



Sigma Anti-bonding Calcium Attenuates Ovariectomy-Induced Bone Loss by Preserving Trabecular Microarchitecture and Suppressing Osteoclastogenesis

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Abstract

Estrogen deficiency-induced osteoporosis is characterized by increased bone resorption and progressive deterioration of trabecular microarchitecture, leading to skeletal fragility. This study investigated the effects of sigma anti-bonding calcium (SAC), a formulation designed to enhance ion release, in an ovariectomized (OVX) mouse model and in RANKL-induced osteoclastogenesis in vitro. Female mice underwent OVX and received oral SAC (100 or 200 mg/kg/day) for 13 weeks following surgery. Body and major organ weights were monitored during the experimental period. SAC administration did not significantly affect body or major organ weights under the conditions examined. Micro-computed tomography demonstrated that SAC attenuated OVX-induced trabecular deterioration, as evidenced by preservation of bone mineral density (BMD), partial restoration of trabecular number, and reduction of structural model index, whereas trabecular thickness and separation were minimally affected. Histological analysis further confirmed preservation of trabecular bone area in SAC-treated mice, whereas marrow adiposity assessed by Oil Red O staining was not markedly altered. Serum analysis revealed that SAC significantly reduced OVX-induced elevation of C-terminal telopeptide of type I collagen (CTX), while formation-associated markers did not show consistent recovery. In vitro, SAC suppressed RANKL-induced osteoclast differentiation in RAW264.7 cells without significantly affecting cell viability at the concentrations examined. SAC dose-dependently reduced the mRNA expression of osteoclast-related genes, including TRAP, NFATc1, Ctsk, and Atp6v0d2, and attenuated NFATc1 protein induction. SAC treatment was associated with reduced RANKL-stimulated p38 phosphorylation without changes in total p38 expression and with decreased MITF protein expression. Collectively, these findings indicate that SAC attenuates estrogen deficiency-associated trabecular bone deterioration and suppresses osteoclast differentiation, accompanied by changes in p38 phosphorylation and NFATc1 and MITF expression. These findings support further investigation of SAC as a potential adjunctive approach for attenuating estrogen deficiency-associated bone loss; however, comparative studies with conventional calcium formulations are required to determine whether the observed effects are specific to SAC.

Keywords Sigma anti-bonding calcium · Osteoporosis · Ovariectomy · Osteoclastogenesis · Trabecular microarchitecture · RANKL signaling

Abbreviations

Atp6v0d2 ATPase H⁺ transporting V0 subunit d2
BALP Bone-specific alkaline phosphatase
BMD Bone mineral density

BS/BV Bone surface to bone volume ratio
CaSR Calcium-sensing receptor
CTX C-terminal telopeptide of type I collagen
Ctsk Cathepsin K

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Extended author information available on the last page of the article

ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
micro-CT	Micro-computed tomography
MITF	Microphthalmia-associated transcription factor
NF- κ B	Nuclear factor kappa B
NFATc1	Nuclear factor of activated T cells c1
OVX	Ovariectomy/ovariectomized
PINP	Procollagen type I N-terminal propeptide
RANK	Receptor activator of nuclear factor- κ B
RANKL	Receptor activator of nuclear factor- κ B ligand
ROI	Region of interest
SAC	Sigma anti-bonding calcium
SMI	Structural model index
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TNF- α	Tumor necrosis factor-alpha

Introduction

Osteoporosis is a systemic skeletal disorder characterized by reduced bone mass and deterioration of bone microarchitecture, resulting in increased fracture susceptibility [1, 2]. With population aging and the associated clinical burden of fragility fractures, preservation of skeletal integrity remains an important therapeutic objective [3, 4]. Postmenopausal osteoporosis is primarily driven by estrogen deficiency, which disrupts the balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation [5]. Estrogen deficiency promotes osteoclast survival and inflammatory osteoclastogenic signaling, thereby accelerating skeletal remodeling and bone loss [6, 7].

Bone strength is determined not only by bone mineral density but also by microarchitecture, material properties, and structural connectivity [8–10]. Micro-computed tomography enables quantitative evaluation of trabecular parameters, including trabecular number, thickness, separation, and structural model index, in preclinical models [11, 12]. Ovariectomized rodent models reproduce key features of postmenopausal bone loss, particularly rapid deterioration of trabecular architecture, and are widely used to evaluate interventions targeting estrogen deficiency-associated osteoporosis [13, 14].

At the cellular level, osteoclast differentiation is orchestrated by receptor activator of nuclear factor- κ B ligand (RANKL) signaling [15]. Binding of RANKL to its receptor RANK on osteoclast precursors activates intracellular pathways, including nuclear factor- κ B (NF- κ B) and

mitogen-activated protein kinases (MAPKs) [16]. These cascades culminate in induction of nuclear factor of activated T cells c1 (NFATc1), the master transcription factor essential for osteoclastogenesis [17, 18]. NF- κ B components such as p50 and p65 are indispensable for precursor survival and differentiation [19], while c-Fos cooperates with NFATc1 to drive terminal osteoclast maturation [20]. Disruption of these signaling axes impairs osteoclast formation and function, underscoring their central role in bone resorption biology.

Calcium-dependent signaling has an established role in RANKL-induced osteoclast differentiation. Sustained intracellular Ca^{2+} oscillations activate calcineurin, which promotes NFATc1 dephosphorylation and nuclear translocation [21, 22]. Extracellular calcium may also influence osteoclast behavior through CaSR-mediated responses [23]. These mechanisms provide a biological basis for considering calcium availability in osteoclastogenesis; however, they do not establish that SAC alters intracellular Ca^{2+} dynamics, calcineurin activity, or CaSR signaling.

Calcium is indispensable for skeletal mineralization and systemic homeostasis [24]. Intestinal calcium absorption occurs through both passive diffusion and active vitamin D-dependent transport pathways [25, 26]. Aging reduces calcium absorption efficiency and alters vitamin D metabolism, contributing to negative calcium balance and increased fracture risk [27]. Experimental studies indicate that estrogen status may also influence calcium absorption [28]. Consequently, adequate dietary calcium intake remains foundational in osteoporosis prevention and management [29]. However, bioavailability varies depending on formulation, solubility, gastric acidity, and patient-specific factors [30, 31]. Epidemiological observations in elderly populations further suggest associations between dietary calcium intake and skeletal health outcomes, although confounding factors must be considered [32].

Beyond mineral supply, calcium functions as an intracellular signaling mediator in bone cells. Calcium/calmodulin pathways regulate osteoblast proliferation and differentiation [33], and certain calcium formulations have demonstrated osteogenic effects in vitro [34]. Emerging data also suggest that molecular regulators coordinate bone formation and resorption to maintain skeletal balance [35]. Therefore, calcium formulations that enhance bioavailability and modulate signaling pathways may have multifaceted effects on remodeling dynamics.

Sigma anti-bonding calcium (SAC) has been described as a modified calcium formulation designed to enhance ion release and aqueous solubility [36]. However, whether these physicochemical characteristics increase calcium absorption or systemic bioavailability has not been established in the present experimental context. Given the established

roles of calcium in skeletal mineralization and cellular signaling, SAC may be relevant to bone remodeling; however, any relationship between its proposed ion-release characteristics and biological effects remains hypothetical. The effects of SAC on bone microarchitecture and RANKL-induced osteoclast differentiation under estrogen-deficient conditions therefore warrant investigation.

Whether SAC differs from conventional calcium supplementation or affects calcium-dependent signaling remains unknown. Therefore, the present study was designed to assess the skeletal effects associated with SAC treatment *in vivo* and its association with RANKL-induced osteoclast differentiation *in vitro*.

Previous studies have reported skeletal effects of SAC in OVX rat models [36], but its effects on trabecular microarchitecture in OVX mice and on RANKL-induced osteoclast differentiation have not been fully characterized. The primary hypothesis of the present study was that SAC treatment would attenuate OVX-associated trabecular deterioration and reduce RANKL-induced osteoclast differentiation. Changes in p38 phosphorylation and NFATc1 and MITF expression were examined as signaling events associated with the cellular response. Altered calcium availability was considered a possible mechanistic explanation but was not directly tested. Accordingly, the study was designed to evaluate skeletal and osteoclastogenic responses to SAC rather than to establish a calcium-dependent mechanism of action.

Materials and Methods

Materials

The SAC solution used in this study was provided by the Calcium & Bone Health Institute (Coquitlam, British Columbia, Canada). No independent physicochemical characterization or measurement of free ionized calcium in the SAC solution was performed as part of the present study. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), Tris-HCl, glycerol, 1-bromo-3-chloropropane, 2-propanol, and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRI reagent[®] was obtained from Molecular Research Center Inc. (Cincinnati, OH, USA). RAW264.7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). RPMI 1640 medium, penicillin-streptomycin, and phosphate-buffered saline (PBS) were purchased from Welgene Inc. (Daegu, Korea). Fetal bovine serum (FBS), a cDNA synthesis kit, and a protein ladder were obtained from Thermo Fisher Scientific (Waltham, MA, USA). qPCRBIO SyGreen Blue Mix Lo-ROX was

purchased from PCR Biosystems Inc. (London, UK). Primers were synthesized by Bioneer Inc. (Daejeon, Korea). Antibodies for Western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA).

OVX-Induced Osteoporosis Mouse Model

Six-week-old female C57BL/6 mice were purchased from Orient Bio, Inc. (Gyeonggi-do, Korea). Mice were housed under a 12 h light/12 h dark cycle with free access to food and water. After a one-week acclimation period, mice were randomly assigned to experimental groups. Osteoporosis was induced by bilateral ovariectomy (OVX) in all groups except the sham-operated group, which underwent a placebo surgery. Mice were allowed a 3-week recovery period following surgery. The 3-week interval was selected to allow recovery from the surgical procedure and to permit the early estrogen deficiency-associated remodeling response to develop before treatment initiation, consistent with the delayed-treatment design used in a previous SAC study employing an OVX model [36]. Thus, the protocol was intended to evaluate SAC as an intervention initiated after the onset of OVX-associated skeletal changes rather than as an immediate prophylactic treatment. Because bone status was not assessed at the 3-week pretreatment time point, the extent of bone loss present at treatment initiation was not directly determined. SAC (100 or 200 mg/kg/day) was then administered orally in 150 μ L of distilled water for 13 weeks (total experimental period: 16 weeks). The doses of 100 and 200 mg/kg/day were selected as lower and higher exposure levels, respectively, with a twofold interval to permit evaluation of a potential dose-related response during repeated oral administration. These doses were intended for experimental efficacy assessment and were not established as optimal, clinically equivalent, or calcium-equivalent doses. No formal dose-ranging study was performed before the present experiment.

Micro-CT Analysis

Femurs were harvested and fixed in 4% paraformaldehyde (Biosesang, Gyeonggi-do, Korea) at 4 °C for 7 days prior to analysis. Micro-computed tomography (micro-CT) scanning was performed *ex vivo* using a SkyScan 1276 system (Bruker, Kontich, Belgium) according to the manufacturer's instructions. Scanning was conducted at a source voltage of 60 kV and a current of 200 μ A with a 0.5 mm aluminum filter, and images were acquired with a rotation step of 0.6° over 360°. The voxel size was approximately 20 μ m. Reconstruction was performed using NRecon software (Bruker), and three-dimensional images were generated using CTvox

software (Bruker). Quantitative analysis was performed using CTAn software (Bruker, Kontich, Belgium; version 1.20.3.0). A standardized trabecular region of interest (ROI) was defined from 1 to 2 mm distal to the growth plate and included the same number of consecutive slices for all specimens. Cortical bone and non-bone regions were excluded from the trabecular ROI. Bone was segmented from background using a fixed global grayscale threshold of 70–255, which was applied identically to all specimens and experimental groups. Segmented images were visually inspected to confirm appropriate separation of mineralized bone from background. Bone mineral density (BMD) and trabecular microarchitectural parameters, including bone volume fraction (BV/TV), bone surface-to-volume ratio (BS/BV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and structural model index (SMI), were quantified using the same analysis settings. All specimens were analyzed by the same investigator using identical reconstruction, ROI, segmentation, and threshold settings. Formal inter- or intra-observer reproducibility testing was not performed. Analyses were performed with the investigator blinded to group allocation. For anatomical reference, three-dimensional reconstruction images of the entire femur were generated from the same datasets. A total of five animals per group were used for micro-CT analysis.

Histological Analysis

Femurs used for micro-CT analysis were subsequently subjected to histological evaluation. Following fixation in 4% paraformaldehyde at 4 °C for 7 days, samples were decalcified in 14% EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 1 week. Decalcified tissues were embedded in paraffin, and serial Sect. (4 µm thickness) were prepared. Sections were stained with hematoxylin and eosin (H&E) for general morphological assessment. Masson's trichrome staining was additionally performed to evaluate collagen deposition and bone matrix organization. For semi-quantitative histological analysis, bone area (%) was measured from H&E-stained femoral sections using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The trabecular bone area within a standardized region of interest (ROI) in the distal femur was quantified and expressed as a percentage of the total tissue area. Histological image acquisition and bone area quantification were performed by an investigator blinded to the experimental group allocation. To evaluate bone marrow adiposity, additional femoral sections were subjected to Oil Red O staining. Paraffin blocks were prepared and sectioned using the same procedure described for histological analysis. Tissue sections were stained with 0.5% Oil Red O solution (prepared in 100% isopropanol) for 10 min at room temperature, rinsed with distilled water,

and counterstained with hematoxylin for 2 min. After washing, the sections were mounted and photographed using a light microscope.

ELISA

Blood samples were collected via cardiac puncture under anesthesia at the time of sacrifice. Serum was obtained by centrifugation at 3,000 rpm for 15 min at 4 °C and stored at −80 °C until analysis. Osteocalcin, procollagen type I N-terminal propeptide (PINP), and C-terminal telopeptide of type I collagen (CTX) levels were measured using ELISA kits from Thermo Fisher Scientific (Waltham, MA, USA), while bone alkaline phosphatase (BALP) was measured using a kit from MyBioSource (San Diego, CA, USA), according to the manufacturers' instructions. All measurements were performed in duplicate.

RAW264.7 Cell Model and Cell Viability Assay

RAW264.7 cells were selected because they provide a reproducible and widely used model of RANKL-induced osteoclast differentiation without requiring additional osteoclastogenic factors such as macrophage colony-stimulating factor. This model was used to examine osteoclastogenic responses and associated signaling changes under standardized experimental conditions. Cytotoxicity of SAC was evaluated using an MTT assay. RAW264.7 cells were seeded in 96-well plates and incubated overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were treated with SAC at concentrations ranging from 0.2 to 20 µL/mL for 24 h. Control cells received an equivalent volume of distilled water. For cell viability assessment under osteoclastogenic conditions, RAW264.7 cells were treated with receptor activator of nuclear factor-κB ligand (RANKL; 50 ng/mL) in the presence or absence of SAC (5–20 µL/mL) or CaCO₃ (100 µg/mL) for 5 days. After treatment, 10 µL of MTT solution was added to each well and incubated for 3 h at 37 °C. Subsequently, 100 µL of solubilization solution (15% SDS) was added to dissolve the formazan crystals, and the plates were incubated overnight. Absorbance was measured at 570 nm using a microplate reader. All experiments were performed as three independent experiments.

Osteoclast Differentiation

RAW264.7 cells were seeded in 6-well plates at a density of 3×10^5 cells/mL and allowed to adhere overnight. Cells were pretreated with SAC (5–20 µL/mL) or CaCO₃ (100 µg/mL) for 30 min, followed by stimulation with RANKL (50 ng/mL) to induce osteoclast differentiation. The culture

medium was replaced every 3 days, and differentiation was maintained for 5 days. After differentiation, cells were used for downstream analyses, including mRNA expression, protein analysis, and tartrate-resistant acid phosphatase (TRAP) staining.

TRAP Staining

RAW264.7 cells were seeded in 24-well plates at a density of 4×10^4 cells/well. After overnight incubation, cells were pretreated with SAC (5–20 $\mu\text{L/mL}$) or CaCO_3 (100 $\mu\text{g/mL}$) for 30 min and then stimulated with RANKL (50 ng/mL) to induce osteoclast differentiation. The culture medium was replaced every 3 days, and differentiation was maintained for 5 days. The differentiated cells were washed with PBS and fixed in 4% paraformaldehyde (Biosesang, Gyeonggi-do, Korea) for 5 min at room temperature. The fixed cells were washed with water and stained using the TRAP staining kit (Cosmo Bio USA, California, USA) for 1 h at 37°C. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using TRI Reagent[®] (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit. Quantitative real-time PCR was performed using gene-specific primers and qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems, London, UK) on a real-time PCR system (Thermo Fisher Scientific). The primer sequences used in this study are listed in Table 1. Gene expression levels were normalized to β -actin as a housekeeping gene and calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Table 1 List of primers

Gene	Direction	Sequences (5'–3')
Nfatc1	F	CCGTGCTTCCAGAAAATAACA
	R	TGTGGGATGTGAACTCGGAA
Trap	F	TCATGGGTGGTGCTGCT
	R	GCCCACAGCCACAAATCT
Atp6v0d2	F	GTGAGACCTTGGAAGTCCTGAA
	R	GAGAAATGTGCTCAGGGGCT
Ctsk	F	CCAGTGGGAGCTATGGAAGA
	R	AAGTGGTTCATGGCCAGTTC
β -actin	F	TGAGCTGCGTTTACACCT
	R	GCCTTCACCGTTCCAGTTTT

Western Blot Analysis

Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg per lane) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature and then incubated with primary antibodies (1:2000 dilution) overnight at 4 °C. After washing with TBST (Tris-buffered saline with 0.1% Tween-20) three times, membranes were incubated with appropriate secondary antibodies (1:2000 dilution) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system. Primary antibodies against NFATc1, p38, phospho-p38, MITF, phospho-c-Fos (#5348), phospho-c-Jun (#9164), c-Jun (#9165), phospho-ERK (#9101), ERK (#4696), phospho-JNK (#9255), and JNK (#4672) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against c-Fos (#SC166940) and β -actin (#SC47778) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test, with the OVX group used as the reference for treatment comparisons. Data normality was assessed using the Shapiro–Wilk test, and no significant deviations from normality were observed. Sample sizes varied among experimental endpoints according to specimen availability, as specified in the corresponding figure legends. No a priori power calculation was performed. The sample sizes were selected with reference to previous studies using OVX rodent models and according to specimen availability for each endpoint. In particular, micro-CT analysis included five animals per group, and histological analyses included four animals per group. These relatively small endpoint-specific sample sizes may have limited the statistical power to detect modest treatment effects. A p -value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA).

Results

Effects of SAC on Body and Organ Weights in OVX Mice

Body weight was monitored throughout the 16-week experimental period. Sham or OVX surgery was performed at week 0, followed by a 3-week post-surgical period and 13 weeks of SAC administration. At the end of the study, major organ weights were measured at the time of euthanasia. As shown in Fig. 1a, OVX mice exhibited a progressive increase in body weight compared with SHAM controls, consistent with metabolic alterations associated with estrogen deficiency. Administration of SAC (100 or 200 mg/kg/day) did not significantly affect body weight relative to the OVX group. Uterine weight was significantly reduced in OVX mice compared with SHAM mice, confirming successful ovariectomy (Fig. 1b). SAC treatment did not significantly alter uterine weight at either dose compared with the OVX group. In contrast, no significant differences in the weights of the liver, kidney, lung, spleen, brain, stomach, or intestine were observed among the experimental groups (Fig. 1c–i). Collectively, SAC administration did not produce significant changes in body weight or the weights of the examined organs under the conditions of this study. Because these measurements provide only limited information regarding systemic toxicity, they should not be interpreted as a comprehensive safety assessment.

Protective Effects of SAC on Trabecular Bone Architecture and Bone Histology in OVX Mice

Micro-computed tomography (micro-CT) analysis was performed to evaluate the protective effects of SAC on OVX-induced bone loss. Representative three-dimensional reconstructions of the entire femur and distal femoral trabecular bone are shown in Fig. 2a. Ovariectomy markedly impaired trabecular architecture, as evidenced by reduced trabecular connectivity and a sparse trabecular network compared with SHAM controls. SAC treatment attenuated these structural alterations and partially preserved trabecular architecture (Fig. 2a). Histological examination of the distal femur further supported the micro-CT findings. H&E and Masson's trichrome staining revealed pronounced trabecular bone loss in OVX mice, whereas SAC-treated mice exhibited preservation of trabecular structures and bone matrix organization (Fig. 2b). Semi-quantitative histological analysis demonstrated that bone area (%) was significantly reduced following OVX and was partially restored by SAC treatment at both 100 and 200 mg/kg/day (Fig. 2c), consistent with the structural improvements observed by micro-CT. To further investigate whether the protective

effects of SAC were associated with alterations in bone marrow adiposity, additional Oil Red O staining was performed. OVX mice exhibited increased Oil Red O staining within the femoral marrow cavity; however, SAC treatment did not substantially alter the overall staining pattern compared with the OVX group (Fig. 2d). Because marrow adiposity was not quantitatively analyzed, no conclusion was drawn regarding the relationship between marrow adiposity and the observed trabecular changes. Quantitative micro-CT analysis demonstrated a significant reduction in bone mineral density (BMD) following OVX (Fig. 2e). SAC treatment at both 100 and 200 mg/kg/day significantly attenuated this decrease. Similarly, bone volume fraction (BV/TV) was markedly reduced in OVX mice and was partially restored by SAC treatment (Fig. 2f). In contrast, the bone surface-to-volume ratio (BS/BV) did not differ significantly among the groups (Fig. 2g). Evaluation of trabecular microarchitectural parameters revealed that OVX significantly decreased trabecular number (Tb.N) and increased trabecular separation (Tb.Sp) (Fig. 2h, i). SAC treatment partially restored Tb.N but did not significantly affect Tb.Sp (Fig. 2h, i). Trabecular thickness (Tb.Th) remained comparable among the experimental groups (Fig. 2j). Furthermore, OVX significantly increased the structural model index (SMI), indicating a shift toward a more rod-like trabecular architecture, whereas SAC treatment significantly reduced SMI relative to the OVX group (Fig. 2k). Collectively, SAC treatment was associated with attenuation of OVX-induced trabecular bone deterioration, as reflected by preservation of bone area and trabecular number and a reduction in SMI.

Effects of SAC on Serum Bone Turnover Markers in OVX Mice

Serum bone turnover markers were analyzed to further evaluate the effects of SAC on OVX-induced alterations in bone metabolism. Bone alkaline phosphatase (BALP), a marker of osteoblastic activity and bone formation, was significantly altered in OVX mice compared with SHAM controls; however, SAC administration did not significantly affect BALP levels relative to OVX (Fig. 3a). Levels of osteocalcin, another marker of bone formation, were significantly lower in OVX mice than in SHAM controls. SAC treatment at 200 mg/kg resulted in an additional reduction compared with OVX, while no significant effect was observed at 100 mg/kg (Fig. 3b). C-terminal telopeptide of type I collagen (CTX), a marker of bone resorption, was markedly elevated in OVX mice compared with SHAM controls (Fig. 3c). SAC treatment significantly attenuated the OVX-induced increase in CTX levels. Procollagen type I N-terminal propeptide (PINP), a marker of collagen synthesis and bone formation, did not differ significantly among

Fig. 1 Effects of SAC on body and organ weights in ovariectomized (OVX) mice.

a Body weight changes in OVX mice. Mice underwent sham-operated (SHAM) or OVX surgery and were orally administered SAC (100 or 200 mg/kg/day) for 13 weeks. Body weight was measured throughout the experimental period in SHAM, OVX, OVX+SAC 100, and OVX+SAC 200 groups. **b** Representative images of uterus (upper panel) and quantitative analysis of uterine weight (lower panel) are shown. **c–i** The weights of the liver (**c**), kidney (**d**), lung (**e**), spleen (**f**), brain (**g**), stomach (**h**), and intestine (**i**) were measured at the time of euthanasia. Data are expressed as mean $\pm$ SD ($n=12$ per group). Individual data points are shown for each animal. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus SHAM group; * $p<0.05$, ** $p<0.01$ versus OVX group

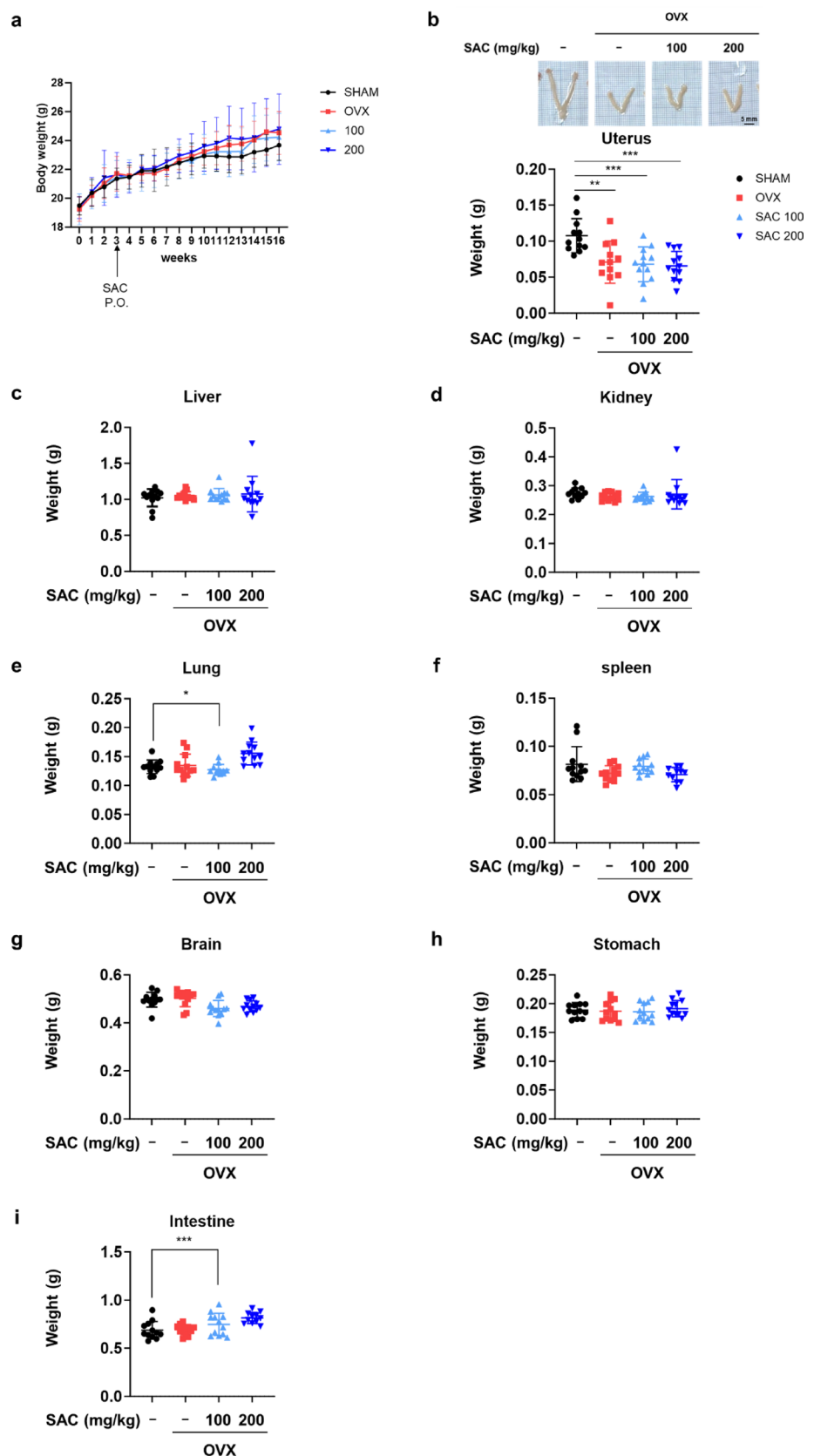
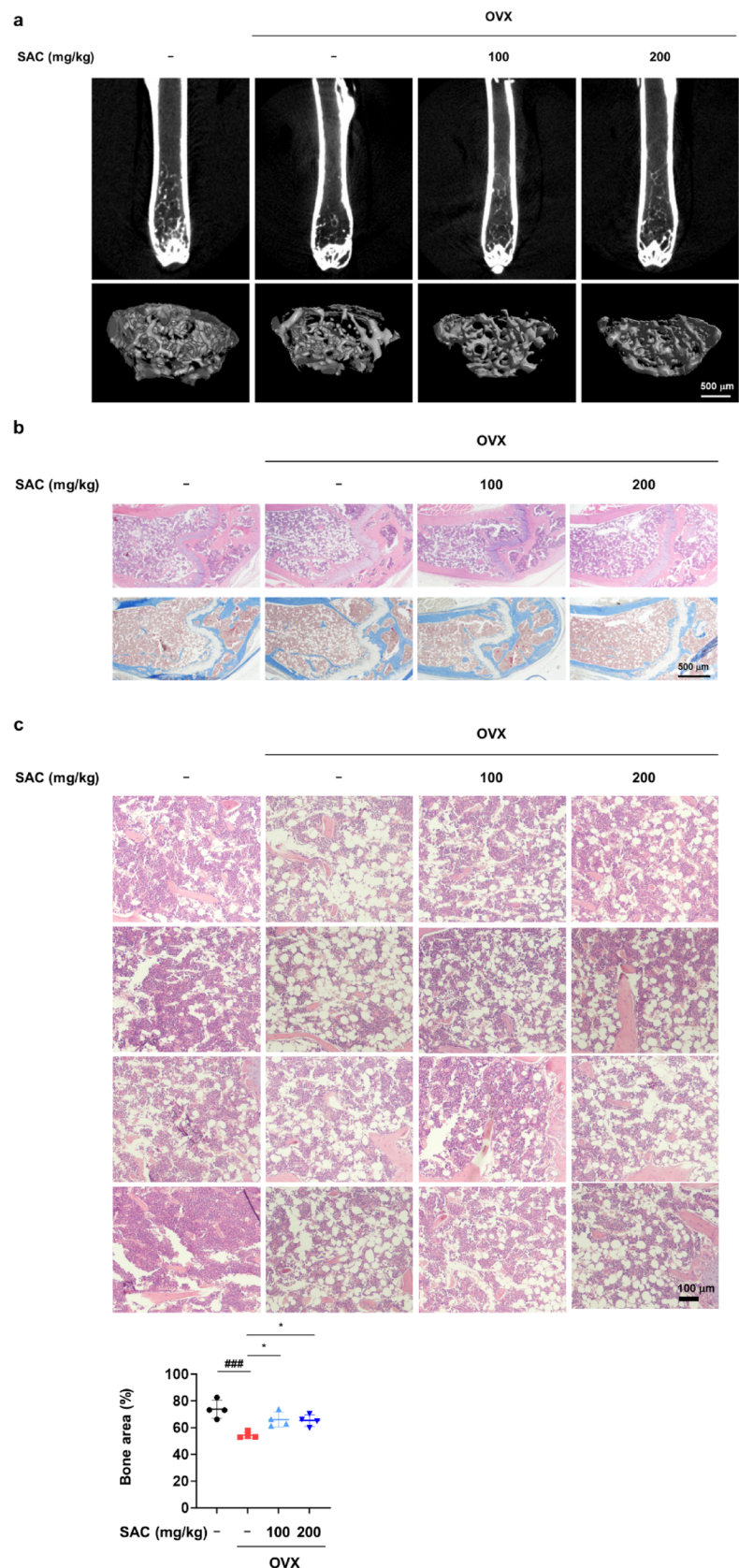


Fig. 2 Protective effects of SAC on trabecular bone microarchitecture and histological changes in ovariectomized (OVX) mice. **a** Representative three-dimensional micro-computed tomography (micro-CT) reconstructions of the distal femur and trabecular bone region. **b** Representative histological images of distal femoral sections stained with hematoxylin and eosin (H&E, upper panel) and Masson's trichrome (lower panel). **c** Semi-quantitative histological analysis of bone area (%) in the distal femur. **d** Representative Oil Red O-stained sections of distal femoral bone marrow. **e** Quantitative analysis of bone mineral density (BMD). **f** Quantitative analysis of bone volume fraction (BV/TV). **g** Quantitative analysis of bone surface-to-bone volume ratio (BS/BV). **h–j** Trabecular morphometric parameters, including trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). **k** Structural model index (SMI), reflecting trabecular structural configuration. Data are presented as mean $\pm$ SD. For micro-CT analyses (**e–k**), $n=5$ animals per group. For bone area analysis (**c**), $n=4$ animals per group. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. ### $p<0.001$ versus SHAM group; * $p<0.05$, ** $p<0.01$ versus OVX group



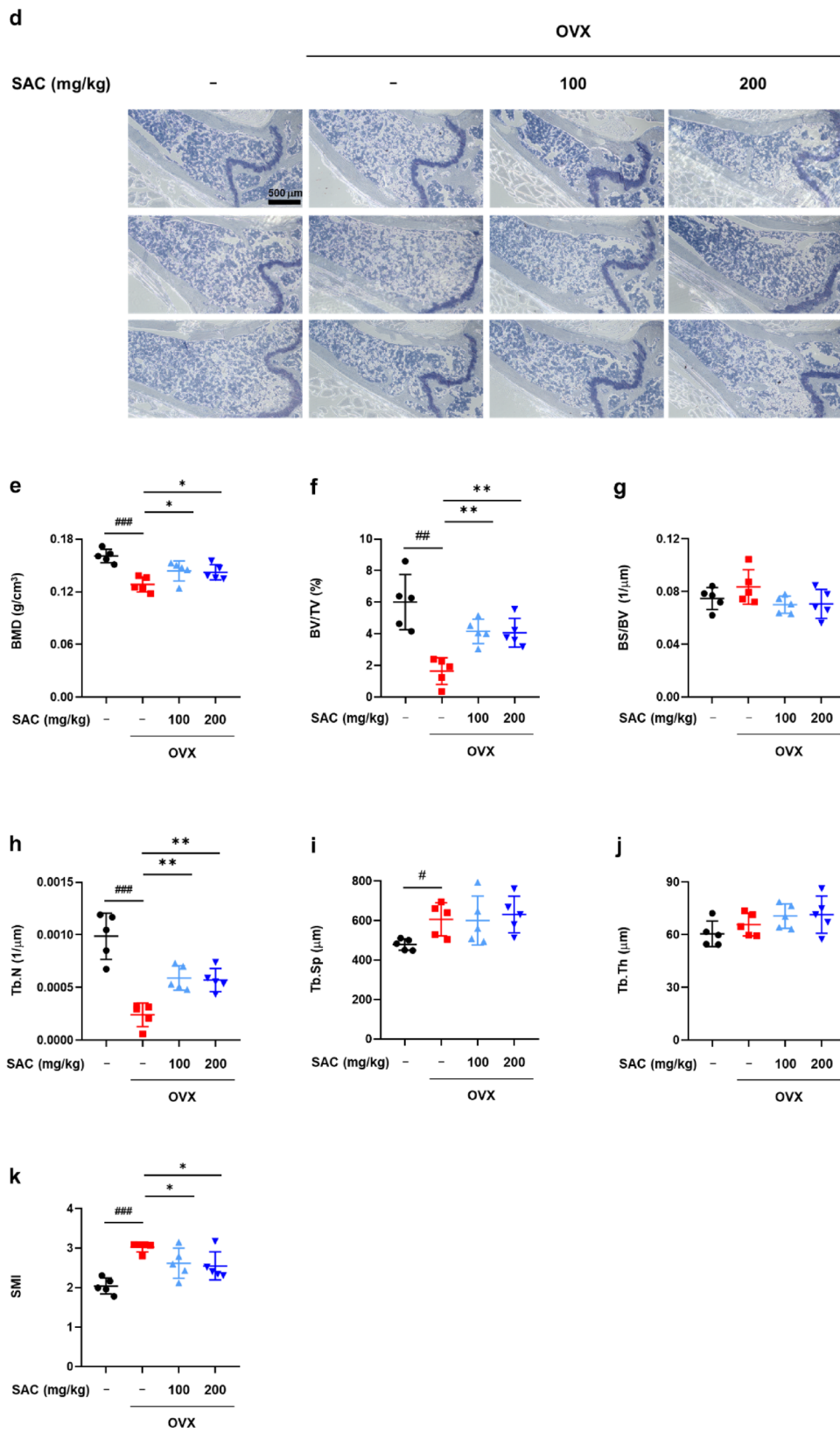
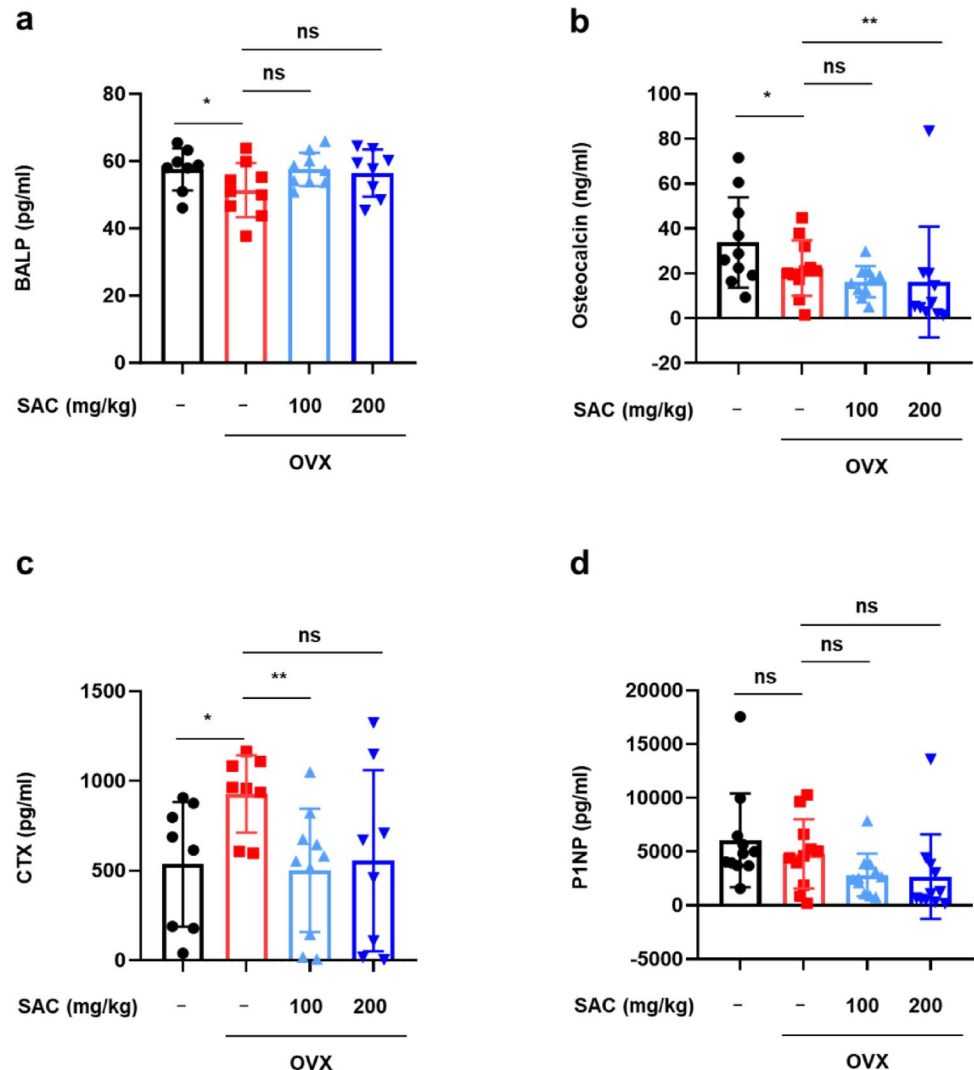


Fig. 2 (continued)

Fig. 3 Effects of SAC on serum bone turnover markers in OVX mice. **a** Serum bone alkaline phosphatase (BALP), a marker of osteoblastic activity and bone formation. **b** Serum osteocalcin levels, another marker of bone formation. **c** Serum C-terminal telopeptide of type I collagen (CTX), a marker of bone resorption. **d** Serum procollagen type I N-terminal propeptide (PINP), a marker of collagen synthesis and bone formation. Data are presented as mean $\pm$ SD ($n=8-11$ per group). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus SHAM group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus OVX group; ns, not significant



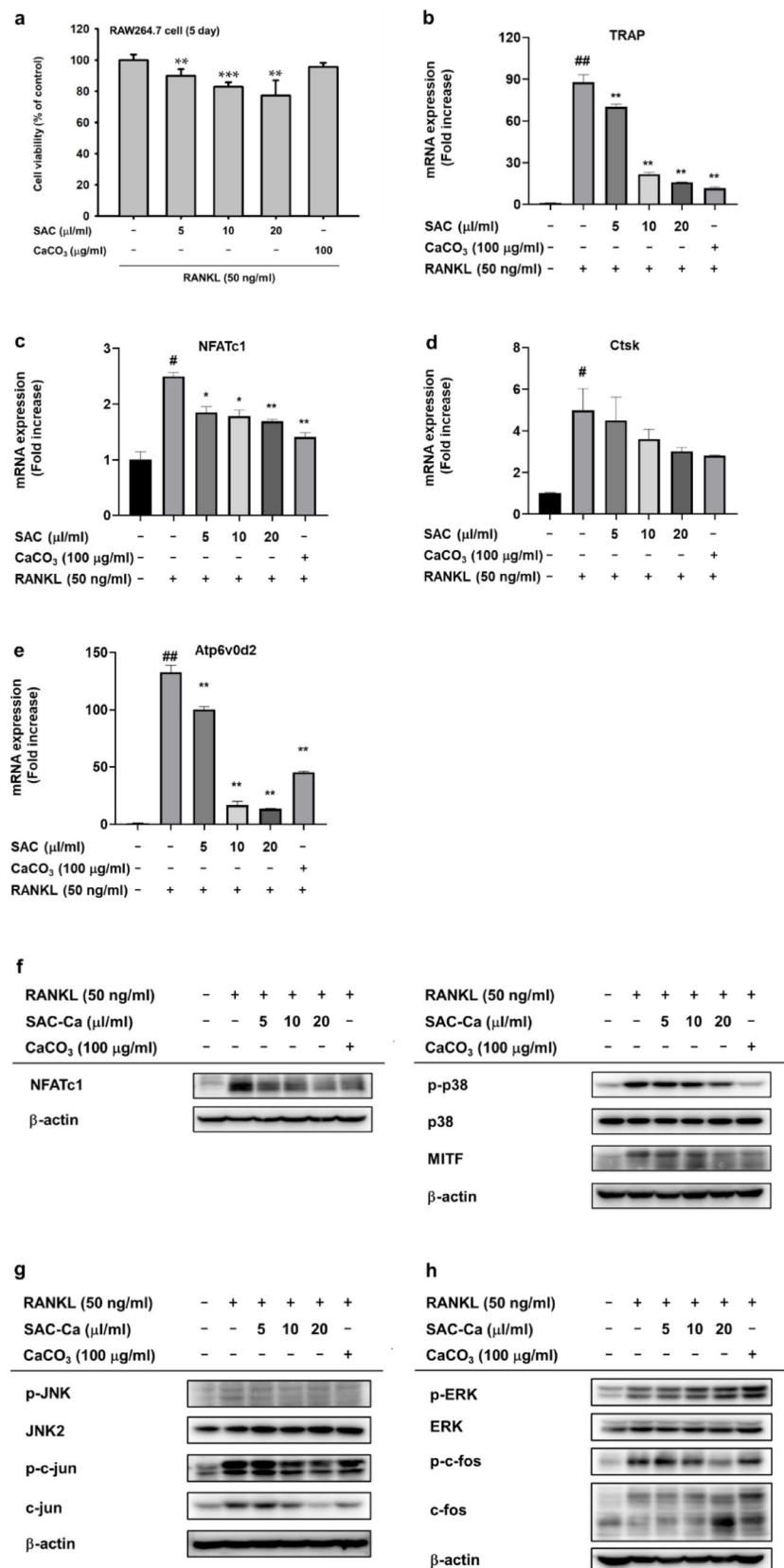
the experimental groups (Fig. 3d). Collectively, SAC treatment was associated with a reduction in the OVX-induced increase in the bone resorption marker CTX, whereas the bone formation-associated markers BALP, osteocalcin, and PINP did not show a consistent treatment-related increase. These serum marker findings prompted further examination of the effects of SAC on osteoclast differentiation in vitro.

Inhibitory Effects of SAC on RANKL-Induced Osteoclastogenesis in RAW264.7 Cells

To examine the effects of SAC on osteoclastogenesis, RAW264.7 cells were stimulated with RANKL (50 ng/mL) in the presence or absence of SAC. Cell viability analysis under RANKL-stimulated conditions showed that SAC (5–20 μ L/mL) did not significantly affect RAW264.7 cell viability over 5 days (Fig. 4a), indicating that the reduction in osteoclastogenic responses was not accompanied by detectable cytotoxicity at the concentrations examined.

RANKL markedly induced the mRNA expression of osteoclast-related genes, including tartrate-resistant acid phosphatase (TRAP), nuclear factor of activated T cells 1 (NFATc1), cathepsin K (Ctsk), and Atp6v0d2 (Fig. 4b–e). SAC treatment (5, 10, and 20 μ L/mL) dose-dependently suppressed the RANKL-induced upregulation of these genes. Calcium carbonate (CaCO_3 , 100 μ g/mL), included as a reference calcium treatment, also reduced the expression of these osteoclastogenic markers. Consistently, RANKL stimulation significantly increased NFATc1 protein expression, whereas SAC attenuated this induction in a dose-dependent manner (Fig. 4f). CaCO_3 also suppressed NFATc1 protein levels. Analysis of MAPK signaling revealed that RANKL increased phosphorylated p38 (p-p38) without altering total p38 expression. Treatment with SAC at 20 μ L/mL and CaCO_3 reduced p-p38 levels, while total p38 remained unchanged (Fig. 4f). Moreover, MITF protein expression was elevated following RANKL stimulation and was decreased by SAC (20 μ L/mL) and

Fig. 4 Effects of SAC on RANKL-induced osteoclastogenesis and associated changes in signaling-related proteins in RAW264.7 cells. **a** Cell viability of RAW264.7 cells treated with SAC (5–20 $\mu\text{L/mL}$) in the presence of RANKL (50 ng/mL) for 5 days, assessed by MTT assay. **b–e** Relative mRNA expression levels of osteoclast-related genes, including tartrate-resistant acid phosphatase (TRAP), nuclear factor of activated T cells 1 (NFATc1), cathepsin K (Ctsk), and Atp6v0d2, in RAW264.7 cells stimulated with RANKL (50 ng/mL) in the presence or absence of SAC (5, 10, and 20 $\mu\text{L/mL}$). **f** Immunoblot analysis of NFATc1, total p38, p-p38, and MITF protein expression following RANKL stimulation and SAC treatment. **g** Immunoblot analysis of JNK signaling pathway components, including p-JNK, JNK2, p-c-Jun, and c-Jun. **h** Immunoblot analysis of ERK signaling pathway components, including p-ERK, ERK, p-c-Fos, and c-Fos. Calcium carbonate (CaCO_3 , 100 $\mu\text{g/mL}$) was included as a reference calcium treatment. Data are presented as mean $\pm$ SD from three independent experiments ($n=3$). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc multiple comparison test. $\#p<0.05$, $\#\#p<0.01$ versus control group; $*p<0.05$, $**p<0.01$ versus RANKL-treated group. Full-length uncropped blots with molecular-weight markers and corresponding loading controls are provided in Supplementary Figure S1



CaCO₃ treatment (Fig. 4f). In addition, SAC treatment was associated with reduced RANKL-induced phosphorylation of JNK and ERK pathway components. RANKL stimulation increased the phosphorylation of JNK and its downstream target c-Jun, whereas SAC treatment reduced these phosphorylation levels without altering total protein expression (Fig. 4g). Similarly, SAC decreased RANKL-induced phosphorylation of ERK and its downstream target c-Fos (Fig. 4h). Collectively, SAC treatment was associated with suppression of RANKL-induced osteoclast differentiation, reduced phosphorylation of p38, JNK, and ERK pathway components, and decreased expression of NFATc1 and MTF. However, these observations do not establish the causal involvement of these signaling changes in the suppression of osteoclast differentiation.

Effects of SAC on RANKL-induced osteoclast formation in RAW264.7 cells

To further evaluate the functional effects of SAC on osteoclast differentiation, TRAP staining was performed in RAW264.7 cells stimulated with RANKL. As shown in Fig. 5a, RANKL treatment markedly increased the formation of TRAP-positive multinucleated osteoclasts compared with control cells. SAC treatment (5, 10, and 20 μ L/mL) reduced the number and size of TRAP-positive multinucleated cells in a dose-dependent manner. Calcium carbonate (CaCO₃, 100 μ g/mL), included as a reference calcium treatment, also decreased osteoclast formation. Quantitative analysis showed that the number of TRAP-positive multinucleated cells was markedly increased by RANKL stimulation and significantly reduced by SAC treatment in a concentration-dependent manner (Fig. 5b). CaCO₃ treatment was also associated with a reduction in osteoclast numbers. Collectively, these results show that SAC treatment was associated with reduced RANKL-induced formation of TRAP-positive multinucleated osteoclasts, together with reductions in osteoclast-related gene expression and changes in signaling-related proteins.

Discussion

The present study provides complementary *in vivo* and *in vitro* evidence that SAC treatment was associated with attenuation of estrogen deficiency-induced trabecular bone loss and suppression of RANKL-induced osteoclast differentiation. Because an *in vivo* conventional-calcium comparator was not included, the present findings do not establish whether these effects are specific to SAC or differ from those of conventional calcium supplementation.

Postmenopausal osteoporosis is fundamentally driven by estrogen deficiency, which disrupts the physiological coupling of bone resorption and bone formation [2, 5]. Under normal conditions, estrogen constrains osteoclast survival and activity through multiple mechanisms. One well-characterized pathway involves estrogen-induced Fas ligand expression in osteoblast-lineage cells, promoting osteoclast apoptosis and thereby limiting resorption [6]. Estrogen deficiency also amplifies immune-mediated osteoclastogenic stimuli; in particular, increased T-cell production of TNF- α enhances osteoclast formation and resorption in estrogen-depleted conditions [7]. Together, these mechanisms shift remodeling toward excessive resorption and net skeletal loss, consistent with the unitary model in which estrogen deficiency contributes to deterioration of trabecular and cortical bone across the lifespan [5]. In the present study, OVX produced the expected phenotype of trabecular deterioration, consistent with prior micro-CT and biochemical marker analyses in OVX mice [13, 14], supporting the suitability of this model for interrogating interventions targeting estrogen deficiency-associated bone loss.

The reduction in serum CTX associated with SAC treatment is consistent with a decrease in bone resorption under the experimental conditions examined. However, the formation-associated serum markers did not provide evidence of enhanced bone formation. Because circulating biomarkers are indirect and time-dependent measures of bone turnover, these findings cannot determine the relative contributions of osteoclast and osteoblast activity to the observed preservation of trabecular microarchitecture. Static and dynamic histomorphometric analyses will be required to clarify these contributions.

Preservation of trabecular microarchitecture may be biologically relevant because trabecular bone is preferentially affected during the early response to estrogen deficiency owing to its high surface area and rapid turnover [14]. However, skeletal strength depends not only on bone mineral density and microarchitecture but also on material properties and structural connectivity [8–10]. Therefore, the micro-CT findings should be interpreted as evidence of structural preservation under the experimental conditions examined and not as evidence of improved mechanical competence. Because biomechanical testing was not performed, whether these structural changes translate into improved bone strength or fracture resistance remains unknown.

Histological analysis was consistent with the micro-CT findings. Semi-quantitative assessment showed that bone area was reduced following OVX and partially restored in SAC-treated mice. Oil Red O staining did not show a marked difference between the OVX and SAC-treated groups. Because marrow adiposity was not quantitatively assessed, these observations do not establish whether

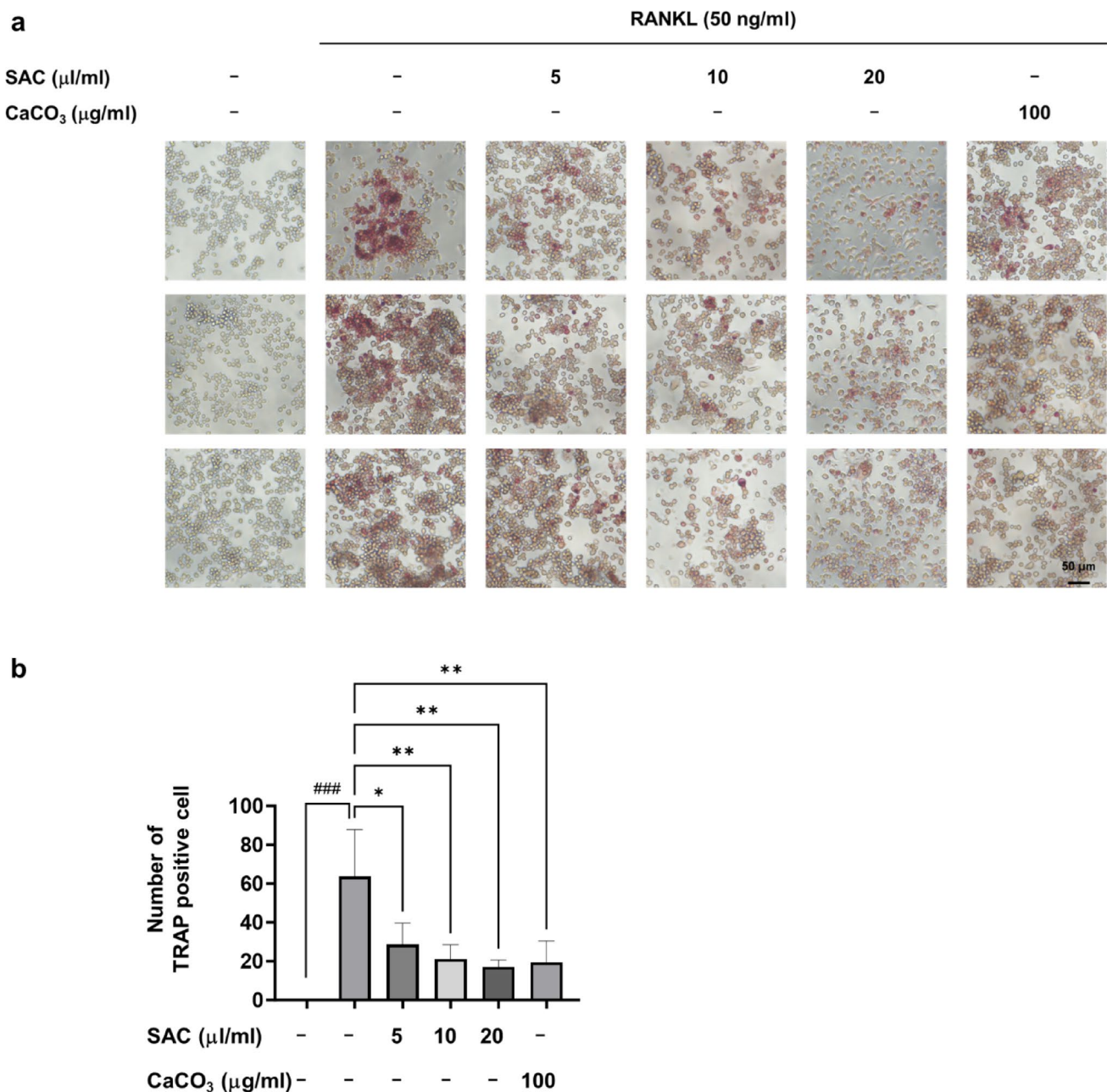


Fig. 5 Effects of SAC on RANKL-induced osteoclast formation in RAW264.7 cells. **a** Representative images of tartrate-resistant acid phosphatase (TRAP)-stained osteoclasts in RAW264.7 cells treated with RANKL (50 ng/mL) in the presence or absence of SAC (5, 10, and 20 $\mu\text{L/mL}$) or CaCO_3 (100 $\mu\text{g/mL}$). TRAP-positive multinucleated cells (≥ 3 nuclei) are indicated by red staining. **b** Quantification

of TRAP-positive multinucleated osteoclasts. Data are presented as mean $\pm$ SD from three independent experiments ($n=3$). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc multiple comparison test. $\#p<0.05$, $\#\#p<0.01$ versus control group; $*p<0.05$, $**p<0.01$ versus RANKL-treated group

changes in marrow adiposity contributed to the preservation of trabecular structure.

At the cellular level, SAC treatment was associated with suppression of RANKL-induced osteoclast differentiation without detectable cytotoxicity at the concentrations examined. RANKL–RANK signaling promotes osteoclastogenesis through NFATc1, a master transcription

factor that coordinates osteoclast-specific gene expression and functional maturation [18, 37]. The concurrent reductions in osteoclast-related markers and TRAP-positive multinucleated cells are therefore consistent with inhibition of the osteoclast differentiation program, although the upstream mechanism responsible for this response remains unresolved.

p38 MAPK and MITF participate in the regulation of osteoclast differentiation and function [38–40]. In the present study, suppression of osteoclast differentiation was accompanied by reduced p38 phosphorylation and MITF expression. However, pathway inhibition and rescue experiments were not performed; therefore, the causal involvement of these signaling changes remains unestablished. CaCO_3 also reduced RANKL-induced osteoclast differentiation and was included only as a reference calcium treatment. Because SAC and CaCO_3 were not compared at calcium-equivalent concentrations and extracellular ionized calcium was not measured, the present findings cannot establish formulation-specific effects or differences in calcium availability. Calcium-equivalent comparisons and direct measurements of ionized calcium will be required to determine whether SAC differs from conventional calcium formulations.

The signaling changes observed following SAC treatment coincided with reduced osteoclast differentiation but do not establish the requirement of any individual pathway. Calcium-dependent signaling, including Ca^{2+} oscillation–calcineurin–NFATc1 activation and CaSR-mediated responses, has an established role in osteoclast biology [21–23]. However, extracellular and intracellular Ca^{2+} dynamics, calcineurin activity, and CaSR activation were not examined in the present study. Consequently, the observed signaling changes cannot be attributed to altered calcium availability or calcium-dependent signaling. Direct calcium imaging, pathway-specific inhibition, and rescue experiments will be required to determine the causal relevance of these pathways to the effects associated with SAC treatment.

Calcium supplementation remains important in osteoporosis management, but its absorption and bioavailability vary according to formulation, solubility, gastric conditions, vitamin D status, age, and estrogen status [24–31]. SAC has been described as a modified calcium formulation designed to enhance ion release and aqueous solubility [36]; however, these proposed physicochemical properties were not evaluated in the present study. Serum ionized or total calcium, intestinal calcium absorption, pharmacokinetics, and tissue calcium levels were also not measured. Therefore, the present findings cannot determine whether SAC increases systemic calcium availability or differs in bioavailability from conventional calcium formulations.

Translation of the administered doses to humans requires caution. Using body surface area conversion, the mouse doses of 100 and 200 mg/kg/day correspond to approximate human-equivalent doses of 8.1 and 16.2 mg/kg/day, respectively [41]. For a 60-kg adult, these values correspond to approximately 486 and 973 mg/day of the SAC formulation. However, these estimates refer to the administered SAC formulation rather than to elemental or systemically available calcium. Because the precise elemental calcium

content, intestinal absorption, and systemic bioavailability of SAC were not determined in the present study, these human-equivalent doses cannot be directly compared with recommended daily calcium intake or the doses of conventional calcium supplements.

At present, SAC should be regarded as an investigational calcium formulation rather than an established treatment for osteoporosis. Its potential clinical role, if supported by further evidence, would be as an adjunct to adequate calcium and vitamin D intake or to established osteoporosis therapy rather than as a replacement for approved anti-osteoporotic agents. Before clinical application can be considered, SAC will require standardized characterization of its composition and elemental calcium content, direct comparison with conventional calcium formulations, dose-ranging studies, comprehensive safety assessment, and controlled clinical evaluation of skeletal outcomes. Its regulatory classification would depend on its intended use and claims and may differ between jurisdictions; therapeutic claims concerning prevention or treatment of osteoporosis would require a substantially higher level of evidence than claims related to nutritional calcium supplementation.

Several limitations are particularly relevant to the interpretation of SAC as a calcium formulation. The study did not include an *in vivo* group receiving a conventional calcium formulation, and SAC and CaCO_3 were not compared at calcium-equivalent concentrations *in vitro*. Serum ionized and total calcium, intestinal calcium absorption, calcium pharmacokinetics, and tissue calcium levels were also not measured. Consequently, the present study demonstrates the effects of SAC only under the tested experimental conditions and does not establish its superiority, comparative bioavailability, or formulation-specific effects relative to conventional calcium supplementation. Safety was not comprehensively evaluated, and the absence of significant changes in body and organ weights does not exclude biochemical, hematological, or histopathological toxicity because clinical chemistry, hematological parameters, and systematic organ histopathology were not assessed. Biomechanical testing was not performed; therefore, whether the observed preservation of trabecular microarchitecture translates into improved bone strength, mechanical competence, or fracture resistance could not be determined [42, 43]. Intracellular calcium dynamics, calcineurin activity, CaSR activation, pathway-specific inhibition, and rescue experiments were not examined. Thus, the present study cannot establish whether the observed changes in p38 phosphorylation and NFATc1 and MITF expression are causally involved in the suppression of osteoclast differentiation. The *in vitro* experiments were conducted exclusively using RAW264.7 cells, and the generalizability of these findings to primary osteoclast precursors remains uncertain. Validation using

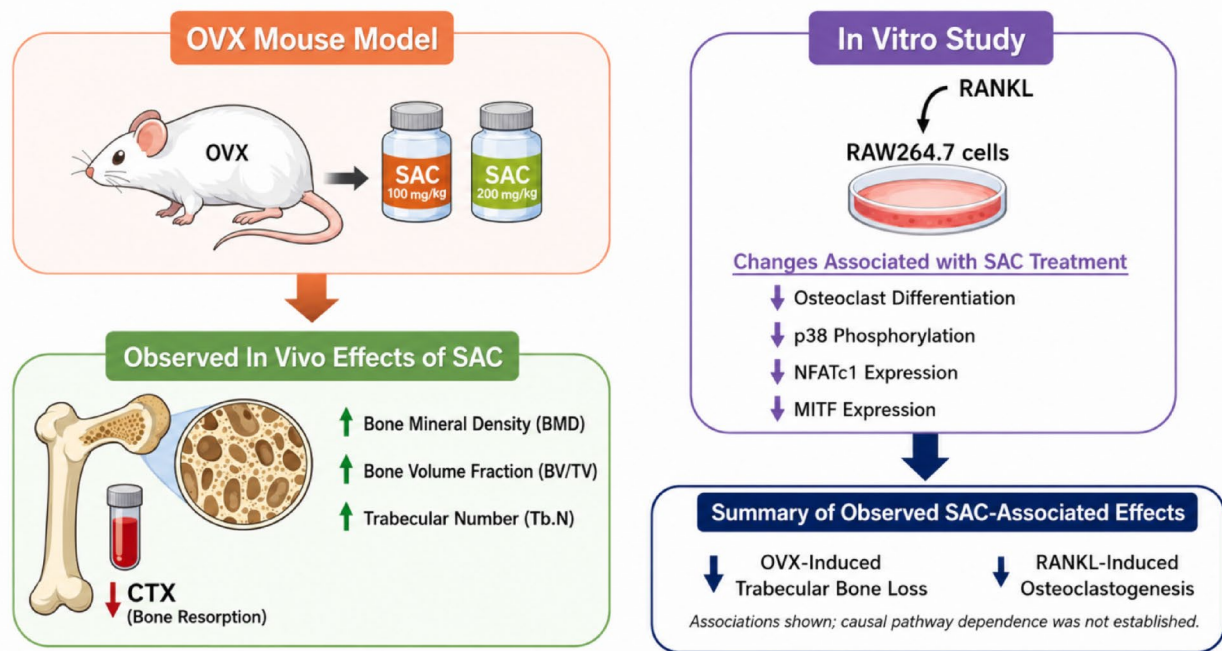


Fig. 6 Schematic summary of the observed effects of SAC on OVX-induced trabecular bone deterioration and RANKL-induced osteoclast differentiation. SAC treatment was associated with reduced phosphor-

ylation of MAPK pathway components and decreased NFATc1 and MITF expression. The schematic illustrates observed associations and does not establish causal pathway dependence

primary bone marrow-derived macrophages and human osteoclast precursors will therefore be required. In addition, no a priori power calculation was performed, and the relatively small numbers of animals included in the micro-CT and histological analyses may have limited the statistical power to detect modest treatment effects. Accordingly, non-significant differences should not be interpreted as definitive evidence of the absence of an effect. Comparative studies with established therapeutic agents and longer-term studies in aged animal models will also be needed to evaluate the relative and sustained effects of SAC [2, 5, 13, 14].

Bone remodeling was not comprehensively evaluated in the present study. Static osteoblast and osteoclast histomorphometry, mineral apposition rate, bone formation rate, double-fluorochrome labeling, and in vivo TRAP staining were not assessed. Therefore, the relative contributions of bone formation and resorption to the observed preservation of trabecular microarchitecture remain uncertain. In addition, Oil Red O staining was not quantitatively analyzed and cannot exclude effects on marrow adiposity or osteoblast-lineage differentiation. Serum estrogen levels were also not measured; although uterine atrophy and trabecular bone loss supported successful establishment of the OVX model, direct hormonal assessment would provide additional validation.

In conclusion, SAC treatment was associated with attenuation of OVX-induced trabecular deterioration and

suppression of RANKL-induced osteoclast differentiation under the experimental conditions examined. Reduced osteoclast differentiation was accompanied by decreased p38 phosphorylation and NFATc1 and MITF expression; however, the causal involvement of these signaling changes remains to be established (Fig. 6). Further studies incorporating conventional calcium comparators, calcium-equivalent dosing, pathway-specific interventions, and direct assessment of calcium bioavailability and calcium-dependent signaling will be required to determine the comparative efficacy, mechanism, and translational relevance of SAC.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University (Approval No. SKKUIACUC2024-10-23-1) and were conducted in accordance with institutional guidelines for animal welfare.

Human and Animal Rights Statement This article does not contain any studies involving human participants. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed Consent Not applicable.

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