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Promotion of osteoblastic Ca^{2+} accumulation by *Eucommia* leaf extract

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Abstract

Eucommia ulmoides is a traditional herbal medicine known to have various systemic effects. In recent years, the bone pharmacological properties of *Eucommia ulmoides* leaf have been reported. However, studies on the effect of *Eucommia ulmoides* leaf on bone-related cells are insufficient. Therefore, this study aimed to investigate the efficacy of *Eucommia* leaf extract (ELE) for osteoblast proliferation and maturation by using primary cultured osteoblasts. Primary osteoblast cultures received constant applications of 10 μg/ml ELE up to 28 days *in vitro* (DIV). Alkaline phosphatase activity and Ca^{2+} accumulation were significantly increased after 28 DIV. In addition, 10 μg/ml ELE application significantly increased the intracellular Ca^{2+} concentration after 14 DIV and significantly increased the expression of *RANKL* and *Col1a2* mRNA after 7 DIV on RT-qPCR. However, ELE application did not affect 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) reduction in a MTT assay. These results suggest that ELE application has no effect on proliferation of osteoblasts but has a regulatory effect on osteoblast ossification. From the standpoint of prophylactic pharmacology, drinking ELE tea as a self-medication potentially could prevent metabolic bone disorders.

Introduction

Eucommia ulmoides is a deciduous tree native to China. The *Eucommia ulmoides* bark is used as a traditional herbal medicine, which is described in the Shennong Bencaojing that was compiled >1600 years ago in China. It is said that *Eucommia ulmoides* bark has no side effects, and it has been used to treat kidney dysfunction and as an analeptic. The *Eucommia ulmoides* leaves are edible, and a decoction of the leaves is drunk as a tea in China, Korea, and Japan for health promotion. *Eucommia ulmoides* is known to have various systemic effects. It is known that various ingredients are contained in *Eucommia ulmoides* [1]. Although the component concentrations are different, the ingredients contained in the *Eucommia ulmoides* bark as a medicine are also contained in *Eucommia ulmoides* leaves as a food. This means that it is possible to easily purchase "natural" active ingredients with no side effects as foods in the market. In addition, it is possible that ingestion of foods considered to be effective for health can be a form of preventive self-medication against possible future diseases.

In recent aging society, it is expected that bone metabolic diseases, such as osteoporosis, will occur more frequently in elderly people. However, bone metabolic diseases were already known in Chinese medicine from much earlier times. *Eucommia ulmoides* bark also is a traditional Chinese medicine that is used to treat bone metabolic diseases. Indeed, applications of the lignan component extracted from *Eucommia ulmoides* bark to postmenopausal osteoporosis model rats by ovariectomy have been reported to prevent osteoporotic lesions [2]. Additionally, application of *Eucommia* leaf extract (ELE), which is a food product, to postmenopausal osteoporosis model rats by ovariectomy has been found to have a preventive effect on osteoporotic lesions [3]. Therefore, it is possible that ingestion of ELE as a food could prevent bone metabolic diseases. However, the effect of ELE on ossification has not been fully clarified at present. This study aimed to investigate the efficacy of ELE on osteoblast proliferation and maturation by using primary cultured osteoblasts.

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Key words: *Eucommia leaf extract (ELE), osteoblast, osteogenesis, prophylactic pharmacology, self-medication*

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Material and Methods

Preparation of ELE

In this study, we used *Eucommia* leaves collected in the Sichuan District of China. To prepare the ELE, fresh *Eucommia* leaves were steamed at 100–110°C and then dried and roasted. Two tons of roasted *Eucommia* leaves were steeped in 10 tons of hot water at 90°C for 1 h, and the extract was then filtered and concentrated. The concentrate was allowed to stand for 1 day. The concentrate was then filtered and concentrated, vacuum-dried, and powdered (yield: 18%) as previously described [4].

Preparation of primary cultured osteoblasts

Appropriate animal experiments that considered animal welfare were conducted according to the "Suzuka University of Medical Science Animal Experiment Guide." All procedures were also performed according to the National Institutes of Health guidelines regarding the principles of animal care (1996). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

Under pentobarbital (Kyoritsu Seiyaku Corporation. Tokyo, Japan) anesthesia conditions, osteoblasts were isolated from the calvaria of 1-to-2-day-old Wistar neonatal rats. In brief, rat calvaria were gently incubated at 37°C for 10 min with 0.2% (w/v) collagenase (Worthington Biochemical Corporation. NJ, USA) in α-modified minimum essential medium (α-MEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), followed by collection of cells in supernatants thus obtained. This incubation was consecutively repeated 5 times. Then, the last 3 digestion supernatants were collected together in α-MEM containing 10% fetal bovine serum (FBS) and 10-units/ml penicillin–10 μg/ml streptomycin (Wako Pure Chemical Industries), followed by centrifugation at 1,500 rpm for 5 min. The pellets were suspended in α-MEM containing 10% FBS. Cells were plated at a density of 1×10^4 cells/ml in appropriate dishes, and then cultured at 37°C for different periods under 5% CO_2 with medium change every 3 days. Throughout the experiments, -MEM containing 10% FBS, 50 mg/ml ascorbic acid (Sigma-Aldrich Co. LLC., MO, USA), 5 mM sodium β-glycerophosphate (Sigma-Aldrich), and 40 mM $NaHCO_3$ (Sigma-Aldrich) were used [5].

ELE treatment protocol

The adjusted primary osteoblast cultures were divided into the control group and the ELE 10 μg/mL application group. Then, the groups were cultured for ≤28 days, and the medium was changed every 3 days in vitro (DIV). Osteoblast cultures of 7 DIV, 14 DIV, and 28 DIV periods were used in each experiment.

MTT reduction assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, yellow tetrazole (MTT) (DOJINDO LABORATORIES, Kumamoto, Japan) assay is a method for examination of the viability and growth rate of cultured cells. The MTT is reduced to violet-colored insoluble formazan in living cells. Then, the obtained formazan is quantified by absorptiometry. In this study, MTT assays using cultured osteoblasts of the control group and ELE application group for 7, 14, and 28 DIV periods were performed. The cultured osteoblasts for different periods were washed in phosphate-buffered saline (PBS). Subsequently, the MTT substrate in PBS (500 μg/mL) was added to osteoblast cells, and the cells were maintained for 1 hour in a CO_2 incubator at 37°C. After the incubation, isopropanol/0.04 N HCl was added to MTT-treated cells, and the cells were agitated to promote elution of formazan. Then, the supernatants were quantified by measuring the absorbance at 550 nm in a SpectraMax M5^e microplate reader (Molecular Devices, Minneapolis, MN, USA) [6].

Determination of ALP activity

Osteoblasts cultured for each period were washed with PBS and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100. The assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM $MgCl_2$, and 10 mM p-nitrophenylphosphoric acid was added at a volume of 200 μl into 10 μl of cell suspensions, followed by a reaction for 30 min at 37°C and subsequent immediate determination of absorbance of p-nitrophenol at 405 nm in a SpectraMax M5^e microplate reader (Molecular Devices, Minneapolis, MN, USA). Simultaneously, the protein concentrations of cell suspension were determined by using the Bradford method, and the obtained alkaline phosphatase (ALP) activities were standardized by the protein concentration and the incubation time of the ALP assay [5].

Determination of Ca^{2+} concentration

Osteoblasts cultured for each period were washed with PBS and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100. HCl at 6 M was added to these cell lysates to make a final concentration of 2 M HCl for calcium release. Then, the cell suspensions were incubated for 16 to 24 hours at room temperature. Finally, the cell suspensions were centrifuged at 15,000 rpm for 5 min, and the Ca^{2+} content was determined in the supernatant by using a calcium E-TEST kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan) [5].

Determination of Ca^{2+} accumulation by Von Kossa staining

Osteoblasts cultured for each period were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were then washed twice with ddH_2O, followed by the addition of 5% silver nitrate (KANTO CHEMICAL CO., INC., Tokyo, Japan) to the cells and exposure to ultraviolet light for 1 hour. Next, the silver nitrate was removed, and the cells were washed twice with ddH_2O followed by addition of 5% sodium thiosulfate (Sigma-Aldrich) to the cells for 5 min to stop the reaction. Thereafter, the cells were washed with ddH_2O and 30% glycerol was then added to the cells. The stained cells were observed on an inverted microscope and photographed to capture the area of mineralization, which was indicated by black staining. Ten images per culture dish well were randomly acquired, and then the mineralized area was quantified by using Image J software [6].

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from osteoblasts cultured for each period by using the standard TriPure Isolation Reagent procedure (Roche Holding AG. Basel, Swaziland). After total RNA extraction, the RNA was subjected to synthesis of the first-strand cDNA by using a ReverTraAce qPCR RT kit (Toyobo CO., LTD., Osaka, Japan). The cDNA (50 ng/μl) were analyzed by RT-PCR using a FastStart Universal SYBER Green Master mix (Roche Holding AG, Basel, Swaziland) and an ABI PRISM 7300 system (Applied Biosystems). Individual cDNAs were amplified in a reaction mixture containing a cDNA aliquot, 2 × master mix of FastStart Universal SYBR Green Master (Rox) (Roche Holding AG) and the relevant sense and antisense primers. The amplification program included an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 59°C–60°C

for 1 min. The oligonucleotide primer sequences are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. Each gene was normalized to GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from the Ct value of the target gene (CT [target]). The relative expression of the target gene was calculated by using sodium dodecyl sulfate v1.2 with RQ software (Applied Biosystems), with ΔCt[target] compared with the ΔCt values of the reference; i.e., ΔΔCt = ΔCt[target] − ΔCt[reference]. The degree of difference (expressed in fold difference) between the target and reference was calculated as $2^{-\Delta\Delta Ct}$ [4].

Statistical analysis

Data analysis results were expressed as the mean ± S.E., and statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's test and/or Dunnett's test.

Results

Efficacy of ELE for osteoblast proliferation

Osteoblast differentiation and maturation were examined by using cell proliferation as determined by MTT reduction rates. Therefore, the MTT reduction was examined for each culture day (Figure 1). The osteoblasts of 7 DIV showed high MTT reduction rates, which indicated that cell proliferation was actively occurring. However, high MTT reduction rates were not observed after 14 DIV in the control group and 10 μg/ml ELE application group. The MTT reduction rates after 14 DIV and 28 DIV were significantly decreased relative to those after 7 DIV, which indicated that the proliferative function of osteoblasts was not as active in both groups (Figure 1). In addition, even if ELE at a concentration of 10 μg/ml was constantly applied, the application did not influence osteoblast proliferation on any of the culturing days (Figure 1).

Efficacy of ELE for ALP activity

It is known that osteoblasts promote ossification by secreting ALP when osteoblasts are hyperactive. Therefore, we examined the effect of ELE application on osteoblast differentiation by using ALP activity as an index. We found that ALP activity increased as the number of culture days progressed, and differentiation was promoted after cell proliferation settled. In the 10 μg/ml ELE application group, significantly increased ALP activity was observed after 14 DIV and 28 DIV relative to that after 7 DIV, which indicated that the ALP activities were significantly increased even when 14 DIV and 28 DIV. Similar tendencies were observed in the control group. Additionally, significant increases in ALP activity were observed between 7 DIV and 28 DIV and between 14 DIV and 28 DIV (Figure 2). As described above, cell proliferation was actively occurring after 7 DIV (Figure 1), and ALP activities were not different between the control group and the 10 μg/ml ELE application group (Figure 2). However, although active cell proliferation was not detected after 28 DIV (Figure 1), a significant increase in ALP activity was detected by 10 g/ml ELE application after 28 DIV (Figure 2).

Efficacy of ELE for Ca^{2+} contents

The changes in intracellular Ca^{2+} concentration required for osteoblasts to cause osteogenesis were investigated by using cultured osteoblasts of each culture day. As a result, in both the control group and 10 μg/ml ELE application group, significant increases in the concentration of Ca^{2+} in the cultured osteoblasts were observed with increasing number of culture days. In addition, after 14 DIV, 10 μg/ml ELE application significantly increased the intracellular Ca^{2+} concentration in the osteoblasts. After 28 DIV, the control group's intracellular Ca^{2+} concentration in the osteoblasts was the same as that in the ELE application group (Figure 3).

Efficacy of ELE for Ca^{2+} accumulation

Von Kossa staining was continuously performed to examine the efficacy of ELE application on Ca^{2+} accumulation. Von Kossa staining is a method that stains calcium phosphate black, which enables detection of the accumulated Ca^{2+}. Therefore, osteoblasts cultured for 14 DIV in which the intracellular calcium concentration had increased were used for Von Kossa staining. Consequently, 14-DIV osteoblasts did not show any significant change in Ca^{2+} accumulation caused by ELE application. However, the 28-DIV osteoblasts showed that significant Ca^{2+} accumulation was induced by 10 μg/ml ELE application (Figure 4).

Efficacy of ELE for osteoblast's differentiation markers

In turn, osteoblast gene samples from each culture day were examined by using various differentiation marker primers of osteoblasts on RT-qPCR. The various differentiation markers changed according to the number of culture days in the early, middle, and terminal stages of differentiation and the dynamics of known differentiation markers could be reproduced. However, the 10 μg/ml ELE application groups did not show any significant changes in the following differentiation marker's mRNA expression levels: *ALP, Runx 2, Colla 1, BMP-2, osteocalcin, osteopontin, Osterix*. On the other hand, the mRNA expression levels of the *RANKL* and *Col1a2* differentiation markers were significantly increased by 10 μg/ml ELE application after 7 DIV (Figure 5).

Discussion

The effect of *Eucommia ulmoides* on bone metabolism *in vivo* have been investigated in ovariectomized rats [3] and granulomas of aged

Table 1. Primers sequence

Gene Name	Accession No	Forward primer (5'- 3')	Reverse primer (5' – 3')
ALP	J03572.1	gatggtatgggcgtctccac	atctccagccgtgtctcctc
RANKL	NM 057149.1	ggccaagatctctaacatga	ccatcagctgaagatagtcc
Runx2	NM 001278483.1	ttcgtcagcgtcctatcagttc	cttccatcagcgtcaacacc
Col1a1	NM 053304.1	tgcaacatggagacaggtcag	cttcttctccttggggtttgg
Col1a2	NM 053356.1	cattctgcagggctccaac	gcaggcgagatggcttattc
BMP-2	NM 017178.1	catgtgaggattagcaggtctttg	gcttccgctgtttgtgtttg
Osteocalcin	NM 013414.1	ctgcattctgcctctctgacc	ccttactgccctcctgcttg
Osteopontin	M 99252.1	ggtgaaagtggctgagtttgg	ctgcttctgagatgggtcagg
Osterix	AY 177399.1	atggcgtcctctctgcttg	gcttctttgtgcctcctttttc

ALP; alkaline phosphatase, RANKL; receptor activator of NF-kappaB ligand, Runx2; Runt-related transcription factor 2, Col1a1; Collagen, type I, alpha 1, Col1a2; Collagen, type I, alpha 2, BMP-2; bone morphogenetic protein 2,;

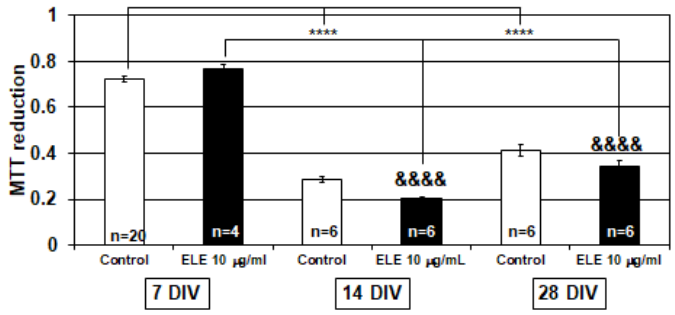

Figure 1. Efficacy of ELE for osteoblast proliferation

The graph shows the MTT reduction level in the control group and ELE 10 μg/ml application group in several culture days' primary osteoblasts. In 7, 14, and 28 days in vitro (DIV), MTT reduction's changes were not observed by ELE 10 μg/ml application. And in 14 and 28 DIV, the cells were significantly decreased the MTT reduction compared with 7 DIV cells. Each value represent the mean ±SEM, $^{****}p<0.0001$, $^{\&\&\&\&}p<0.0001$. (Dunnett)

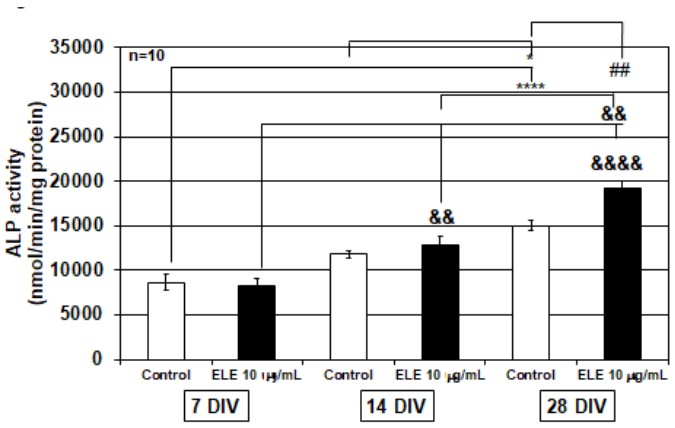

Figure 2. Efficacy of ELE for ALP activity

The graph shows the alkaline phosphatase (ALP) activity in the control group and ELE 10 μg/ml application group in several culture days' primary osteoblasts. In the control group, the ALP activity was significantly elevated from 7 DIV to 28 DIV and from 14 DIV to 28 DIV, whereas the ALP activity significantly increased in the ELE 10 μg/ml application group in association with the increase in the number of culture days. In addition, it was observed that the ALP activity was significantly increased by ELE 10 μg/ml application as compared with the control group on 28 DIV. Each value represent the mean ±SEM, $^{*}p<0.05$, $^{****}p<0.0001$, $^{\&\&}p<0.01$, $^{\&\&\&\&}p<0.0001$, $^{\#\#}p<0.01$. (Turkey)

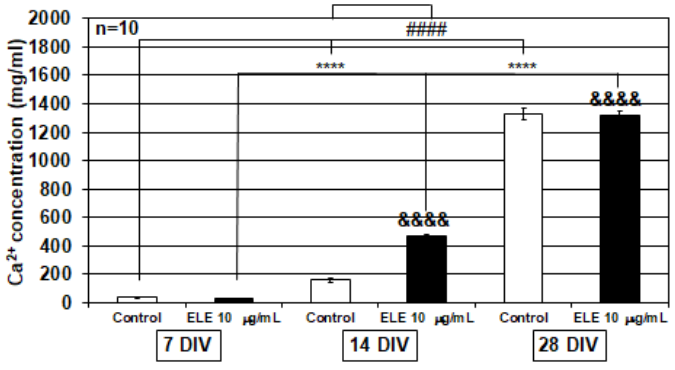

Figure 3. Efficacy of ELE for Ca^{2+} contents

In both the control group and the ELE 10 μg/ml application group, a significant increase in the Ca^{2+} concentration of the cultured osteoblasts was observed with the increase in the number of culture days. In addition, on the 14 DIV, the Ca^{2+} concentration of cultured osteoblasts was significantly increased by ELE application compared with the control group. Each value represent the mean ±SEM, $^{****}p<0.0001$, $^{\&\&\&\&}p<0.0001$, $^{\#\#\#\#}p<0.0001$. (Tukey)

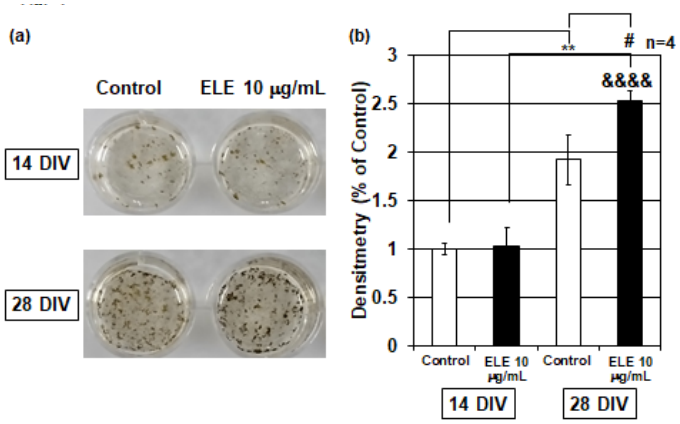

Figure 4. Efficacy of ELE for Ca^{2+} accumulation

(a) The photographs show the Von Kossa staining results in the control group and ELE 10 μg/ml application group. Black spots are silver phosphate precipitates, which are caused by reaction of silver ions with phosphate of calcium phosphate. (b) The graph shows the Ca^{2+} accumulation levels in the control group and ELE 10 μg/ml application group in several culture days' primary osteoblasts. No change in Ca^{2+} accumulation due to the ELE 10 μg/ml application was observed at 14 DIV compared to the control group. However, a significant increase in Ca^{2+} accumulation was observed by ELE 10 μg/ml application at 28 DIV. Each value represent the mean ±SEM, $^{**}p<0.01$, $^{\&\&\&\&}p<0.0001$, $^{\#}p<0.05$. (Turkey)

model rats [7]. On the other hand, the effect of *Eucommia ulmoides* on bone metabolism *in vitro* have been investigated in osteoblast-like cell lines [8] and co-cultured osteoblasts with osteoclasts [9]. In a report on the effect of *Eucommia ulmoides* leaf as a food on bone metabolism, the collagen content in granulomas of aged model rats was increased by administration of ELE [7] and the osteoporosis-like symptoms were prevented in ovariectomized rats by ELE administration [3]. In addition, it has been reported that ELE exerted an antioxidant effect by suppressing oxidative stress, and the effect increased with age and maintained the function of osteoblasts [8]. However, studies on the effect of ELE on bone-related cells are insufficient. According to the MTT assay results in this study, ELE did not affect osteoblast proliferation. This finding is in contrast to the finding in a previous study in which the lignan component contained in the *Eucommia ulmoides* bark promoted osteoblast proliferation [2]. This discrepancy might have been caused by differences between the components of the *Eucommia ulmoides* bark and *Eucommia ulmoides* leaves. Indeed, the main components of the *Eucommia ulmoides* leaves are not lignans [1,10]. On the other hand, the present study showed that ALP activity was significantly increased by 10 μg/ml ELE application in 28 DIV osteoblasts, and the accumulation of calcium phosphate was also significantly increased by 10 μg/ml ELE application. In addition, the increase in intracellular Ca^{2+} concentration caused by 10 μg/ml ELE application was already significantly increased in 14 DIV osteoblasts, and it is inferred that the preparation for calcium accumulation is significantly advanced. These results suggest that ELE has an accelerating effect on the osteogenesis of osteoblasts. The RT-qPCR results suggested that the differentiation of osteoblasts by ELE application might have an accelerating effect at an early stage of osteoblasts. Osteoblasts differentiate from mesenchymal stem cells and progressively differentiate into osteoprogenitor cells, pre-osteoblasts, osteoblasts, mature osteoblasts, and osteocytes [11]. In the present study, *Col1a2* was observed during osteoblast differentiation to be significantly induced by ELE application in the osteoprogenitor cell stage. *Col1a2* is a type I collagen known as a bone matrix [12]. In addition, promotion of osteoblast calcification was observed by ELE application. These findings suggest that the promoting effect of osteoblast calcification by ELE application is facilitated by expression of *Col1a2* in the osteoprogenitor cell stage and increased

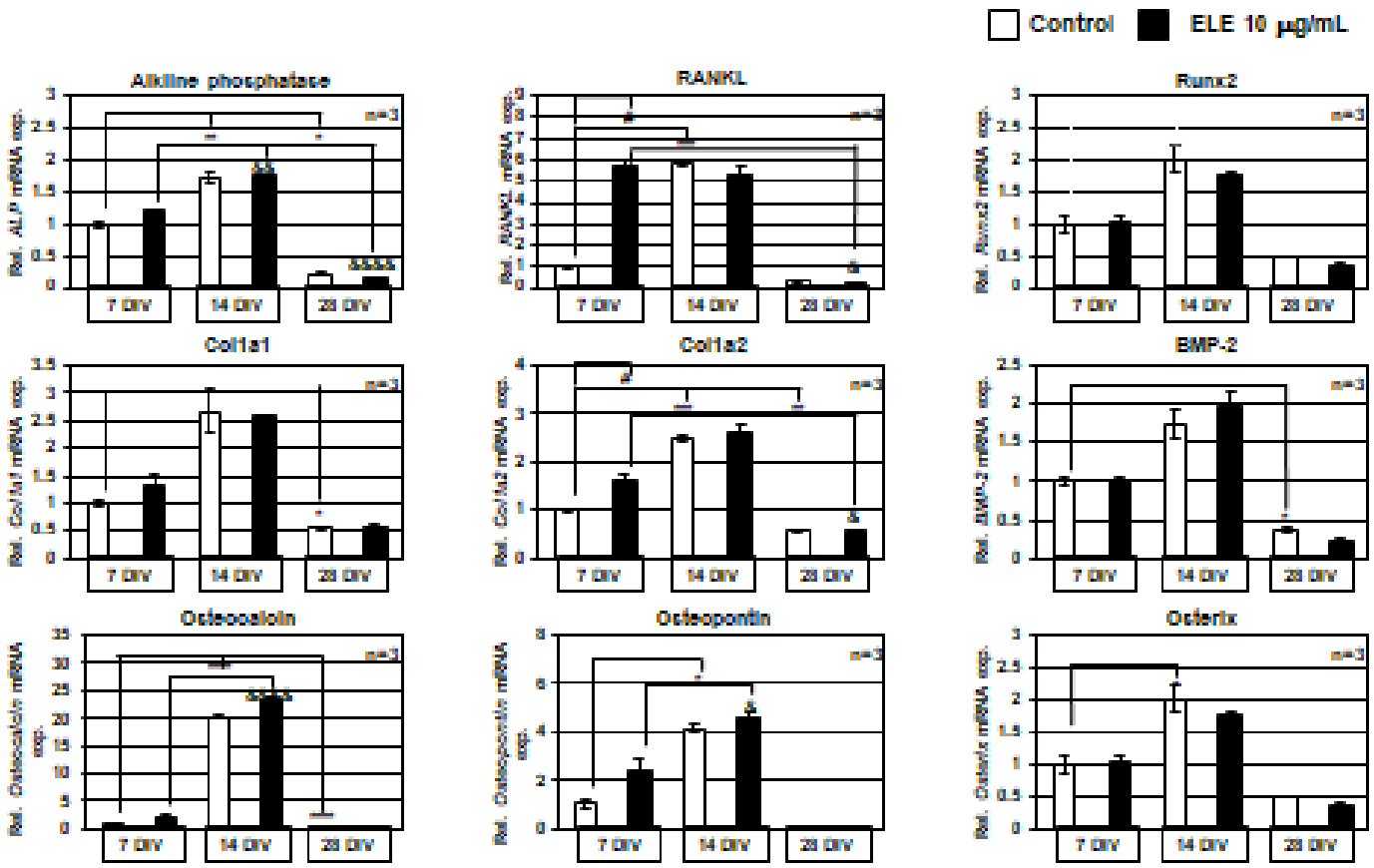

Figure 5. Efficacy of ELE for osteoblast's differentiation markers

The graphs show the RT-qPCR results of mRNA expression level changes of osteoblast differentiation marker by ELE 10 μg/ml application. The mRNA expression level changes of ALP, Runx 2, Col1a1, BMP-2, Osteocalcin, Osteopontin, and Osterix were not observed by ELE 10 μg/ml application at every culture days. However, in RANKL and Col1a2, the mRNA expression levels were significantly increased at 7 DIV. Each value represent the mean ±SEM, $^{**}p<0.01$, $^{\&\&\&\&}p<0.0001$, $^{\#}p<0.05$. (Tukey)

Ca^{2+} accumulation in the collagen matrix in the mature osteoblasts stage. This possibility is consistent with previous findings that ELE application increased collagen content [13,14]. On the other hand, a significant increase in *RANKL* mRNA, which activates osteoclasts in the early stage of osteoblast differentiation, was observed in the present study. This suggests the possibility that remodeling is promoted by renewing bone metabolism.

According to recent reports, *Eucommia ulmoides* bark and *Eucommia ulmoides* leaf have shown the following various effects on health: hepatoprotective [15,16], neuroprotective and hypnotic [17-22], anti-obesity and anti-metabolic syndrome [10,23-26], antibacterial [27], antiviral [28], anti-inflammatory [29,30], inhibitory action against diabetic complications [31-33], antioxidative [8,34-37], antihypertensive [38-41], anticancer [42,43] and bone metabolism effects [2,3,7-9,13,14,44-47].

Conclusion

These various findings clearly show that *Eucommia ulmoides* is a traditional herbal medicine that has systemic effects. In addition, *Eucommia ulmoides* leaves are treated as food, not pharmaceuticals, and can be purchased as a tea at neighborhood supermarkets at low prices. The results of the present study suggest that continued ingestion of ELE from an early age promotes ossification and may lower the risk of future bone metabolic disorders. From the standpoint of prophylactic pharmacology, ingestion of ELE is considered very useful for preventive self-medication.

Acknowledgments

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Conflict of interest

There are no conflicts of interest to declare.

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