 10)

Group	Dose (mg/kg)	U-Ca/Cr (mmol/mmol)	U-P/Cr (mmol/mmol)	ALP (U/L)	OC (mmol/L)
Control	-	0.14±0.04[*]	3.3±0.3[*]	102.5±11.6[*]	6.9±0.4[*]
OVX	-	0.53±0.03	6.7±0.2	244.2±27.5	16.5±0.3
E2	0.025	0.31±0.03[*]	4.1±0.3[*]	132.6±15.4[*]	12.6±0.3[*]
L-CLJE	150	0.35±0.02[*]	5.6±0.3	172.4±17.1[*]	13.8±0.4
M-CLJE	300	0.33±0.02[*]	4.2±0.2[*]	166.2±15.3[*]	12.1±0.3[*]
H-CLJE	600	0.25±0.03[*]	3.4±0.3[*]	135.4±16.4[*]	8.6±0.3[*]

[*]$P < 0.05$ and [**]$p < 0.01$ versus OVX group. L-CLJE: low dose CLJE, M-CLJE: medium dose CLJE, H-CLJE: high dose CLJE

Microarchitecture of L4 vertebrae

Analysis of the representative samples (Table 3) indicate that OVX resulted in the deterioration of the trabecular bone microarchitecture, as demonstrated by the reduced Tb-N and Tb-Th compared with the sham group (both $p < 0.05$). In contrast, Tb-Sp was significantly increased in response to OVX compared to the sham group ($p < 0.05$). Higher dosage of CLJE treatment (300 or 600 mg/kg/day) significantly improved the microarchitecture deterioration mentioned above ($p < 0.05$), while E$_2$ also reversed these parameters to the similar degree as that in the H-CLJE group ($p > 0.05$).

Biochemical profile of rat serum and urine

Data presented in Table 4 show the effects of CLJE on biochemical parameters in the serum and urine of OVX rats. Serum U-Ca/Cr, U-P/Cr, ALP, and OC levels were significantly increased in the OVX group compared to the sham group ($p < 0.05$). All three CLJE doses significantly decreased the U-Ca/Cr and ALP levels ($p < 0.05$) in a dose-dependent manner. Higher dosage of CLJE treatment (300 or 600 mg/kg/day) decreased the U-P/Cr and OC levels ($p < 0.05$). E2 administration also reversed these increases which were statistically significant. It is worth noting that the reductions in U-P/Cr, ALP, and OC in the H-CLJE group were similar to those observed for E2 (all $p > 0.05$).

DISCUSSION

High incidence, serious complications, financial burden and dramatically decreased living quality, characterize the severity of osteoporosis in humans. Despite the pharmacological and clinical advantages of HRT as a widely accepted therapeutic strategy for osteoporosis, serious side effects of long-term application have also been reported. Therefore, the development of new preventive and therapeutic drugs for osteoporosis is urgently needed. In recent decades, Chinese medicinal herbals have been extensively investigated for their pharmacological effects related to bone protection.

Bone remodeling is the biologic process that mediates changes in the traits that influence bone strength [1]. Any reasons such as menopause will disturb the balance between formation and resorption and cause bone mass loss [22]. Therefore, we used OVX rats as animal model for human osteoporosis in vivo experiments. It has been reported that statistically significant bone loss can be seen after 30 days [23], so treatment was initiated 4 weeks after OVX. Consistent with other studies, OVX caused significantly higher body weights in our present study, which may be attributed to fat deposition caused by the lack of estrogen [24]. Previous studies suggest that estrogen plays an important role in stimulating the differentiation of progenitor cells through the osteoblast lineage but not the adipocyte lineage [25].

Decreased BMD is one of the major factors jeopardizing bone strength, resulting in increased susceptibility to fractures [26]. Thus, BMD measurement can best predict fracture risk [27]. The results in the present study showed that OVX reduced BMD in the right femurs and L4 vertebrae, which are rich in trabecular bone, while treatment with high-dose dose-dependently prevented the decreases in BMD. Although BMD is among the strongest predictors of facture resistance, both empirical observations and theoretical analyses show that the biomechanical properties of bone and trabecular microarchitecture influence trabecular bone strength as well [28-30]. Three-point bending tests of the left femurs in our study indicated that the higher crocin doses (300 or 600 mg/kg/day) prevented the OVX-induced tendency toward decreased biomechanical parameters.

Moreover, measurements of structural parameters using micro-CT also showed that treatment with CLJE effectively restored the trabecular micro architectural properties compared to the OVX group. In addition, measurement of bone markers plays a role in osteoporosis diagnosis and treatment. Bone mass loss, as evidenced by enhanced levels of ALP, OC, U-Ca/Cr, and U-P/Cr, indicated upregulation of bone turnover by OVX. The bone turnover markers above were dose-dependently reversed by CLJE, indicating a reduction in bone turnover rate after treatment of CLJE. The results suggest that CLJE's potential effectiveness in treating osteoporosis in menopausal women.

CONCLUSION

The findings of this study show that CLJE, at high doses over a 16-week period, prevents OVX-induced osteoporosis in rats. Thus, CLJE has the potential to be further developed as a natural alternative for postmenopausal osteoporosis management in future.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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