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Original Research Article

Effect of Eucommia ulmoides extract on osteoblast proliferation

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Abstract

Purpose: *To evaluate the effect of Eucommia ulmoides extract (EUE) on osteoblast proliferation as well as investigate its probable mechanisms of action.*
Methods: *EUE was pharmacologically evaluated at three doses. Osteoblast cells were divided as follows: Group I: negative control; groups II–IV: received EUE (180, 360 and 540 µg/ml, respectively). We performed 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay to determine osteoblast viability following treatment. Alkaline phosphatase (ALP), osteocalcin, and collagen I levels in osteoblasts were quantified using commercially available kits. Thereafter, mRNA and protein expression of ALP, collagen I, osteocalcin, transforming growth factor-β1 (TGF-β1) were measured using real-time quantitative PCR (qPCR) and western blot, respectively.*
Results: *EUE significantly ($p < 0.01$) promoted osteoblast proliferation at three treatment doses (180, 360, and 540 µg/mL). Furthermore, ALP, osteocalcin, collagen I and TGF-β1 expression at both mRNA and protein levels increased significantly (all $p < 0.05$) following EUE treatment.*
Conclusion: *The results suggest that EUE may promote osteoblast cell proliferation and that ALP, osteocalcin, collagen I and TGF-β1 gene expression may be involved in the mechanism of action.*

Keywords: *qPCR, collagen I, Bone, Liver, Eucommia ulmoides extract*

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INTRODUCTION

Osteoporosis reduces bone mass by destroying bone microstructure, leading to increased bone fragility. This systemic bone metabolic disease increases the risk of bone fracture [1,2].

Eucommia ulmoides is a perennial deciduous tree that is endemic to China and protected by the government. This plant is often used for medicinal purposes. *Eucommia ulmoides* has been used in several pharmacological applications, such as treating back and knee pain, addressing Qi deficiencies (Bu Zhong), and strengthening bone [3]. *Eucommia ulmoides* has also been used to treat numbness, certain types of liver deficiencies, tinea cruris, and other conditions. Modern pharmacological research shows that *Eucommia ulmoides* can remove metabolic byproducts from the body, enhance cellular metabolism, prevent skeletal muscle aging, balance blood pressure and cholesterol, reduce body fat, restore vascular elasticity, treat diuresis, exert broad-spectrum anti-bacterial activity, excite the central nervous system, and improve immune response [4,5].
Here, the effects of *Eucommia ulmoides* extract on cell viability and ALP, collagen I, osteocalcin,

and TGF-β1 mRNA and protein levels in osteoblasts were evaluated.

EXPERIMENTAL

Materials

We collected *Eucommia ulmoides* plants from XiangYang City, HuBei Province, China in 2015. Dr. Chen identified the taxonomy of the plant, and we deposited a voucher specimen (no. 20150611) at the Chinese National Herbarium (Chinese Academy of Sciences). *Eucommia ulmoides* extract was prepared in our laboratory as follows. Dried *Eucommia ulmoides* bark was boiled with eight volumes of water for 2 h. The sample was centrifuged at 14,000 *g* for 15 min. We collected the supernatant, evaporated it under vacuum, and lyophilized it (yield 84.6 g).

The Animal Ethical Care and Use Committee of Dalian Medical University, China approved all experiments (Ethical permit no. 04/S0356/711). Experiments complied with institutional guidelines and the National Research Council Guide for the Care and Use of Laboratory Animals [6].

MTT assay

Extract was dissolved in DMSO (512 µg/mL). Extract was serially diluted two-fold twelve times (concentration range, 0.25 – 512 µg/mL). Osteoblasts were treated with extract for 72 h. The microtiter plates were sealed with EVA Capmats™ and allowed to incubate in humidified conditions at 37 °C and 5 % CO_2. Unsealed plates of treated cells were also set up as a control. Following treatment, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was prepared in EMEM medium to a final concentration of 1 mg/mL and pipetted in wells. Then, we incubated plates for another 2 h at the above culture conditions. DMSO (0.15 mL) was added to each well, and plates were rocked until the crystals in the culture medium dissolved completely. Using a microplate reader, we measure the absorbance (OD) at 570 nm.

Quantitation of ALP, collagen I, and osteocalcin levels in osteoblasts

Osteoblasts were treated for 72 h as described above. We measured ALP, osteocalcin, and collagen I levels using commercially available kits, according to the manufacturers' protocols.

PCR analysis

Osteoblasts were treated for 72 h as described above. Total RNA was extracted according to the manufacturer's instructions using the QIAamp DNA stool Kit (Qiagen, Hilden, Germany) and stored at -80 °C. cDNA was synthesized using a reverse transcription kit. RNA samples were heated to 42 °C for 30 min then to 95 °C for 3 min and plated in 96-well reaction plates in triplicate. Each reaction included 10 µL 2× SYBR Premix Ex Taq, 1 µL forward primer, 1 µL reverse primer (Table 1), 3 µL cDNA, and 5 µL nuclease-free water. We performed RT-qPCR using a Light Cycler 480 II (Roche Diagnostics) and measured cycle threshold (Ct) values to calculate the relative amounts of amplified PCR products. β-actin was used as a reference.

Table 1: Primers and their sequences

Name	Sequence of forward primers	Sequence of reverse primers
TGF-β1	CCACCTGCAAGAC CATCGAC	TGCTTCCCGAATG TCTGACG
Collagen I	GTGAGACAGGCGA ACAAG	AACCAGGAGAAC CAGGAG
Osteocalcin	CCTCAGTCCCCAG CCCAGATCC	CAGGGCAGAGAG AGAGGACAGG
ALP	ACACCTTGACTGTG GTTACTGCTGA	CCTTGTAGCCAG GCCCGTTA
β-actin	TGTGTCCGTCGTG GATCTGA	TTGCTGTTGAAGT CGCAGGAG

Western blot

Osteoblast were treated with extract for 48 h, washed with cold PBS three times, and lysed in *RIPA* buffer for 30 min on ice. Samples were centrifuged (12000 rpm, 4 °C, 20 min). The total protein concentration of the clarified lysate was determined using the BCA protein assay (Pulilai, China). The samples were resuspended in protein-loading buffer and loaded onto a 10 % SDS-PAGE gel. Approximately 80 µg of total protein was loaded per lane. We performed electrophoresis at 100 mV and transferred proteins to a polyvinylidene fluoride membrane at 300 mA. The membrane was blocked with 5 % skim milk, probed with primary antibodies overnight, and washed. We incubated the membrane with a 1:2000 dilution of anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) (Pulilai, China) for 2 h. The bands were evaluated using an enhanced chemiluminescence detection system. Densitometric analysis was performed using a gel image scanner (ChemiDoc XRS, Biorad, USA).

Statistical analysis

Experimental data are expressed as mean ±

standard error of the mean. SPSS version 20 was used for analyses. Among-group differences were analyzed using Analysis of Variance (ANOVA) and Dunnett's *t*-test. A p value < 0.05 denoted statistical significance.

RESULTS

Compared with the normal control group, *Eucommia ulmoides* extract treatment significantly increased osteoblast growth in a dose-dependent manner (all $p < .05$). Additionally, osteoblast growth rate increased significantly with prolonged *Eucommia ulmoides* extract treatment (Table 2).

ALP, osteocalcin and collagen I levels in osteoblasts increased significantly (all $p < .05$) following treatment with *Eucommia ulmoides* extract (Table 3). Further, a dose-dependent effect was observed. The effect was especially obvious at high extract concentrations.

Similarly, ALP, collagen I, osteocalcin, and TGF-β1 mRNA levels in osteoblast increased significantly (all $p < .05$) and a dose-dependent effect was observed following treatment with *Eucommia ulmoides* extract (Table 4). Again, the effect was more pronounced at higher extract concentrations.

Protein expression levels confirm the above results. As before ALP, osteocalcin, TGF-β1, and collagen I protein levels increased significantly (all $p < 0.05$) following *Eucommia ulmoides* extract treatment, and the same dose-dependent effect was observed (Figure 1).

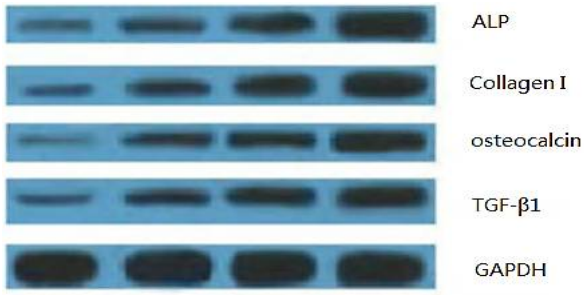

Figure 1: Effect of *Eucommia ulmoides* extract treatment on ALP, osteocalcin, collagen I, and TGF-β1 protein levels in osteoblast cells

DISCUSSION

Bone tissue can renew itself by mineralizing an organic matrix. The process of bone remodeling is a delicate balancing act between osteoblast bone formation and osteoclast bone resorption [7,8].

When this balance is disrupted, the result is osteopathic diseases, including Paget's disease and osteoporosis [9]. Older women commonly

Table 2: Effect of *Eucommia ulmoides* extract (EUE) treatment on osteoblast growth

Group	MTT		
	24 h	***48 h***	***72 h***
NC	0.1853±0.0131	0.3275±0.0242	0.3371±0.0264
EUE (180 μg/mL)	0.1951±0.0138	0.3281±0.0283	0.3516±0.0251 *
EUE (360 μg/mL)	0.2028±0.0127	0.3482±0.0241*	0.3718±0.0283**
EUE (540μg/mL)	0.2112±0.0166	0.3629±0.0242**	0.4172±0.0306**

*$P < .05$, **$p < .01$ compared with the normal control (NC) group

Table 3: Effect of *Eucommia ulmoides* extract (EUE) treatment on ALP, osteocalcin, and collagen I levels in osteoblasts

Group	ALP (μKat/g)	Osteocalcin (ng/10^6)	Collagen I (ng/mL)
NC	12.52 ± 1.08	2.97 ± 0.18	10.37 ±0.93
EUE (180 μg/mL)	14.27 ± 1.14	3.31 ±0.24 *	12.03 ± 1.05
EUE (360 μg/mL)	16.38 ± 1.09*	3.49 ±0.22**	16.29 ± 1.17**
EUE (540μg/mL)	17.34 ± 1.13*	4.15 ± 0.31**	22.71 ± 1.82 **

* $p < .05$, **$p < .01$ compared with the normal control (NC) group

Table 4: Effect of *Eucommia ulmoides* extract (EUE) treatment on the relative mRNA expression level of ALP, collagen I, osteocalcin, and TGF-β1 in osteoblasts

Group	ALP mRNA	Collagen I mRNA	Osteocalcin mRNA	TGF-β1 mRNA
NC	1.01 ± 0.03	1.03 ± 0.01	1.04 ± 0.02	1.02 ± 0.01
EUE (180 μg/mL)	0.99 ± 0.02	1.08 ± 0.03	1.11 ± 0.04	1.13 ± 0.02*
EUE (360 μg/mL)	1.18 ± 0.05	1.18 ± 0.04*	1.26 ± 0.04**	1.29 ± 0.04**
EUE (540 μg/mL)	1.25 ± 0.03*	1.21 ± 0.05**	1.34 ± 0.03 **	1.42 ± 0.06**

*$P < .05$; **$p < .01$ compared with the normal control (NC) group. Values normalized to β-actin

develop osteoporosis because their estrogen levels decrease following menopause

Traditional Chinese medicine considers *Eucommia ulmoides Oliv*. to be valuable because of its anti-bacterial, anti-inflammation, immune-boosting, and anti-osteoporosis effects [10]. One study showed that consumption of *Eucommia ulmoides* bark extract inhibits estrogen-deficiency-induced bone loss in ovariectomized rats. Specifically, the extract slowed bone resorption and increased osteoblast activity [11].

Alkaline phosphatase (ALP) is an enzyme involved in osteoblast and osteoblast differentiation. It plays a key role in calcification *in vitro*. Alkaline phosphatase can hydrolyze organic phosphatase, which increases the local concentration of PO_4^{3-}. The combination of PO_4^{3-} and Ca^{2+}, initiates calcification. Osteocalcin, a major non-collagenous protein in the bone matrix, is the only bone extracellular matrix protein produced exclusively by osteoblasts. Osteocalcin contributes to bone formation, making it a specific marker of osteoblast function. TGF-β is commonly expressed in numerous tissues, and the TGF-β1 subtype is highly expressed in bone tissue. Type 1 collagen is the primary component of the extracellular matrix because it serves as a framework for the hydroxyapatite deposition reaction.

Our results showed that *Eucommia ulmoides* extract promoted osteoblast proliferation and increased ALP, collagen I, osteocalcin, and TGF-β1 levels of both mRNA and protein in osteoblasts.

Limitation of the study

There is one limitation to this study. The bone-promotion effects of *Eucommia ulmoides* extract in ovariectomized animals, such as bone loss inhibition, were not examined. We will address this limitation in our future studies.

CONCLUSION

This study demonstrates that *Eucommia ulmoides* extract has beneficial effects on osteoblast growth. Specifically, *Eucommia ulmoides* extract treatment significantly promotes osteoblast growth and increases ALP, collagen I, and osteocalcin secretion. These results indicate that *Eucommia ulmoides* extract may affect osteoblast cell differentiation and stimulate the growth of osteoblast cells by increasing ALP, osteocalcin, collagen I, and TGF-β1 levels. *Eucommia ulmoides* extract could possibly be developed as a therapy for osteoporosis.

DECLARATIONS

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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