Int J Clin Exp Pathol 2017;10(4):4514-4521
www.ijcep.com /ISSN:1936-2625/IJCEP0048305

Original Article

Eleutherococcus senticosus Inhibits RANKL-induced osteoclast formation by attenuating the NF-κB and MAPKs signaling pathway

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Received January 7, 2017; Accepted February 20, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Natural plant derived eleutherococcus senticosus (ES) has potential therapeutic and preventative effects on treating bone metabolism diseases such as: arthritis and osteoporosis. However, the mechanisms of ES effects on the osteoclastogenesis remains to be investigated. The current study is the first time to explored the effect of ES on RANKL mediated marrow derived macrophages (BMMs) and mice macrophage RAW 264.7 cells *in vitro*. Here, we found ES can significantly decrease RANKL-induced osteoclast differentiation and bone resorption compared with the control in dose dependent manner and showed no cytotoxicity. ES also downregulated the expression of osteoclastic genes such as TRAP and OSCAR. Moreover, ES markedly inhibited the phosphorylation of early signaling pathway such as NF-κB and MAPKs activity. Furthermore, ES extract significantly decreased the expression of osteoclast key transcriptor c-Fos/NFATc-1. Our results collectively demonstrating that as a natural compound, ES may serve as a useful drug in the prevention of bone resorption disease.

Keywords: Eleutherococcus senticosus, RANKL, osteoclast

Introduction

Bone homeostasis is maintained by a delicate balance between the activities of osteoblasts and those of osteoclasts [1]. Excessive osteoclast activity relative to osteoblast activity leads to bone diseases such as osteoporosis [2, 3]. Osteoclasts are unique bone cells that can control the amount of bone tissue by resorbing mineralized bone matrix [4]. Osteoclasts are characterized by tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (MNCs) originate from hematopoietic precursors. Osteoclast formation requires two essential cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) [5]. Binding of M-CSF to its transmembrane receptor c-Fms induces receptor activation of nuclear κB (RANK) expression on osteoclast precursor cells by activating the Ets transcription factor PU.1 and thereby mediates the proliferation and survival of osteoclasts [6, 7]. Moverover, binding of RANKL to its receptor RANK induces nuclear factor kappaB (NF-κB) pathway and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 [8, 9]. These signaling pathways ultimately lead to induction and activation of transcription factors including c-Fos, PU.1, and nuclear factor of activated T cells c1 (NFATc1), which have a critical role in osteoclastogenesis. Subsequently, activated NFATc1 induces the expression of target genes, including TRAP, cathepsin K, and osteoclast-associated receptor (OSCAR), which are important for osteoclast differentiation or function [10].

Eleutherococcus senticosus (ES) also known as Siberian ginseng or eleuthero, is a shrub that belongs to the family Araliaceae Davydov. Previous studies showed ES process diverse pharmacologic effects such as: anti-inflamma-

Table 1. Primer sequences used for real-time RT-PCR analysis

Gene Name	Primer Sequence (5'-3') Forward	Primer Sequence (5'-3') Reverse
c-Fos	5'-CTGGTGCAGCCCACTCTGGTC-3'	5'-CTTTCAGCAGATTGGCAATCTC-3'
NFATc1	5'-CTCGAAAGACAGCACTGGAGCAT-3'	5'-CGGCTGCCTTCCGTCTCATAG-3'
TRAP	5'-CTGGAGTGCACGATGCCAGCGACA-3'	5'-TCCGTGCTCGGCGATGGACCAGA-3'
OSCAR	5'-CTGCTGGTAACGGATCAGCTCCCCAGA-3'	5'-TCC AGG CAG TCT CTT CAG TTT-3'
Cathepsin K	5'-CAC TGC TCT CTT CAG GGCTT-3'	5'-ACG GAG GCA TTG ACT CTG AA-3'
GAPDH	5'-TCA AGA AGG TGG TGA AGC AG-3'	5'-AGT GGG AGT TGC TGT TGA AGT-3'

tory and neuro-protective [12]. Moreover, the most recently study demonstrated ES extract has the prevent effects on ovariectomized-induced rat osteoporosis model [13]. However, there is lack of knowledge on ES extract therapeutic influence on chronic inflammatory osteolysis. In this study, we aimed to investigate the effects of ES extract on signaling pathways involved in osteoclast differentiation, activation, and function *in vitro.*

Materials and methods

Reagents and antibodies

ES extract were provided by Dr. Park, ES extract prepared and identified as previously described [13]. Human RANKL and M-CSF was obtained from Peprotech (HuShang Biological Tec Co., China). The XTT assay kit was obtained from Roche (Indianapolis, IN, USA). Reagents and antibodies including: c-Fos, NFATc-1 and western blot antibodies for phosphor-p65, p-65, phosphor-ERK, ERK, phosphor-JNK, JNK, phosphor-p38, p38, and I-κB were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); β-actin antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Osteoclast differentiation

All animal care and experimental procedures were approved by Animal Care Committee of Hong-Hui Hospital, Xi'an Jiaotong University College of Medicine (Animal Ethics Approval #201501059) and conducted strictly followed by "the institutional guidelines for the care and use of laboratory animals at the Jiaotong University College of Medicine". Primary bone marrow macrophages (BMMs) were isolated from 7 week-old mice by flushing the tibiae and femora. Cells were co-cultured by α-MEM containing 10% inactivated FBS, 100 U/ml penicillin/streptomycin with recombinant human M-CSF (20 ng/ml) in 100 mm tissue culture dishes for 3 days. After 3 days incubation, floating cells were removed and adherent cells on dish bottoms were isolated by trypsin and classified as BMMs. Freshly isolated BMMs were cultured in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 4 days with or without ES extract. TRAP positive multinucleated cells have three or more nuclei were counted to determine osteoclast number.

Bone absorption assay

Approximately 200 BMMs were seeded onto bovine bone slices with three replicates. After culturing for 48 h at 37°C, cells were stimulated with 50 ng/mL RANKL and 20 ng/mL M-CSF with or without ES extract treatment (0, 0.3, 0.6, or 1.2 μM) until mature osteoclasts formed. Cells were removed by mechanical agitation and sonication. Resorption pits were visualized by Philips XL30 and the percentage of bone resorption area was quantified using Image J software (NIH, Bethesda, MD, USA).

F-actin ring immunofluorescence

F-actin ring formation, ES extract treated cells cultured on bovine bone discs were fixed in 4% paraformaldehyde after washing in phosphate-buffered saline (PBS). After that, cells were incubated with 2 units/ml rhodamine phalloidin, 25°C for 1 h. Cells mounted with ProLong Gold anti-fade mounting medium. Finally, Leica fluorescence microscope was used to observe the F-actin ring formation.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with QIAzol reagent (QIAGEN, Valencia, CA, U.S.A.) according to the manufacturer's instructions RNA (1 μg) was reverse transcribed using oligo dT primers (10 μg) and dNTPs (10 mM). The mixture was incu-

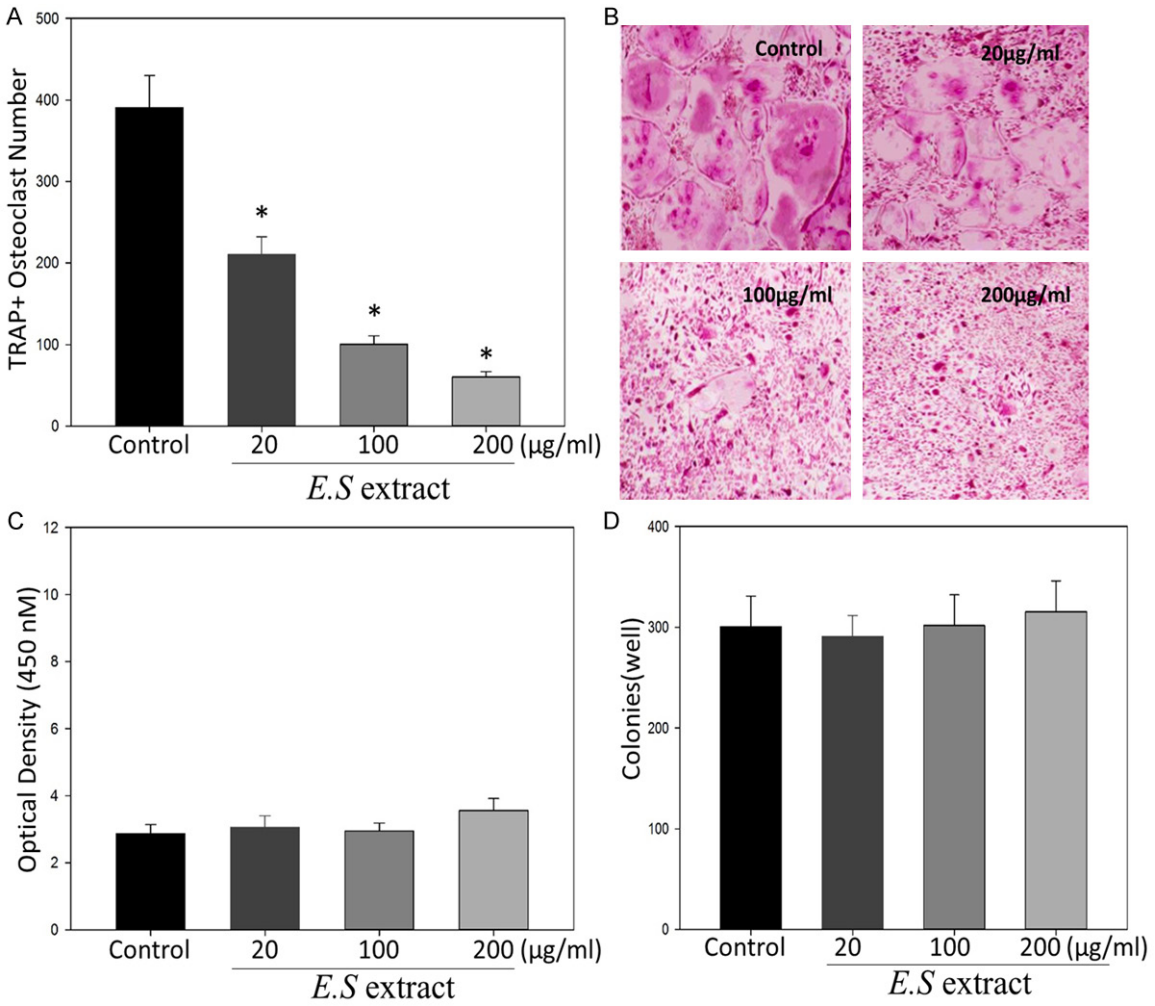

Figure 1. Effect of ES extract on RANKL-induced osteoclast differentiation. A. TRAP-positive cells were counted as osteoclasts. Asterisk indicates a statistically significant difference ($P<0.05$) between control and treated. B. BMMs were cultured for 4 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) in the presence of varying concentrations of ES extract. C. Cytotoxicity of ES extract on BMMs. D. RAW 264.7 cell colonies cultured with the indicated concentrations of ES extract for 4 days.

bated at 65°C for 5 min, and cDNA was produced by incubating at 42°C for 50 min with first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 100 mM DTT, RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). Primers employed for amplification were showed in **Table 1**. The mouse GAPDH gene was used as internal control. The amplification parameters consisted of an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The specificity of the SYBR green assays was confirmed by melting-point analysis. Expression data were calculated from the cycle threshold (Ct) value using the Ct.

Western blot analysis

BMMs or osteoclasts were lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. The lysates were centrifuged at 14,000×g for 20 min and supernatants were collected. Protein concentrations of supernatants were determined. Cellular proteins (30 μg) were resolved by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes (Milipore, Bedford, MA, USA). Non-specific interactions were blocked with 5% skim milk for 2 h and

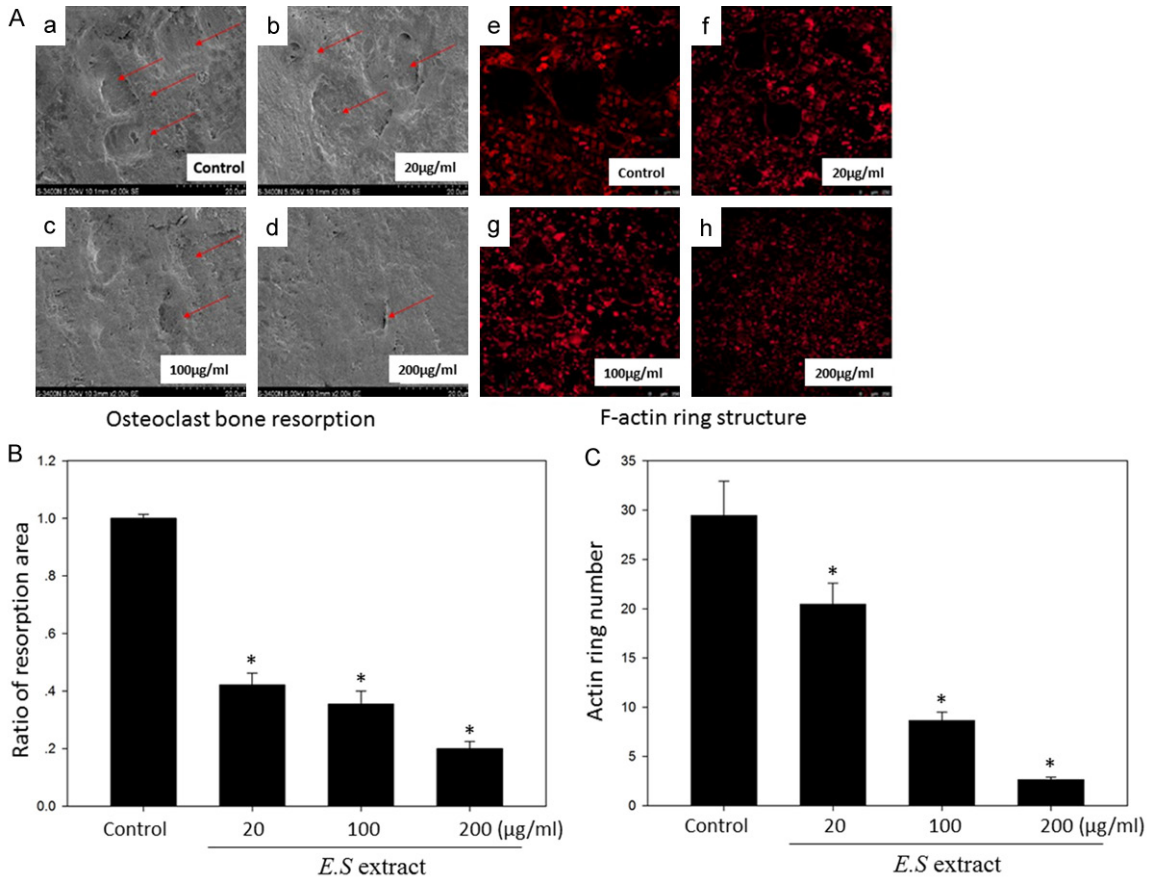

Figure 2. ES extract extract inhibited osteoclast bone resorption and F-actin ring structure. A. a-d: BMMs were stimulated with M-CSF (20 ng/mL) and RANKL (50 ng/mL) for three days. Later, cells were cultured in the presence of the indicated concentrations of ES extract with M-CSF (20 ng/mL) and RANKL (50 ng/mL) for another 48 h. e-h: Bone marrow-derived monocytes/macrophages (BMMs) were treated with various concentrations of HP followed by M-CSF (20 ng/mL) and RANKL (50 ng/mL) stimulation for five days. Cells were then fixed with 4% PFA and stained for the formation of F-actin ring. B. Resorption pit area. C. Number of actin ring. All experiments were performed at least three times, and the significance was determined as indicated in methods (*P < 0.05).

were then probed with the appropriate primary antibodies. Membranes were incubated with the appropriate secondary antibodies attached to horseradish peroxidase, and immunoreactivity was detected with enhanced chemiluminescence reagents. Densitometric values were quantified for each band with the Image Pro-plus program version 4.0.

Statistical analysis

All data are expressed as means ± standard deviation (SD). Statistical analysis was done using SPSS software package ver. 11.0 (SPSS, Chicago, IL); one-way ANOVA was used for comparison among the different groups. *Post hoc* testing of differences between groups was performed by using Duncan's test when the ANOVA was significant. All results were considered to be significant at the 5% critical level ($P < 0.05$).

Results

ES extract inhibits RANKL-Induced OCs differentiation

To verify the effects of ES extract in osteoclastogenesis, RAW 264.7 cells and BMMs were treated with various concentrations of ES extract in the presence of M-CSF (20 ng/ml) plus RANKL (50 ng/ml). Treatment with ES extract inhibited the formation of TRAP-positive multi nucleated osteoclasts from BMMs, which were subjected to RANKL-induced osteoclastogenesis, in a dose-dependent manner (IC50 = 0.6 μM) (**Figure 1A**, **1B**). Moreover, in order to demonstrate the downregulation effects of ES extract on bone marrow cells was not due to cytotoxicity, we performed the XTT assay. As the results shown in **Figure 1C**, ES extract demonstrated no cytotoxic effects at the same

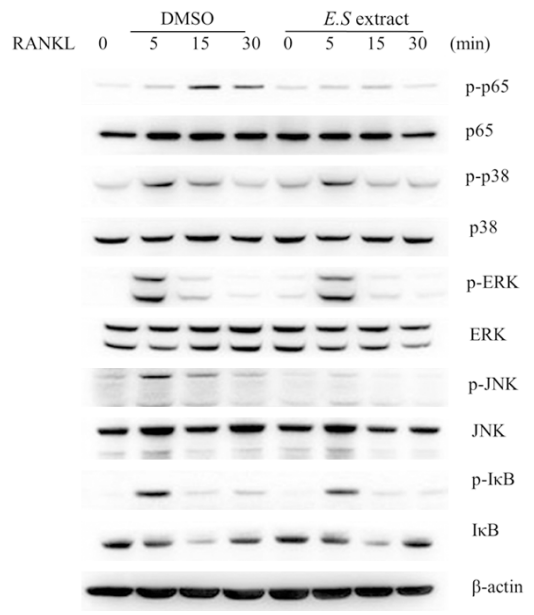

Figure 3. Effect of ESextract on RANKL-induced MAPKs and NF-κB activation. ES extract inhibits the p38, ERK, JNK, p65 phosphorylation and I-κB degradation in RANKL-stimulated BMMs. BMMs were pretreated with or without ES extract (100 ug/ml) for 1 h prior to RANKL stimulation (100 ng/ml) at indicated time periods.

doses which effectively downregulated OCs formation. Furthermore, compared with control group, ES extract did not shown the inhibited effects on RAW 264.7 cells colony formation (**Figure 1D**), indicating that e inhibitory effect of ES extract on OCs differentiation was not due to cellular toxicity or cell proliferation.

ES extract decreases osteoclastic bone resorption in vitro

To investigate the effects of ES extract on osteoclastic bone resorption, BMMs were cultured onto bovine bone slices, after attachment, cells culturing without or with various concentrations of ES extract (20 ug/ml; 100 ug/ml and 200 ug/ml). Post 48 h incubation, bone slices were retrieved for bone resorption pits electron microscopy scanning. As results shown, large bone resorption pits were observed on the surface of bone slices in the control group. However, the resorption area was markedly decreased after treatment with ES extract, respectively ($P < 0.05$) (**Figure 2Aa-d**).

Moreover, F-actin ring formation is a key indicator of osteoclast formation. Consist with resorption pits electron microscopy scanning results, after treatment with ES extract, both number and morphology of F-actin ring were down regulated (**Figure 2Ae-h**). Collectively, those results suggest ES extract decreases osteoclastic bone resorption in a dose dependent manner in vitro.

RANKL induced c-Fos, NFATc1 expression is reduced by ES extract

The c-Fos and NFATc-1 genes are essential transcription factors in RANKL-mediated OCs formation [14, 15]. Therefore, we examined the effects of ES extract on the expression of c-Fos and NFATc-1 both at the mRNA and protein level. Cells were pretreated with ES extract and further stimulated with RANKL at various time points. In agreement with previous studies, our results showed that c-Fos and NFATc-1 expression was markedly increased in BMMs by RANKL stimulation. However, c-Fos and NFATc-1 mRNA expression was significantly suppressed by ES extract (**Figure 3A**). Moreover, ES extract downregulated c-Fos and NFATc-1 protein expression (**Figure 3B**). Our results suggest that the inhibitory effect of ES extract on osteoclastogenesis is mediated, at least in part, by suppressing c-Fos and NFATc-1 in BMMs.

ES extract inhibits a variety of signals transduced by RANKL

To examine upstream signaling events involved in the inhibitory effect of ES extract on osteoclastogenesis, we examined the effect of ES extract on variety of signal transducers such as: mitogen-activated protein (MAP) kinase (p38, JNK, ERK), transcription factor NF-κB and I-κB signaling pathway, which has been known to regulate the c-Fos and NFATc1 expression. Ostoclast precursors were treated with ES extract at various time points in the presence of RANKL and M-CSF. Different early signaling pathways were detected. The results demonstrated the activation of ERK, JNK, p38, NF-κB p65 and degradation of I-κB by RANKL were all significantly inhibited by ES extract (**Figure 4**).

RANKL induced TRAP, and OSCAR mRNA expression is reduced by ES extract

Osteoclasts formation and function are regulated by the production of various osteoclastic

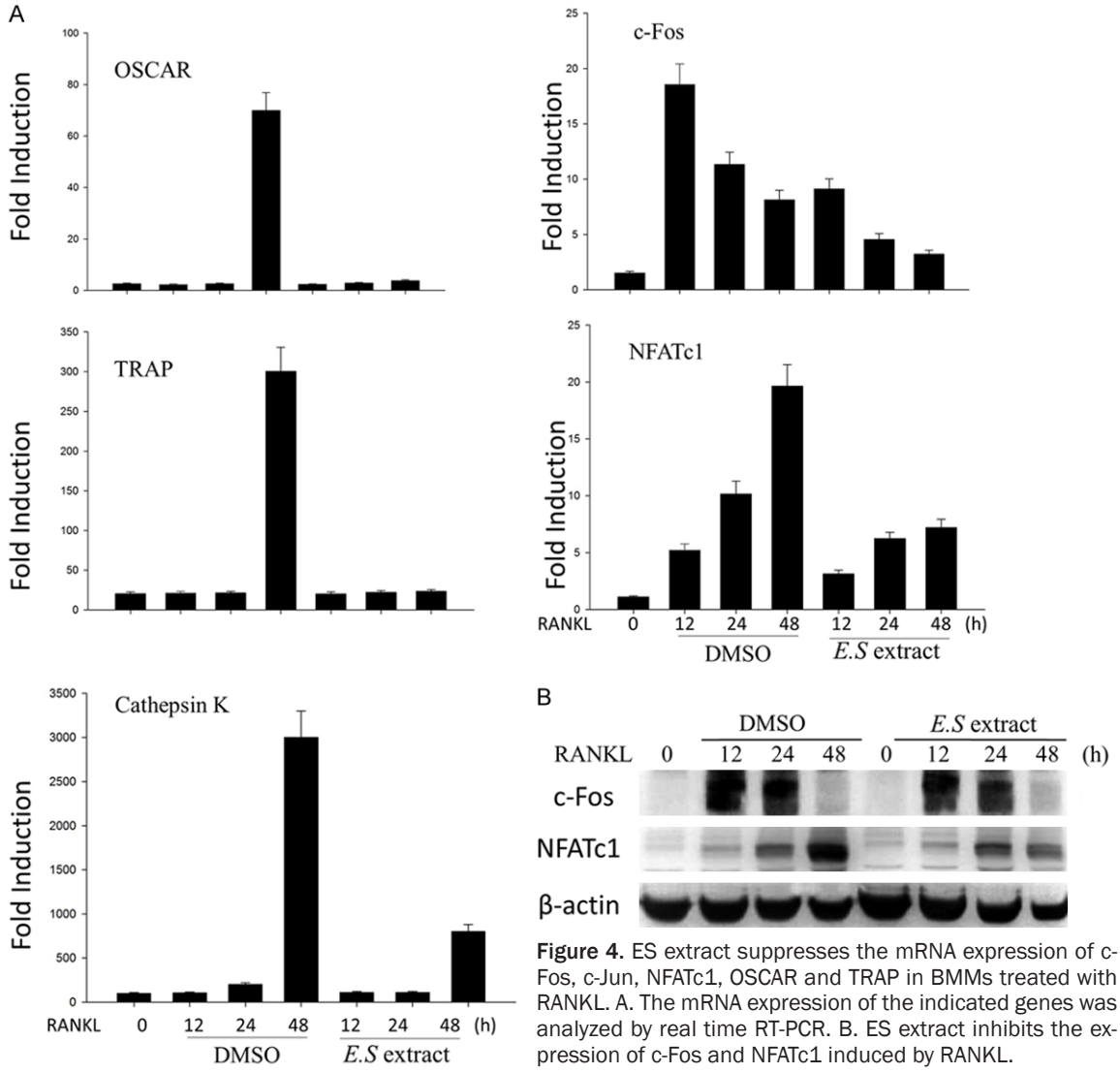

Figure 4. ES extract suppresses the mRNA expression of c-Fos, c-Jun, NFATc1, OSCAR and TRAP in BMMs treated with RANKL. A. The mRNA expression of the indicated genes was analyzed by real time RT-PCR. B. ES extract inhibits the expression of c-Fos and NFATc1 induced by RANKL.

markers mediated by RANKL and RANK binding [16]. ES extract showed the down-regulation effects on the RANKL-stimulated c-Fos and NFATc-1 expression, to further confirm the down regulation effects of ES extract on TRAP and OSCAR expression, the osteoclat gene expression profile was investigated by real-time RT-PCR analysis. Results revealed that osteoclast specific genes TRAP and OSCAR mRNA expression was markedly suppressed by ES extract (**Figure 3A**). Moreover, expression of cathepsin K, which is related to bone-resorbing activity, was also dramatically dowregulated byES extract.

Discussion

Excessive bone resorption plays a critical role in pathologic chronic inflammatory osteolysis [17]. Thus, down regulation osteoclast differentiation could be a promising choice in the treatment for osteolysis. ES extract, a traditional medicine with a long therapeutic history, has been used clinically for the treatment of several diseases including gastric ulcers, ischemic heart disease, hypertension, rheumatism, and allergy. Recently, Kim et al. [9] reported that a component of ES extract: suppresses the LPS-induced induced adhesion of monocytes to endothelial cells. The results demonstrated that ES extract exerts anti-inflammatory activity via the suppression of LFA-1 and Mac-1, lending itself as a potential therapeutic galenical for the prevention and treatment of various inflammatory diseases. Moreover, the most recently study demonstrated ES extract has the prevent effects on ovariectomized-induced rat osteoporosis model [13]. However, the effect of ES

extract on RANKL-induced osteoclast formation, especially RANKL-mediated intracellular signal pathway still is an interesting question that remains to be investigated. Our results showed that ES extract suppressing RANKL-induced osteoclastogenesis without significant cytotoxicity.

After RANKL binds to RANK receptor subsequently causes trimerization of TRAF6 cascades down signalling pathway of MAPKs and NF-κB. MAPK is primarily composed of ERK, JNK and p38 in mammalian cells. RANKL and RANK receptor binding expressed in osteoclast precursors provides a link between distinct signaling molecules such as ERK, JNK and p38 MAPK [5]. Previous research has reported ERK activated c-Fos for osteoclastogenesis, and the suppression of ERK has been shown to down-regulation osteoclast differentiation. Meanwhile, dominant-negative JNK decrease RANKL-mediated osteoclastogenesis [19], while p38 signaling is particularly important in the early stages of osteoclast generation as it promotes the activity of microphthalmia-associated transcription factor (MITF) and TRAP expression. Ourpresence study investigated the effects of ES extract on MAPKs signaling pathway. Our results showed ES extract significantly inhibited the phosphorylation of ERK, JNK and p38 signaling. These results collectively indicated that phosphorylation of MAPKs may contribute to the anti-osteoclastogenic effect of ES extract.

Additionally, NF-κB signaling is a critical signal pathway for inflammatory and immune reactions. NF-κB plays a key transcription factor for RANKL-activated osteoclastogenesis [19]. Classical NF-κB signalling pathway involves I-κB kinase (IKK) complex activation. Previous study has reported, I-κB is attached to NF-κB preventing it from migrating into the nucleus, subsequently phosphorylate IKK into two proteins, further leading I-κB ubiquitination and degradation. Finally, the degradation of I-κB allows the transfer of NF-κB into the nucleus and transcription of the target gene [20]. Although previous study showed ES extract decreases NF-κB luciferase activity in a reporter assay in THP-1 cells, we also confirmed that ES extract inhibits NF-κB/I-κB protein expression in BMMs. These results are manifested the suppression of the NF-κB transactivation is one of the mechnisims involved in the therapeutic effects of ES extract in treat inflammatory osteoclastogenic diseases (**Figure 2**).

Under normal conditions, the RANKL-RANK axis appears to be essential for osteoclastogenesis, and costimulatory immunoreceptors lead to robust induction of c-Fos and NFATc1, which are the necessary and sufficient transcription factors for osteoclast differentiation. NFATc1 is well known as the master regulator of osteoclastogenesis both in vitro and in vivo. NFATc1 autoamplifies and up regulates the expression of series osteoclast specific genes including: TRAP, calcitonin receptor, OSCAR, and cathepsin K [21, 22]. c-Fos as a main component of transcription factor AP-1, induces NFATc1 expression. Moreover, NFATc1 rescued RANKL-induced osteoclastogenesis in osteoclast precursor cells lacking c-Fos, suggesting the induction of NFATc1 may be cooperatively up-regulated by c-Fos, which make up each other for the induction [23, 24]. Our study demonstrates the reduced c-Fos expression in ES extract treated BMM cells (**Figure 3**), which suggested cause by the impaired activation of NFATc1 responsible for the inhibition of RANKL-induced osteoclast formation. Furthermore, mRNA levels of major osteoclast marker TRAP and OSCAR was also inhibited by ES extract (**Figure 3**). These data suggest that the c-Fos/NFATc1 transcription factor is the targets of ES extract t mediated inhibition of osteoclastogenesis.

In conclusion, we demonstrated the inhibitory effects of ES extract on osteoclastogenesis in primary precursor cells in the present study.

Disclosure of conflict of interest

None.

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