Int J Clin Exp Med 2016;9(2):2148-2156
www.ijcem.com /ISSN:1940-5901/IJCEM0016478

Original Article

Anti-osteoporotic activity of the ethanol extracts of eucommia ulmoides in glucocorticoid-induced osteoporosis male rats through the activation of androgen receptor signaling

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Received September 20, 2015; Accepted December 4, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Objective: The present study systematically investigated the in vivo effect of ethanol extracts of eucommia ulmoides (EE-EU) on bone deteriorations in GIOP rat, which was a secondary osteoporosis experimental animal model. This animal model has typical pathologic manifestation and good replication, and could be used to study the effect of drugs. Methods: Forth 6-week-old male Sprague-Dawley rats were randomly divided into vehicle-operated group, prednisone acetate (PA) group, PA with EE-EU of graded doses (100 mg/kg/day or 500 mg/kg/day). The biomarkers in serum and urine were measured, tibias and femurs were taken for the measurement histomorphology, biomechanical parameters, genes and protein expression. Results: High concentrations of EE-EU could significantly prevent the increase in urine calcium and urine phosphorus levels in GIOP rats, meanwhile, EE-EU at a dose of 500 mg/kg/day could dramatically reverse an increase in TRAP-5b and PINP, and a decrease in ALP and FGF-23, which was induced by PA administration. Moreover, treatment with DZCE at higher doses (500 mg/kg/day) was found to be able to significantly prevent PA-induced decrease in biomechanical quality and BMD of femur. Furthermore, the decreased thickness of newly formed cartilage of the GIOP mice was effectively reversed by high concentrations EE-EU. Intriguingly, high concentrations EE-EU treatment could reverse PA-induced low mRNA, protein expression of BMP2, serum testosterone and AR protein expression. Conclusions: The present study demonstrated the anti-osteoporotic effects of EE-EU against bone deteriorations and cartilage degradations in experimentally DIOP rats, and the underlying mechanism was mediated, at least partially, through the activation of androgen receptor signaling.

Keywords: Osteoporosis, eucommia ulmoides, prednisone acetate, cartilage degradations

Introduction

Glucocorticoids are widely used for the treatment of autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, tumors, and organ transplantation in clinical practice [1]. However, the therapeutic use for immunosuppression after organ transplantation or for inflammatory diseases of glucocorticoids is always accompanied by substantial adverse outcomes such as diabetes, obesity, and bone deteriorations [2-4]. It is believed that endogenous glucocorticoid is essential for the proliferation and differentiation of osteoblast in skeleton development, while long-term use of external glucocorticoid inhibits bone formation. Bone deteriorations are the most common side effects by the treatment of glucocorticoids, which are the most common inducement for secondary osteoporosis in adults. Bone loss induced by glucocorticoids occurs early and progresses at a fast rate becoming significant within the first 6 months [5]. Previous studies demonstrate that the long-term administration of glucocorticoids results in the development of osteoporosis in approximately 50% in patients with asthma [6]. Glucocorticoids are known to be associated

with thinner trabeculae, low trabecular number, low connectivity density, low bone volume to tissue volume (BV/TV) and high trabecular separation, intuitively linking glucocorticoids to poor microarchitectural bone quality [7]. The underlying molecular mechanisms accounting for glucocorticoids-induced bone deteriorations can be summarized as a decrease in bone formation directly by inhibiting osteoblasts from producing new bone and an increase in bone resorption by increasing osteoclast activity [8, 9]. In vitro studies show that glucocorticoids can induce osteoblasts and osteocyte apoptosis, and osteoprotegerin (OPG) can prevent glucocorticoids-induced osteocyte apoptosis and increase in both trabecular number and trabecular width in rodent [2, 10-12]. Studies also show that bone mesenchymal stem cells (BMSCs) proliferation, osteogenic differentiation, and reactive activity to an osteogenic inductor are reduced in glucocorticoids-induced osteoporosis (GIOP) rats [13, 14]. Moreover, decreased OPG/RANKL ratio and increased nuclear factor kappa B ligand (RANKL) mRNA expression in GIOP mice [15].

Eucommia ulmoides, also called Du-Zhong in China, is an ancient medicinal plant native from China, Japan, Korea and other countries. It is used widely to antihypertension [16], prevent miscarriages and improve the tone of liver [17], which has been attributed to its high triterpenoid content. It is also a kidney tonifying herbal medicine with a long history of safe use for treatment of bone fractures and osteoarthritis [18]. In vivo and in vitro pharmacological studies have shown that Du-Zhong cortex extracts can promote osteogenesis [19-21]. Eucommia ulmoides can induce primary osteoblastic cell proliferation and differentiation, and osteoclastogenesis is inhibited through an increase in OPG and a decrease RANKL expression in vitro [22]. Moreover, the alcohol extracts of Eucommia ulmoides prevent ovariectomy (OVX)-induced osteoporosis in rats [23]. However, the antiosteoporotic activity of eucommia ulmoides extracts in GIOP animal model remains largely unknown.

Recent studies suggest that the extracts of Eucommia ulmoides have protective effect on osteopenia induced by estrogen deficiency. But still no scientific literature has so far been reported that it can protect against GIOP. GIOP rat is a well-established experimental model for the study of the mechanisms of bone deteriorations and for evaluation of potential therapeutic approaches to ameliorate the bone loss [1]. Therefore, a safe and effective anti-osteoporotic agent is urgently demanded. Traditional Chinese herbal medicines which contain a large number of active compounds could provide a choice for this kind of agent. The present study is aimed to determine the effects of the ethanol extracts of Eucommia ulmoides on glucocorticoid-induced osteoporotic rat. It is hoped that this study will further increase our understanding on the anti-osteoporotic actions of Eucommia ulmoides, which might be useful in secondary osteoporotic patients.

Materials and methods

Animal treatment

Six-week-old male Sprague-Dawley rats (Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) were allowed to acclimate to the environment for 1 week. All experimental procedures were carried out in accordance with the guidelines of the Laboratory Animals of Guangdong Laboratory Animal Monitoring Institute under by National Laboratory Animal Monitoring Institute of China. All experimental protocols were approved by the Academic Committee on the Ethics of Animal Experiments of the Guangzhou University of Traditional Chinese Medicine, Guangzhou, China. All chemicals and reagents were purchased from Sigma (Oakville, Ontario, Canada), except where noted.

After one week of acclimatization, a total of 40 male SD rats were randomly divided into four groups (10 rats per group, n = 10): (1) Vehicle group; (2) rats were orally administered with prednisone acetate (PA) concentration of 5 mg/kg/day (PA group); (3) rats were orally co-administered with prednisone acetate (5 mg/kg/day) and ethanol extracts of Eucommia ulmoides (EE-EU, 100 mg/kg/day, EU100 group); (4) rats in the EU500 group received orally prednisone acetate at a dose of 5 mg/kg/day combined with EE-EU at a dose of 500 mg/kg/day for 12 weeks (EU500 group).

Extraction and concentration of ethanol extracts of eucommia ulmoides

Eucommia ulmoides was obtained from Guangdong province of China in January 2014 and

Table 1. Body weight (BW) throughout the study. BW was recorded every three weeks during experimental period

	0 W	3 W	6 W	9 W	12 W
Vehicle	219 ± 6.8	236 ± 8.5	255 ± 10.7	273 ± 14.6	288 ± 15.3
PA	221 ± 5.5	231 ± 8.9	237 ± 14.5*	241 ± 13.4**	244 ± 15.7**
EU100	218 ± 3.9	235 ± 10.4	248 ± 9.4	265 ± 10.9$^{\#}$	277 ± 14.3$^{\#\#}$
EU500	220 ± 5.2	240 ± 9.3	258 ± 13.5$^{\#}$	274 ± 15.7$^{\#\#}$	292 ± 20.2$^{\#\#\#}$

Values are expressed as mean ± SEM, n = 6-8 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus PA group.

authenticated according to a method listed in Chinese Pharmacopeia. A voucher specimen was deposited in the First Affiliated Hospital of the Guangzhou University of Traditional Chinese Medicine, Guangzhou, China. The dried and powdered (2 kg) crude Eucommia ulmoides cortex was extracted with 70% ethanol elution and concentrated by vacuum freeze drying method.

Chemistries in serum and urine

The concentrations of calcium (Ca), phosphorus (P) and creatinine (Cr) from serum and urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The level of urine Ca was corrected by the concentration of urine Cre. Serum levels of alkaline phosphatase (ALP), fibroblast growth factor-23 (FGF-23), tartrate resistant acid phosphatase-5b (TRACP-5b) and propeptide of type 1 procollagen (PINP) were detected using rat bioactive ELISA assay (Immutopics, Inc., San Clemente, CA, USA) with ELISA reader (MD SpectraMax M5, USA).

Histological analysis and BMD

After fixation in 75% alcohol for 7 days, the femurs (n = 3/group) specimens were dehydrated in graded ethanol and embedded in methylmethacrylate. Section of 5 μm were cut and stained with von Kossa, and visualized under a microscope (Leica DM 2500). Moreover, the femurs were decalcified in 0.5 M EDTA (pH = 8.0) and then embedded in paraffin by standard histological procedures. Section of 5 μm were cut and stained with safranin O, and visualized under a microscope (Leica DM 2500).

The bone mineral density (BMD) of femurs in each group was measured by dual-energy X-ray absorptiometry (DEXA) (LUNAR DPXIQ, GE Healthcare, USA).

Biomechanical parameters

Femurs were placed on the Instron machine (Instron Microtester 5848, Instron Corp., USA) in a three-point bending configuration. The load was applied at the mid-diaphysis in an anteroposterior direction with a loading speed of 5 mm/min until the femur fractured. The load, stress, and elastic modulus curves were automatically calculated by the computer using the Bluehill software. The femora were kept moist at all times during the testing. The parameters measured were load, stress and modulus curves.

Quantitative real-time PCR

The proximal tibias of each animal were crushed under liquid nitrogen conditions and RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total-RNA using All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia., USA) and oligo (dT) primers for the analysis of osteoclast apoptosis-related genes, including the following: Runx2, forward CCTGACTCTGCACCAAGTC-3' and reverse 5'-GAGGTGGCAGTGTCATCATC-3'; ALP, forward 5'-GCTGAACAGGAACAACGTGA-3' and reverse 5'-AGACTGCGCCTGGTAGTTGT-3'; OPG, forward 5'-TGCTCCTGGCACCTACCTA-3' reverse 5'-ACTCCTGCTTCACGGACTG-3'; RANKL, forward 5'-GGAAGCGTACCTACAGACTA-3' reverse 5'-AGTACGTCGCATCTTGATCC-3'; BMP2, Forward 5'-GAGTTGGATCGTTCTAGTACTG-3' and Reverse 5'-CAACCTTGTATCATACGGAATCAG-3'; GAPDH, forward 5'-CACCATGGAGAAGGCCGGGG-3' reverse 5'-GACGGACACATTGGGGGTAG-3'. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The proximal tibias were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, trans-

Table 2. Biochemical parameters in serum and urine

	Vehicle	PA	EU100	EU500
S-Ca (mmol/L)	2.55 ± 0.17	2.33 ± 0.37	2.41 ± 0.26	2.40 ± 0.29
S-P (mmol/L)	1.65 ± 0.10	1.71 ± 0.15	1.68 ± 0.14	1.70 ± 0.18
U-Ca/Cr (mmol/mmol)	0.17 ± 0.015	0.33 ± 0.027*	0.36 ± 0.045	0.24 ± 0.031#
U-P/Cr (mmol/mmol)	4.25 ± 0.32	5.68 ± 0.49*	5.16 ± 0.63	4.41 ± 0.45#
ALP (U/L)	1.79 ± 0.16	1.28 ± 0.21*	1.62 ± 0.24#	1.71 ± 0.18#
FGF-23 (ng/L)	478 ± 35	314 ± 46*	358 ± 54	452 ± 44#
PINP (μg/L)	13.6 ± 1.3	20.8 ± 3.4*	15.2 ± 2.2#	11.6 ± 2.9##
TRAP-5b (pg/L)	1364 ± 89	2050 ± 231*	1537 ± 168#	1306 ± 209##

Values are expressed as mean ± SEM, n = 6-8 in each group. *$P < 0.05$ versus vehicle group; #$P < 0.05$, ##$P < 0.01$ versus PA group.

ferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked and then incubated with primary antibodies specific for BMP2 and AR. β-actin was used as protein loading control. The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized and detected by chemiluminescence (Thermo, USA).

Statistical analysis

Data are presented as the mean value ± SD. Statistical analysis was performed with ANOVA using SPSS16.0 software. *P*-values < 0.05 were considered statistically significant.

Results

Basic parameters and biomarker in serum and urine

Four groups of rats had a similar initial mean body weight. However, the body weight significantly decreased in PA group when compared with that of the vehicle group on week 4 after prednisone acetate administration. The body weight of PA group continued to be significantly lower than vehicle group throughout the study (**Table 1**). At the endpoint, the mean body weight of the rats was decreased by 15.2% after treated with prednisone acetate at 5 mg/kg/day. EE-EU, the concentration of 500 mg/kg/day, completely prevented the decrease in body weight associated with glucocorticoid treatment and returned body weight to the level maintained by vehicle group (**Table 1**).

The serum calcium and serum phosphorus had no obvious differences among four groups, however, the urine calcium and urine phosphorus in PA group were significantly higher compared with vehicle group (**Table 2**). High concentrations (500 mg/kg/day) of EE-EU could significantly prevent the increase in urine calcium and urine phosphorus levels in EU group, whereas, EE-EU at the low dose (100 mg/kg/day) could not influence the levels of calcium and phosphorus in urine (**Table 2**). Serum concentrations of bone turnover markers, like ALP and FGF-23 as a bone formation marker, TRAP-5b and PINP as a bone resorption marker, was determined. The results showed that the serum TRAP-5b and PINP levels were significantly increased, and the serum ALP and FGF-23 levels were significantly decreased in PA group when compared with that of the control group (**Table 2**). However, high concentrations (500 mg/kg/day) of EE-EU could dramatically reverse an increase in TRAP-5b and PINP, and a decrease in ALP and FGF-23, which was induced by prednisolone acetate administration.

Biomechanical parameters and BMD

As for the biomechanical parameters, there was significant difference between the vehicle and PA groups, the significantly lower maximum load, fracture load, elastic modulus and maximum stress parameters for the PA group as compared with the vehicle group (**Figure 1A-D**). We also found that the femoral BMD values in PA group lower than vehicle group (**Figure 1F**). The 12 weeks treatment with EE-EU at 500 mg/kg/day significantly improved the biomechanical strength of femur and increased the femoral BMD compared with the PA group (**Figure 1A-F**).

Histological analysis and bone metabolism-related genes expression

Undecalcified histological analysis on trabecular bone in proximal tibial head of rat was performed by von Kossa staining. The results revealed that EE-EU-treated increased the osteoid surface (OS) and osteoid width (O.Wi) in both low and high concentration treatment group as compared with prednisolone acetate

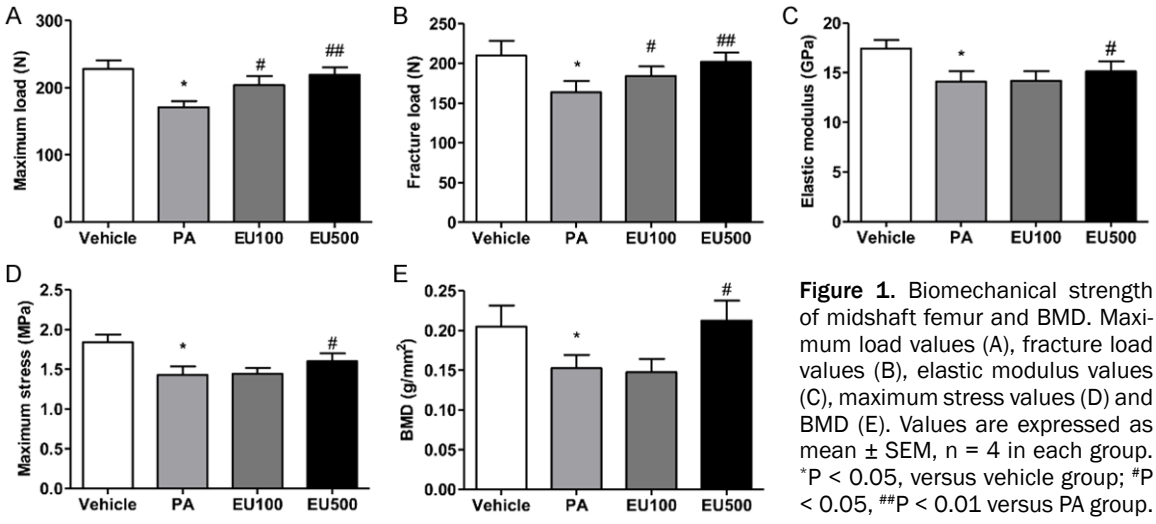

Figure 1. Biomechanical strength of midshaft femur and BMD. Maximum load values (A), fracture load values (B), elastic modulus values (C), maximum stress values (D) and BMD (E). Values are expressed as mean ± SEM, n = 4 in each group. *P < 0.05, versus vehicle group; #P < 0.05, ##P < 0.01 versus PA group.

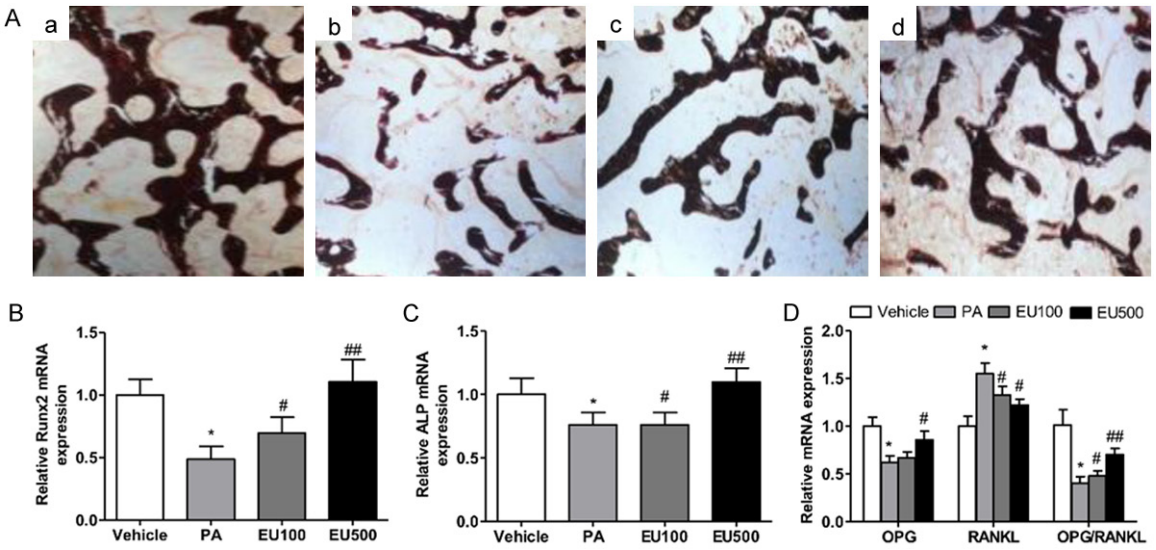

Figure 2. Undecalcified histological sections stained with von Kossa (a. Vehicle group; b. PA group; c. EU100 group; d. EU500 group; A. Magnification, ×100). The mRNA expression of Runt-related transcription factor (Runx2, B), alkaline phosphatase (ALP, C), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) and the quantitative ratio of OPG/RANKL (D). Values are expressed as mean ± SEM, n = 6 in each group. *P < 0.05 versus vehicle group; #P < 0.05, ##P < 0.05 versus PA group.

single treatment group (**Figure 2A**). To determine the changes of the osteoblast-specific genes which are responsible for osteoblasts-involved bone resorption, the mRNA expression of Runx2 and ALP was measured. The results (**Figure 2B** and **2C**) showed that the mRNA expression of Runx2 and ALP was significantly decreased in PA group compared with vehicle group. EE-EU treatment could promote the expression Runx2 and ALP in the proximal tibia of rat. Importantly, the maturation and formation of osteoclasts were mainly regulated by the balance of extracellular OPG and RANKL levels, thus, the ratio of OPG/RANKL expression in proximal tibia was determined in our study. The real-time PCR result showed that OPG and the ratio of OPG/RANKL was significantly decreased in rat treated by prednisolone acetate administration as compared with the vehicle group, on the contrary, the expression of RANKL was increased in PA group (**Figure 2D**). Compared to PA group, treatment with 500 mg/kg/day EE-EU reversed the above mentioned findings at the same degree, all were statistically significant.

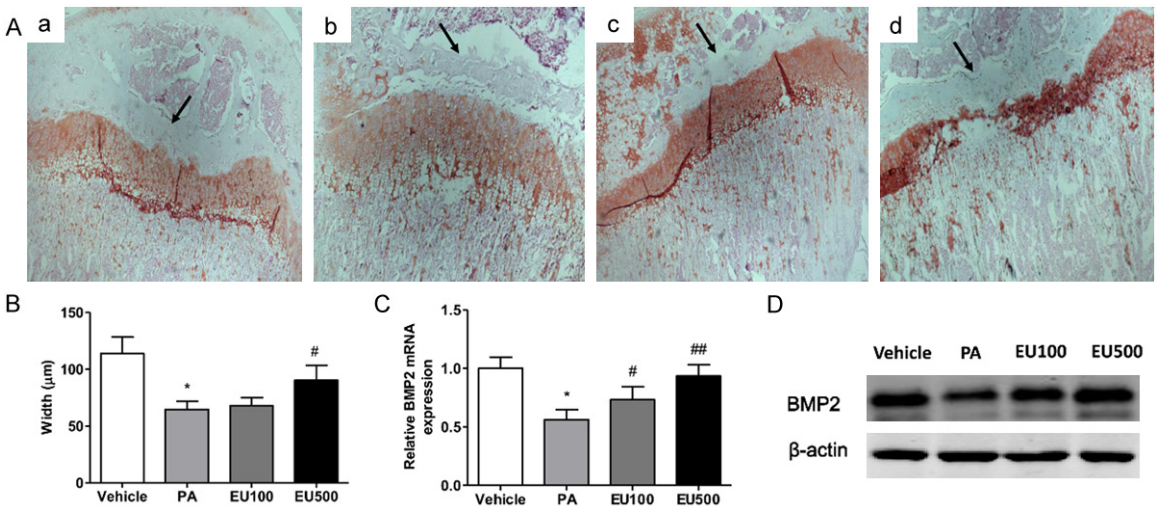

Figure 3. Cartilage adjacent to the epiphyseal plate was shown by Safranin O staining of the proximal metaphysis of the tibia (A. Magnification, ×50) and the width of the articular cartilage is quantified (B). The mRNA (C) and protein (D) expression of BMP2 are respectively measured by Quantitative real-time PCR and western blotting. Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ versus PA group.

Safranin O staining was performed to observe the upper epiphyseal cartilage of proximal tibias. The thickness of cartilage adjacent to the epiphyseal plate was reduced in the proximal tibia of the PA group (shown by arrow) suggesting the occurrence of delayed formation of new cartilage on the upper epiphyseal plate caused by prednisolone acetate. The decreased thickness of newly formed cartilage of the GIOP mice was effectively reversed by high concentrations EE-EU (**Figure 3A** and **3B**). BMP2 is used as the target gene for the treatment of local osteoporosis in rat femurs. Recent studies have confirmed that BMP2 is capable of promoting ossification. The mRNA and protein expression of BMP2 in the proximal tibias of rat were performed by real-time PCR and western blotting respectively. The results indicated that the mRNA and protein expression of BMP2 were inhibited by prednisolone acetate treatment, however, EE-EU-treated could increased the mRNA and protein expression of BMP2 in both low and high concentrations EE-EU treatment group (**Figure 3C** and **3D**).

Testosterone and androgen receptor

Androgen receptor (AR) plays an important role in the homeostasis of the male skeleton. Global deletion of AR in male mice results in high bone turnover and increased resorption, as well as decreased trabecular and cortical bone volume. In our study, PA group showed significantly lower testosterone in serum and AR protein expression in the proximal tibias compared to vehicle group (**Figure 4A** and **4B**). Importantly, both low and high concentrations EE-EU treatment could reverse prednisolone acetate-induced low testosterone and AR protein expression (**Figure 4A** and **4B**).

Discussion

An increasing amount of evidence suggests a role for the extracts of eucommia ulmoides cortex in the treatment of postmenopausal bone loss in rodents [20, 22, 23]. Menopause results in accelerated bone remodeling, an increase in bone resorption and bone formation, which may be different with a decrease in bone formation and an increase in bone resorption by glucocorticoid treatments [1, 23]. Proof of efficacy, however, in the treatment of GIOP in comparison with currently available treatments is still lacking. The present study was designed to systematically evaluate the effect of EE-EU on the protection against glucocorticoid-induced bone loss in male rats.

Glucocorticoid treatment is suggested to influence bone physiology and remodeling and disturbance of calcium homeostasis [1]. As expected, prednisone acetate was continuously administered for 12 weeks in rats, which result-

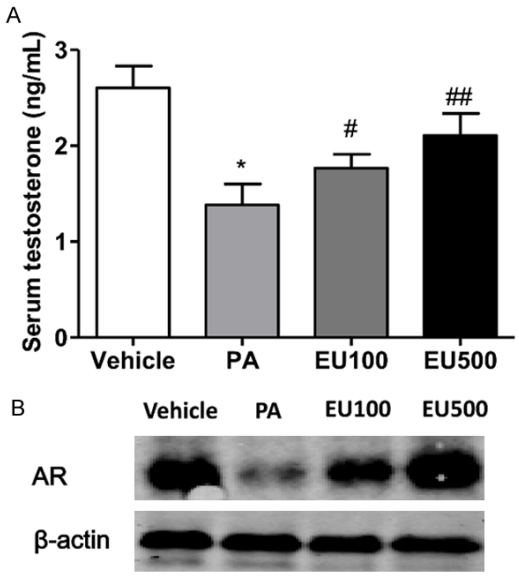

Figure 4. Serum testosterone was measured by ELISA analysis (A). The expression of androgen receptor (AR) was measured by western blotting (B). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ versus PA group.

ed in significant decrease in the femoral biomechanical strength and BMD. This loss of bone mass was accompanied by a significant decrease in bone remodeling, which was evidenced by the reduced levels of the bone formation markers ALP and FGF-23. Treatment with EE-EU could prevent the decreases in femural BMD and biomechanical strength, which were reflected by the decreases in serum TRAP-5b and PINP levels. In addition, an increase in urine calcium and urine phosphorus excretions was demonstrated in GIOP rats. Therefore, these results shown that a decrease in calcium and phosphorus reabsorption efficiency might contribute to the reduction of BMD and biomechanical strength in the femur of rats. From these basic biochemical and biomechanical parameters, it was well shown that EE-EU exerted protective effects on PA-induced bone deteriorations in rats. Moreover, safranin O staining results shown that the micro-architecture of trabecular bone was improved by EE-EU in PA-induced osteoporosis, the preservation of trabecular microarchitecture significantly contributes to bone strength and may reduce fracture risk irrespective of BMD [24].

In this study, we had demonstrated that EE-EU enhanced bone mass and bone strength in a rat model of osteoporosis by altering bone metabolism-related gene expression. The expression of osteoblast-specific genes, Runx2 and ALP, was suppressed in the proximal tibia of GIOP rats. Runx2 is as an osteoblast master regulator that is required for bone formation. Initially identified based on its interaction with the bone specific osteocalcin promoter [25]. After further studies found that inhibition of Runx2 in vitro abrogates expression of osteoblast markers, and its forced expression in non-osteoblasts induces bone-like cellular phenotypes [26]. Intriguingly, over-expression of Runx2 in osteoblasts results in increased osteoclast differentiation, and Runx2-mediated increase in RANKL secretion, which is attributable in part to a decrease in OPG expression [27, 28]. The decreased mRNA expression of OPG and the ratio of OPG/RANKL in tibia indicated that prednisone acetate could stimulate osteoclastogenesis in GIOP mice. The present study was demonstrated that EE-EU at higher doses (500 mg/kg/day) significantly prevented the above mentioned findings in GIOP rats. The anti-osteoporotic effects of EE-EU appear to be related to high contents of the polyphenolic compounds such as lignans, phenolic acid, and flavonoids [20, 22, 23].

There have been numerous studies indicate that bone morphogenetic proteins (BMPs) are strong inducers of osteoblast differentiation and bone formation [29]. Overexpressed lentiviral vector-mediated human BMP2 gene in the femoral bone marrow stromal cells can improve local osteoporosis in rat femurs [30]. In $OPG^{-/-}$ mice, the mRNA expression of BMP2 and BMP4 is upregulated after the treatment of Icariin [31]. In this study, the mRNA and protein expression of BMP2 were increased after the treatment of EE-EU in GIOP mice. A new research from Korea finds that eucommia ulmoides extracts significantly increased BMP-2 expressions in the proliferative and hypertrophic zones by immunohistochemical staining [32]. Moreover, eucommia ulmoides extracts increase longitudinal bone growth rate by promoting chondrogenesis and stimulating chondrocyte differentiation through the upregulation of BMP-2 [32].

Testosterone deficiency is the most common cause of male osteoporosis. Testosterone deficiency elicits an imbalance in bone remodeling, whereby bone formation is decreased and bone

resorption is increased, resulting in trabecular bone loss [33]. Lower testosterone in serum and AR protein expression in the proximal tibias compared to vehicle group were demonstrated in this study. Importantly, both low and high concentrations EE-EU treatment could reverse prednisolone acetate-induced low testosterone and AR protein expression. In addition to well-known phytoestrogens, the existence of phytoandrogens is reported in eucommia ulmoides [34]. The anti-osteoporotic activity of eucommia ulmoides may be attributed to a phenomenal tripartite synergism existing between the sex steroid receptors (androgen and estrogen receptors), their cognate steroidal ligands and lipidic augmenters isolated from eucommia ulmoides, and phytoandrogenic activity of eucommia ulmoides is mediated by plant triterpenoids binding cognately to the androgen receptor (AR) ligand binding domain [34].

In conclusion, the present study clearly demonstrated that EE-EU protected against osteoporosis associated with glucocorticoid use. On the basis of the present results, EE-EU might represent a therapy with bone-forming as well as an anti-resorptive activity in GIOP mice. Our findings provide evidence to support the role of E-EU as an effective therapeutic approach in the management of glucocorticoid-induced bone loss.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81373665) and the Special Fund Project of Industrial Technology Research and Development in Guangdong Province (No. 2013B021800226). Province natural science fund of Guangdong (No. 2015A030310203); the Project of Youth Scientific Research Personnel Training in Guangdong Province (No. 2015QN01).

Disclosure of conflict of interest

None.

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