Sclareol prevents ovariectomy-induced bone loss in vivo and inhibits osteoclastogenesis in vitro via suppressing NF- κ B and MAPK/ERK signaling pathways

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Abstract

BACKGROUND/AIMS:

Postmenopausal osteoporosis (PMO) is a progressive disease occurring in elderly postmenopausal women that is characterized by low bone mass and impaired bone quality. Suppressing osteoclastogenesis is considered an effective therapeutic approach for PMO. Sclareol is a natural product (initially isolated from the *Salvia sclarea*) that possesses immune-regulation and anti-inflammatory effects, but its role in osteoclastic formation and function as well as the PMO remains unknown. In the current study, we aimed to elucidate whether sclareol affects osteoclastogenesis and the progression of PMO.

METHODS:

BMMs, that act as osteoclast precursors, were cultured with M-CSF (50 ng/ml) and RANKL (50 ng/ml) for 5 days to generate osteoclasts. Osteoclast differentiation under different treatments was measured by TRAP staining. Osteoclast bone resorption was detected on the hydroxyapatite-coated plate. The expression of osteoclast-related genes were analyzed by RT-PCR and western blotting. The changes of downstream signalling pathways induced by RANKL were confirmed through western blotting and intracellular Ca^{2+} measurement. Furthermore, an ovariectomy (OVX)-induced bone loss mouse model was established to test the ability of sclareol to prevent bone loss.

RESULTS:

Sclareol not only inhibited osteoclast formation but also suppressed osteoclast function. The expression of RANKL-induced osteoclast marker gene and protein was also reduced by sclareol treatment. In addition, we found that attenuating the activation of the NF- κ B and MAPK/ERK pathways contributed to the inhibitory effect of sclareol against osteoclastogenesis. Moreover, the *in vivo* experiment showed that sclareol exerted a protective effect against bone loss in OVX mouse.

CONCLUSION:

Sclareol suppressed RANKL-induced osteoclast formation and function in vitro by targeting the NF- κ B and MAPK/ERK signalling pathways, and prevent the bone loss in OVX mouse. These findings suggest that sclareol has potential value as a therapeutic agent for PMO.

Keywords: Sclareol, Osteoclast, NF- κ B, MAPK/ERK, Ovariectomy.

Introduction

Bone shaping is the continuous process of coordinated and balanced resorption and formation activities of osteoclasts and osteoblasts[1]. Disorder of the balance between bone formation and bone resorption may lead to metabolic bone diseases, such as postmenopausal osteoporosis (PMO). PMO is a common disease in elderly women and is defined as a skeletal disorder characterized by low bone mass and impaired bone quality, which predisposes a person to an increased risk of fracture[2]. During the progression of PMO, osteoclasts are not kept under control by oestrogen and therefore become overactive, which leads to the temporary uncoupling of bone resorption and formation, resulting in an overall net bone loss[3]. Therefore, agents that inhibit the formation and function of osteoclasts and thus can be a promising therapeutic target for PMO are sought.

Osteoclasts are multinucleate giant cells derived from haematopoietic precursor cells of monocyte/macrophage lineage. The formation and differentiation of osteoclasts are regulated by osteoblasts through the synthesis of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)- κ B ligand (RANKL)[4]. M-CSF promotes the proliferation and survival of osteoclast precursor cells and upregulates the expression of RANK[5]. RANKL binds to its receptor RANK, and sequentially recruits TNF receptor-associated factor 6 (TRAF6) in the osteoclast precursor cells. TRAF6 activates downstream signalling pathways, including NF- κ B and mitogen-activated protein kinases (MAPKs)[6, 7]. Ultimately, the activation of NF- κ B and MAPKs contribute to the expression of transcription factors such as c-Fos and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which are crucial for the differentiation of osteoclast precursors[8, 9]. Thus, blocking these crucial signalling pathways induced by RANKL is a potential therapeutic target for treating PMO.

Sclareol, a phytochemical labdane diterpene isolated from *Salvia sclarea*, has been reported to possess a series of functions, such as immune-regulatory[10],

anti-inflammatory[11] and anticancer[12] effects. However, the influence of sclareol on osteoclastogenesis still remains unknown. Recently, sclareol was found to ameliorate lipopolysaccharide-induced pulmonary inflammation through the inhibition of NF- κ B and MAPK[13]. As mentioned above, NF- κ B and MAPK play an indispensable role in the formation and differentiation of osteoclasts. Therefore, in our study, we investigated the inhibitory effect of sclareol on RANKL-induced osteoclastic formation and function as well as the underlying mechanism. Furthermore, an ovariectomy (OVX)-induced bone loss mouse model was established to mimic PMO, and the protective role of sclareol against bone loss from PMO was examined.

Materials and methods

Ethics Statement

The study was approved by the Animal Care and Use Committee at the Wenzhou Medical College. All surgical interventions, treatments, and post-operative animal care procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Reagents

Sclareol (purity>98%) was purchased from Nantong Feiyu Biological Technology Co, Ltd. (Nantong, China). Sclareol was dissolved in DMSO as a 100 mM stock solution and stored at -20 °C. Further dilution was performed in cell culture medium. The primary antibody of NFATc1, c-Fos, Integrin- β 3, CTSK, V-ATPase d2 and β -actin were obtained from Santa Cruz Biotechnology (San Jose, CA). I κ B- α , ERK/p-ERK and P38/p-P38 antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). The MTS assay kit and luciferase assay system were purchased from Promega (Madison, WI, USA). A Leukocyte acid phosphatase staining kit was purchased from Sigma-Aldrich (Sydney, Australia). Recombinant macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN). Recombinant GST-rRANKL protein was synthesized and purified by Jiake Xu as

previously describe[14]. The cell culture medium, alpha modified minimal essential medium (α -MEM) and fetal bovine serum (FBS), were purchased from Thermo Fisher Scientific (Scoresby, Vic, Australia).

Cell Culture

Bone marrow macrophages (BMMs) was flushed from tibiae and femurs, which were isolated from 6-week-old C57/BL6 mice. Then BMMs were cultured for 3 days in α -MEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotic mixture of penicillin, streptomycin and 50 ng/mL M-CSF, and incubated at 37°C and humidified at 5% CO₂. Culture medium was changed every 2 days, and cells were passaged when 90–100% confluence had been attained.

TRAP staining

BMMs were seeded into 96-well plates at a density of 5×10^3 cells per well and allowed to adhere overnight. Following, RANKL (50 ng/ml) were used for osteoclast induction, which was treated in the presence or absence of different concentrations of sclareol. The medium was replaced every two days. After 5 days, cells were fixed with 4% glutaraldehyde and TRAP staining assay was then performed using a leukocyte acid phosphatase kit (Sigma) according to the manufacturer's instructions. The number of TRAP-positive multinucleated cells (nuclei > 3) were counted under the microscope (Nikon Corporation, Tokyo, Japan).

Cytotoxicity assays

The passage 2 BMMs were planted in 96-well plates (5×10^3 cells/well) and incubated in α -MEM with 10% FBS and 50 ng/mL M-CSF at 37°C for 24 h. Then, the cells were treated without or with different concentration (0, 1, 2.5, 5 and 10 μ m) of sclareol for 48 hours. After 48 hours, MTS solution (20 μ L/well) was added and incubated with cells for 2 h. The absorbance at 490 nm was read with a microplate reader (ThermoFisher, Waltham, MA, USA)

Resorption pit assay

To measure the influence of sclareol on osteoclasts function, BMMs were seeded into 6-well plate with 50 ng/mL RANKL and 50 ng/mL M-CSF until mature osteoclasts were generated. Then mature osteoclasts were gently detached from the wells using cell dissociation solution (Sigma, St. Louis, MO, USA), and cultured into hydroxyapatite coated 96-well plates containing RANKL (50 ng/mL), M-CSF(50 ng/mL) and different concentrations of sclareol. After 48 hours, resorption areas were captured using a standard light microscopy and quantified using ImageJ software (NIH, Bethesda, MD, USA).

Fibrous Actin Ring Observation

BMMs were seeded into a 96-well plate at a density of 5×10^3 cells per well and incubated in α -MEM with 10% FBS, 50 ng/mL M-CSF, 50 ng/mL RANKL and the indicated concentration of sclareol for 5 days. The medium was replaced every 2 days. After the treatment period, cells were fixed with paraformaldehyde (4%) for 15 min at room temperature. After washing with FBS three times, cells were permeabilized with 0.25% Triton X-100 and blocked with 3% BSA in PBS. Next, the F-actin rings were stained with rhodamine conjugated phalloidin (Eugene, OR, USA), while the cell nuclei were stained with DAPI. Then images were collected using confocal laser scanning microscopy (Nikon, Tokyo, Japan)

RNA Isolation And Quantitative RT-PCR Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyze specific gene expression during osteoclastogenesis, such as TRAcP, MMP9, CTSK and V-ATPase d2. Briefly, BMMs were seeded at 8×10^4 /well in 6-well plates. The cells were cultured with 50 ng/ml M-CSF and 50 ng/ml RANKL, with or without 10 μ M sclareol for 0, 1, 3, or 5 days. Total RNA was isolated from cell cultures using Trizol reagent according to the manufacturer's instructions (ThermoFisher Scientific, Scoresby, Australia). The complementary DNA (cDNA) was synthesized using 1 μ g of RNA template. Then cDNA was amplified using a PCR Premix Kit (Invitrogen, Carlsbad, CA, USA). The cycling parameters using for RT-PCR reactions were 94 °C

for 40 s, 60 °C for 40 s, 72°C for 40 s, 72°C for 5 min, and were performed for 40 cycles. Relative levels of tested genes were normalized to that of HPRT. Primers for real-time PCR are listed in Table 1.

Luciferase Reporter Assays

The effects of sclareol on RANKL-induced NF- κ B and NFATc1 activation were measured using RAW264.7 cells. RAW264.7 cells were stably transfected with either an NF- κ B-responsive luciferase construct or an NFATc1-responsive luciferase reporter construct. Briefly, BMMs were seeded in 48-well plates at a density of 5×10^3 cells/well overnight. The next day, cells were pretreated with different concentrations of sclareol for 1 h. Following, pre-treatment cells were subsequently stimulated with RANKL (50 ng/mL) (6 h for NF- κ B and 24 h for NFATc1) in the presence of sclareol. Luciferase activity was measured using the luciferase reporter assay system according to the manufacturer's protocol (Promega, Sydney, Australia).

Western Blotting

Western blotting was performed using routine protocols. The protein was extracted from treated cells using RIPA buffer. Cellular proteins were resolved by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Silverwater, Australia). Then membrane was blocked with 5% skim milk for 2 h at room temperature and incubated overnight at 4 °C with the primary antibody. After washed in TBST for three times, the membrane was incubated with an secondary antibody. Protein bands were then visualized using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Sydney, Australia).

Intracellular Ca²⁺ Measurement

Intracellular Ca²⁺ measurement was performed to detect the activation of calcium signalling pathway induced by RANKL. Briefly, BMMs were seeded into 48-well plates with M-CSF (50 ng/mL) at a density of 1×10^4 cells per well for 24 h; next, the

cells were treated with or without sclareol (10 μ M) and RANKL (50 ng/mL) in the presence of M-CSF (50 ng/mL) for another 24 h. Then, the cells were washed twice with assay buffer (Hanks' balanced salt solution supplemented with 1 mM probenecid and 1% FBS) and stained with Fluo4 staining solution (Fluo4-AM dissolved in 20% Pluronic F127 (w/v) in DMSO diluted in assay buffer) in the dark at 37°C for 45 min. Next, the intracellular free calcium was detected by an inverted fluorescence microscope (Nikon Ti-U) at an excitation wavelength of 488 nm. To obtain the changes of Ca^{2+} oscillation, images of different group were captured every 2 sec for 3 min, and the results were recorded and analysed by Nikon Basic Research Software (cells with two or more oscillating peaks were included). The average amplitude of each oscillating cell under different treatments was analysed.

Animal Experiments

Seven-week-old female C57BL/6 mice were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China, and randomly divided into sham group (n=10), OVX group (n=10), and sclareol group (n=10). The mice were kept for at least 7 days before bilateral ovariectomy or sham operation. The bilateral OVX procedures were performed in OVX group and sclareol group using dorsal approach, and the rat in sham group were subjected to the same surgical procedure with the ovary exposed for 1 min, but did not undergo the ovariectomy. After the surgery, the animals were allowed to recover from surgery for one week prior to the experiments. Sclareol group received sclareol (10 mg/kg/day) dissolved in CMC by intragastric administration on a daily basis until the mice were sacrificed. Meanwhile, mice in sham group and OVX group were administered an equivalent volume of CMC. All mice were humanely euthanized after 8-weeks following treatment, and their femur bone samples were collected. The bone loss and microstructure of the distal femur were observed using micro-CT scanning.

Micro-CT Scanning

The micro-architecture on the trabecular bone of distal femur was determined using a

high-resolution micro-CT (Skyscan 1176; Bruker, Belgium). Briefly, the trabecular parameters were evaluated by micro-CT Skyscan CTAn software, including the bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). The three-dimensional (3D) bone structure images were reconstructed by the Mimics 18.0 software (Materialise, Leuven, Belgium).

Statistical Analysis

All experiments were performed independently for at least three times. All results were expressed as the mean $\pm$ standard error of the mean (SEM). Intergroup comparisons were made using one-way ANOVA, followed by the Tukey's test. Probability values of $P < 0.05$ were considered statistically significant.

Results

Sclareol suppressed RANKL-induced osteoclastogenesis in vitro

To investigate the inhibitory effect of sclareol on osteoclast formation, BMMs were treated with various concentrations (0, 1, 2.5, 5 and 10 μ M) of sclareol in the presence of M-CSF and RANKL. As shown in Figure 1A and Figure 1C, sclareol exerted a dose-dependent inhibition on RANKL-induced osteoclastogenesis compared to the RANKL-induced group, which is measured by counting TRACP positive multinucleated cells (≥ 3). Furthermore, in order to clarify at what stage sclareol play the most important role, the BMMs were treated with sclareol during different periods (1-3 days, 3-5 days, 5-6 days). We found the process of osteoclastogenesis was more suppressed while treated with sclareol during 1-3 days, and the inhibitory effect decreased during 3-5 and 5-6 days (Fig. 1B, C). These results indicated that sclareol dose-dependently inhibited RANKL-induced osteoclastogenesis and mainly acted at the early stage during osteoclastogenesis. Then, cell viability assays was performed to evaluate the toxicity of sclareol on osteoclastogenesis. As indicated in Figure 1D, sclareol has no obvious toxicity on osteoclastogenesis at the concentration up to 10 μ M. Therefore, the inhibitory effect of sclareol on osteoclastogenesis is not due to the

cytotoxic effect.

Sclareol inhibits RANKL-induced F-actin ring formation in osteoclasts

To further evaluate the inhibitory effect of sclareol on osteoclastogenesis, we examined how sclareol affected RANKL-induced osteoclast actin ring formation, which is a prerequisite for osteoclast bone resorption and is the most obvious characteristic of mature osteoclasts during osteoclastogenesis[15]. The F-actin rings were stained with rhodamine conjugated phalloidin, and the cell nuclei were stained with DAPI. The result indicated that BMMs treated with higher concentrations of sclareol grew into smaller osteoclasts with fewer nuclei (Fig. 2). The results showed that sclareol attenuated RANKL-induced F-actin ring formation in osteoclasts.

Sclareol attenuates RANKL-induced osteoclast bone resorption

To determine the effect of sclareol on osteoclast function, BMMs were cultured in α -MEM with RANKL and M-CSF until mature osteoclasts formed. Then cells were seeded into hydroxyapatite coated plates for bone resorption pit assay. A decrease in bone resorption was observed with an increase in sclareol concentration (Fig. 3A, C). After analysing the results, the area resorbed per OC also diminished in a dose-dependent manner (Fig. 3E). Thus, these data indicated that sclareol inhibited osteoclast bone resorption.

Sclareol down-regulates osteoclast relative gene expressions

Osteoclast differentiation is regulated directly by the expression of a series of related marker genes, such as CTSK, NFATc1, TRAcP and calcitonin receptor (CTR)[16, 17]. Then qRT-PCR was performed to clarify the expression of relative marker genes in osteoclasts which were treated with or without various concentrations of sclareol. As shown in Figure 4, the mRNA expressions of CTSK, TRAcP, NFATc1 and CTR were significantly down-regulated by sclareol in a dose-dependent manner.

Sclareol inhibits RANKL-induced NFATc1 activation and downstream protein expression in BMMs

It is well-known that NFATc1 is a key transcription factors during RANKL-induced osteoclastogenesis, which also is a master switch for regulating terminal differentiation of osteoclasts[17]. Luciferase reporter assay was performed to confirm the effect of sclareol on the expression of NFATc1. The results revealed that the NFATc1 luciferase activity was suppressed by the treatment of sclareol, especially at concentration of 10 μ M (Fig. 5A). In addition, we performed western blotting to measure the expression level of NFATc1 and NFATc1 downstream protein. As shown in Figure 5B, the up-regulation protein level of NFATc1 induced by RANKL was reduced by sclareol treatment during the osteoclast differentiation. Similarly, the expression of NFATc1 downstream protein including c-Fos, and V-ATPase-d2 was inhibited on day 3 and 5, while the expression of CTSK, and integrin β was inhibited on day 5.

Sclareol inhibits osteoclastogenesis by suppressing NF- κ B and MAPK/ERK pathways

The differentiation of osteoclast precursors into mature osteoclasts is associated with RANKL-stimulated NF- κ B activation, which play a crucial role in osteoclastogenesis. Western blotting and luciferase reporter assay were conducted to detect the effect of sclareol on NF- κ B signaling pathway. The results showed that NF- κ B luciferase activity was significantly suppressed by sclareol in a dose-dependent manner, especially at the concentration of 5 and 10 μ M (Fig. 6A). Furthermore, according to western blotting result, sclareol significantly attenuated RANKL-induced degradation of I κ B α , which is an inhibitory subunit of NF- κ B and serves as a key factor in NF- κ B signaling pathway (Fig. 6B). Besides NF- κ B, RANKL also stimulated MAPK signaling pathway during osteoclast differentiation. As shown in Figure 6C, consistent with previous reports, RANKL stimulated the phosphorylation of ERK and P38 within 20 min (Fig. 4). However, the treatment of sclareol obviously suppressed the phosphorylation of ERK while have no obvious inhibitory effect on the phosphorylation of P38. Furthermore, we performed intracellular Ca²⁺ measurement

to evaluate the effect of sclareol on calcium signalling pathway. The results indicated that sclareol failed to alter Ca^{2+} oscillation induced by RANKL (Fig. S1). Overall, sclareol inhibited osteoclastogenesis mainly by suppressing NF- κ B and MAPK/ERK pathways.

Sclareol prevents OVX-induced bone loss in vivo

To examine whether sclareol prevents OVX-induced bone loss, we used the OVX mouse model to mimic menopause-induced bone loss in women. A significant decline of bone volume and trabecular number with increased trabecular space was observed in the OVX group compared to the sham group by micro-CT scanning and 3D reconstruction (Fig. 7A). However, sclareol administration reversed all these alterations induced by OVX, with markedly increases in BV/TV and Tb.N, and a decrease in Tb.Sp (Fig. 7B-D). As with most researches, the value of Tb.Th kept unchanged in all groups (Fig. 7E).

Discussion

PMO, characterized by low bone mass and micro-architectural alterations, is a common disease in women after menopause that results in bone fragility and an increased risk of fractures. PMO is due to progressive and excessive bone resorption by osteoclasts, which is attributable to the dearth of oestrogen[3]. Thus, the search for drugs suppressing the formation and function of osteoclasts can be a valuable treatment for this pathological bone loss. Numerous pharmacological agents and therapies are clinically applied for the treatment of PMO, but with serious adverse effects[18-20]. For example, hormone replacement therapy (HRT) is a treatment for PMO that enhances the level of oestrogen and thus reduces bone loss. Nevertheless, long-term HRT has been found to increase the risk of heart attack, stroke, and breast and uterine cancer[21]. In recent years, TCMs have garnered increasing attention for their efficacy against various diseases with low side effects[22, 23]. Sclareol, mainly isolated and purified from the leaves and flowers of *Salvia Sclarea*, has been reported to exert many effects on anti-tumour, anti-inflammatory and immune-regulatory

activity[10-12]. However, sclareol's influence on osteoclastogenesis still remains unknown. In this study, for the first time, we demonstrated that sclareol inhibited RANKL-induced osteoclast formation and function and had a protective effect against bone loss in OVX mice.

Osteoclasts, which serve as the only bone-resorptive cells in the body, play a dispensable role in the balance between bone formation and bone resorption. The differentiation of osteoclasts mainly depends on the presence of two key factors, macrophage colony stimulation factor (M-CSF), and receptor activator of NF- κ B ligand (RANKL). M-CSF plays a crucial role in osteoclast precursor cell proliferation, whereas RANKL induces the differentiation of osteoclast precursor into osteoclasts[24]. In our study, we found that sclareol had little effect on M-CSF-induced osteoclast precursor cell proliferation by an MTS assay, while sclareol dose-dependently inhibited RANKL-induced osteoclastic formation and function.

The binding of RANKL to its receptor RANK leads to the recruitment of TRAF6 and the subsequent activation of a broad range of downstream signalling pathways, including NF- κ B, MAPKs and calcium[7, 25, 26]. It is well established that the activation of NF- κ B is involved in the phosphorylation of I κ B α and the p65 subunit as well as the subsequent degradation of I κ B α . Once liberated from I κ B α , the NF- κ B p65 subunit translocates from the cytoplasm to the nucleus to conduct transcriptional activity[27]. Our data revealed that sclareol not only inhibited the degradation of I κ B α protein but also suppressed the transcriptional activity of NF- κ B. In addition to the NF- κ B signalling pathway, activation of the MAPK also plays a crucial role in osteoclastogenesis[5, 28]. The extracellular-signal-regulated kinase (ERK) pathway is one of the major signalling MAPK signalling pathways, which plays an important role in the survival, apoptosis and differentiation of osteoclasts. The inhibition of ERK protein kinase reportedly attenuates RANKL-induced osteoclastogenesis[9, 29, 30]. In our study, we found that sclareol significantly suppressed the phosphorylation of ERK

but had no effect on P38. Calcium signalling pathway also was reported to play an important role in the osteoclastogenesis[26]. RANKL-induced Ca^{2+} oscillation could lead the expression of transcription factors in osteoclast differentiation. However, the results of intracellular Ca^{2+} Measurement indicated that sclareol had no significant effect on Ca^{2+} oscillation. Taken together, these data indicated that inhibiting or delaying the activation of the NF- κ B and MAPK/ERK pathways contributed to the inhibitory effect of sclareol against osteoclastogenesis (Fig. 8).

Activation of the NF- κ B and MAPK signalling pathways promotes the expression of several transcription factors such as c-Fos, activator protein 1 (AP1) and NFATc1[31-33]. NFATc1 is the most strongly induced transcription factor gene by RANKL stimulation and serves as a 'master regulator' to drive the expression of osteoclast-specific genes such as TRAP, CTR, Cathepsin K, and V-ATPase-d2, most of which also participate in the process of osteoclastic bone resorption[34]. In the present study, we found that the RANKL-induced upregulation of c-Fos, CTSK, V-ATPase-d2, NFATc1 and Integrin β 3 was inhibited by sclareol at the protein level. The mRNA levels of TRAcP, NFATc1, CTSK and the CTR were also reduced by sclareol in a dose-dependent manner.

In this study, an ovariectomy-induced osteoporosis mouse model was examined for further investigation of the therapeutic effect of sclareol in vivo. By using micro-CT scanning and 3D reconstruction, we found that the bone loss and micro-architecture change induced by OVX were markedly reversed by sclareol administration. Taken together, the in vivo and in vitro experiments suggested that sclareol possesses potential therapeutic value in preventing the progression of PMO.

In conclusion, for the first time, our findings demonstrate that sclareol suppressed RANKL-induced osteoclast formation and function in vitro by targeting the NF- κ B and MAPK/ERK signalling pathways. Moreover, the oral administration of sclareol was effective in suppressing bone loss in OVX mice. These results suggest that

sclareol could be a potential agent for the treatment of osteoclast-related diseases, such as PMO.

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Conflict of interest

The authors declare no conflict of interest.

Table. 1 Primer sequences used in qRT-PCR

Gene	Forward primer	Reverse primer
TRAcP	5'-TGTGGCCATCTTTATGCT -3'	5'-GTCATTTCTTTGGGGCTT -3'
NFATc1	5'-CAACGCCCTGACCACCGATAG-3'	5'-GGCTGCCTTCCGTCTCATAGT -3'
CTSK	5'-GGGAGAAAAACCTGAAGC-3'	5'-ATTCTGGGGACTCAGAGC-3'
Calcitonin receptor	5'-TGGTTGAGGTTGTGCCCA-3'	5'-CTCGTGGGTTTGCCTCATC-3'
HPRT	5'-GTTGGGCTTACCTCACTGCT-3'	5'-TAATCACGACGCTGGGACTG-3'

Figure Legends

Figure 1. Sclareol suppressed RANKL-induced osteoclastogenesis in vitro. (A) Representative TRAcP staining images of RANKL-induced osteoclast formation treated with different concentrations (1, 2.5, 5, and 10 μ M) of sclareol for 5 days as well as with 10 μ M sclareol on indicated days. Scale bar, 400 μ m. Scale bar in enlarged images, 100 μ m. (B) The schematic diagram of 10 μ M sclareol treatment time in A. (C) Quantification of TRAP⁺ multinucleated cells (nuclei>3) shown in A. (D) Cell viability under different concentrations of sclareol was evaluated by MTS assay. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group. #P<0.05, ##P<0.01 relative to the RANKL-induced group. n=3.

Figure 2. Sclareol inhibits RANKL-induced F-actin ring formation in osteoclasts. (A) Cells after the treatment were fixed and stained with rhodamine (red) for F-actin rings, and Hoechst (blue) for nuclei. Scale bar, 200 μ m. (B) Quantification of the osteoclasts (nuclei $\geq$ 3) in each field. (C) Quantification of the average nucleus number in each group. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group. #P<0.05, ##P<0.01 relative to the RANKL-induced group. n=3.

Figure 3. Sclareol attenuates RANKL-induced osteoclast bone resorption. (A,B) Representative images of resorbing pits and TRAcP stained osteoclasts on hydroxyapatite-coated plate. The white areas are where the osteoclasts have resorbed the hydroxyapatite-coated plate. Scale bar, 400 μ m. Scale bar in enlarged images, 100 μ m. (C,D) The osteoclasts number per well, resorbed areas in total of each well and

per cell were quantified using ImageJ software. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group. n=3.

Figure 4. Sclareol down-regulates osteoclast relative gene expressions. (A-D) Expression of mRNA for RANKL-induced osteoclast marker genes, TRAP, NFATc1, Ctsk, and calcitonin receptor, at day 5 of different groups were examined by qRT-PCR analyses. HPRT were used as loading controls. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group. #P<0.05, ##P<0.01 relative to the RANKL-induced group. n=3.

Figure 5. Sclareol inhibits RANKL-induced NFATc1 activation and downstream protein expression in BMMs. (A) The effect of sclareol on RANKL-induced transcriptional activity of NFATc1 were examined using a luciferase reporter gene assay. (B) Expression levels of the RANKL-induced osteoclast marker proteins, NFATc1, c-Fos, CTSK, V-ATPase d2, and Integrin- β 3, were examined by western blotting analyses on the indicated days. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group. #P<0.05, ##P<0.01 relative to the RANKL-induced group. n=3.

Figure 6. Sclareol inhibits osteoclastogenesis by suppressing NF- κ B and MAPK/ERK pathways. (A) The effect of sclareol on RANKL-induced transcriptional activity of NF- κ B were examined using a luciferase reporter gene assay. (B-D) Representative images of western blotting and quantitative analysis demonstrating the effect of sclareol on I κ B α degradation, phosphorylation of ERK1/2, and phosphorylation of P38 during RANKL-induced osteoclastogenesis time course. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the control group, #P<0.05, ##P<0.01 relative to the RANKL-induced group. &P <0.05,&&P<0.01 relative to the RANKL-induced group on the same indicated time. n=3.

Figure 7. Sclareol prevents OVX-induced bone loss in vivo. (A) Representative

reconstructed trabecular and cortical bone structure images of distal femoral bone in different group. (B-E) Quantitative analysis of distal femoral bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) in each group. Data are presented as mean $\pm$ SEM, *P<0.05, **P<0.01 relative to the sham group. #P<0.05, ##P<0.01 relative to the OVX group, n=6.

Figure 8. Schematic model of the signalling mechanisms underlying the inhibitory effect of sclareol on RANKL-induced osteoclastogenesis.

Figure S1. Sclareol has little effect on Ca^{2+} oscillation induced by RANKL. (A-C) Representative images of Ca^{2+} oscillation under the different treatments. (D) Quantitative analysis showing that sclareol had no significant effect on RANKL-induced Ca^{2+} oscillation.

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FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. S1