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Capsaicin-activated autophagy protects BMSC function under oxidative stress: mechanisms and therapeutic implications

Yurong Chen^{1,2,3,*}, Qian Peng^{4,*}, Dongmei Lan^{1,2,3} , Chao Yao^{1,2,3}, Xiang Chen^{2,3}, Yan Wang^{1,3,5} and Shengcai Qi^{1,2,3} 

¹Medical College, Anhui University of Science and Technology, Huainan, China

²Department of Prosthodontics, Shanghai Stomatological Hospital & School of Stomatology, Fudan University, Shanghai, China

³Shanghai Key Laboratory of Craniomaxillofacial Development and Diseases, Fudan University, Shanghai, China

⁴Department of Plastic and Reconstructive Surgery, Hubei NO. 3 People's Hospital of Jiangnan University, Wuhan, China

⁵Department of Preventive Dentistry, Shanghai Stomatological Hospital & School of Stomatology, Fudan University, Shanghai, China

Correspondence should be addressed to Y Wang: kq_wangyan@fudan.edu.cn or to S Qi: dentistqi@163.com

*Y Chen and Q Peng contributed equally to this work)

Abstract

Bone marrow stromal cells (BMSCs) play an important role in bone regeneration, but their functional activity is affected by oxidative stress, which is a key pathological feature of osteoporosis. The aim of this study was to investigate the effects of capsaicin on the proliferation and differentiation of BMSCs under oxidative stress. We assessed cell viability and osteogenic potential of capsaicin in promoting BMSC survival and enhancing osteogenic capacity under oxidative stress by cell counting kit-8 (CCK-8), reactive oxygen species fluorescence staining, alkaline phosphatase (ALP) staining, Alizarin Red S (ARS) staining, Western blot (WB), and real-time PCR (RT-PCR). Our results indicate that capsaicin improves cell viability, antioxidant capacity, and osteogenic differentiation in rat BMSCs treated with hydrogen peroxide (H₂O₂). In addition, immunohistochemistry (IHC) analysis revealed that the surface of BMSCs expressed the capsaicin receptor transient receptor potential vanilloid protein 1 (TRPV1). More importantly, capsaicin increased Ca²⁺ influx and autophagy and inhibited phosphorylation of the PI3K/AKT/mTOR signaling pathway. In conclusion, capsaicin protects BMSC function during oxidative stress, possibly through inducing TRPV1-mediated Ca²⁺ influx and PI3K/AKT/mTOR-activated autophagy. The results suggest the potential of capsaicin as a therapeutic agent for osteoporosis.

Keywords: capsaicin; TRPV1; BMSCs; PI3K/AKT/mTOR pathway; Ca²⁺; autophagy

Introduction

Osteoporosis affects millions of individuals globally (Wu *et al.* 2021). Epidemiological studies indicate that osteoporosis affects over 200 million individuals worldwide, leading to diminished quality of life, increased fracture risk, and substantial healthcare costs (Clynes *et al.* 2020). Current clinical treatments, such as bisphosphonates and hormone replacement therapy,

often have limited efficacy and may cause adverse side effects, including osteonecrosis of the jaw and an increased risk of cancer (Ezra & Golomb 2000, Cauley *et al.* 2003). During osteoporosis progression, the functional balance between osteoclasts and bone marrow mesenchymal stem cells (BMSCs) is disrupted, resulting in bone loss (Wawrzyniak & Balawender 2022).

BMSCs play crucial roles in bone regeneration and repair (Wang *et al.* 2013). As multipotent cells, BMSCs can migrate to injury sites, suppress local immune responses, and promote bone regeneration (Qin *et al.* 2014). Therefore, enhancing the osteogenic potential of BMSCs under osteoporotic pathological conditions may be critical for the treatment of osteoporosis.

Oxidative stress is a critical factor contributing to the dysfunction of BMSCs during osteoporosis, leading to various bone diseases and complications (Banfi *et al.* 2008, Zhao *et al.* 2022a). It is characterized by an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defenses, resulting in cellular damage and impaired function of BMSCs (Afzal *et al.* 2023). ROS directly damage bone matrix proteins, such as collagen and osteocalcin, through oxidative stress response, while inhibiting osteogenic differentiation and activating osteoclast activity, resulting in an imbalance between bone formation and resorption (Lin *et al.* 2018, Chen *et al.* 2021). BMSCs play a crucial role in bone formation through their osteogenic differentiation potential (Lan *et al.* 2022a). However, oxidative stress promotes apoptosis and inhibits osteogenic differentiation of BMSCs, thereby exacerbating conditions such as osteoporosis and fractures (Ardura *et al.* 2020, Yang *et al.* 2021). Importantly, our previous studies found that natural antioxidants such as quercetin, tocopherol, and leonurine have shown therapeutic promise by scavenging ROS, protecting BMSCs from oxidative damage, and restoring the balance of bone metabolism by rescuing the osteogenic function of BMSCs (Lan *et al.* 2022a,b, Zhao *et al.* 2022b), suggesting natural antioxidants could be useful in antiresorptive therapies for bone loss in osteoporosis.

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide, C₁₈H₂₇NO₃; Fig. 1) (Waldvogel & Waldvogel 2001), a natural bioactive alkaloid, is a small-molecule vanilloid compound with a molecular weight of 305.4 Da (Gonzalez-Mondragon *et al.* 2011). As the main active ingredient in peppers, capsaicin has garnered extensive research owing to its pharmacological properties, including antioxidant, anti-inflammatory, antitumor, and gastroprotective effects (Basith *et al.* 2016, Lu *et al.* 2020). It primarily exerts its biological functions through activation of the transient receptor potential vanilloid 1 (TRPV1) receptor, a key modulator of calcium signaling and various cellular processes (Zhai *et al.* 2020, Oz *et al.* 2023). In addition, capsaicin demonstrates

ROS-scavenging capacity and exhibits therapeutic potential against osteoarthritis through activation of the Ca²⁺/CaMKII/Nrf2 pathway while simultaneously inhibiting M1 macrophage polarization (Lv *et al.* 2021). Excessive accumulation of ROS can damage cell membranes, proteins, and DNA, leading to mitochondrial dysfunction and apoptosis (Liguori *et al.* 2018, Jun *et al.* 2019). To mitigate these effects, cells activate autophagy, a conserved lysosome-dependent degradation process that eliminates damaged organelles, misfolded proteins, and cytotoxic aggregates (Kim & Lee 2014). Furthermore, the natural antioxidant compound capsaicin can mitigate oxidative damage in cells by enhancing autophagy or induce autophagic cell death in cancer cells (Huang *et al.* 2021, Qiao *et al.* 2021). Although the protective effects of natural antioxidants such as leonurine and quercetin on BMSCs under oxidative stress conditions have been well documented (Lan *et al.* 2022b, Zhao *et al.* 2022b), the therapeutic potential and underlying mechanisms of capsaicin in modulating oxidative stress responses and osteogenic differentiation in BMSCs remain unexplored. Therefore, we hypothesize that capsaicin may protect BMSCs by coordinating TRPV1-mediated autophagy, thereby maintaining osteogenic capacity during oxidative stress.

The aim of this study was to investigate the effect of capsaicin on the function of BMSCs under oxidative stress and its potential mechanism. The cell viability of BMSCs under 200 μ M H₂O₂ stimulation was determined by CCK-8. The osteogenic differentiation capacity, TRPV1 receptor expression, and autophagy of BMSCs were detected. Meanwhile, the PI3K/AKT/mTOR pathway was analyzed.

Materials and methods

Isolation and culture of primary BMSCs

BMSCs were isolated from the humeri of 3-week-old male Sprague-Dawley rats by flushing the marrow cavity with α -MEM (Gibco, USA) using a syringe, followed by centrifugation at 161 g for 10 min. The pelleted cells were cultured in α -MEM supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO₂, with medium changes every 48–72 h to remove non-adherent cells, and passages 2–5 BMSCs were used for subsequent experiments.

TRPV1 immunohistochemistry (IHC) analysis in primary BMSCs

BMSCs were plated in 12-well plates (1 \times 10⁵ cells/mL, 1 mL/well) for 24 h. Following PBS washes, cells were fixed with 4% paraformaldehyde (Servicebio, China) for 20 min at room temperature (RT) and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 3% BSA

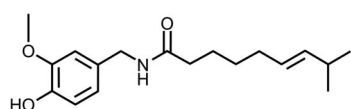


Figure 1

Chemical structure of capsaicin.

(RT, 30 min), cells were incubated overnight at 4°C with rabbit anti-TRPV1 primary antibody (1:100; Abmart, China; Cat: M047783F; Lot: 334467) or PBS instead of primary antibody (negative control). Following PBS washes, HRP-conjugated goat anti-rabbit IgG (1:200; BioSharp, China) was applied (RT, 1 h) in the darkness. DAB development for 3–5 min was monitored microscopically and stopped with distilled water. Nuclei were counterstained with hematoxylin (BioSharp) for 3 min, followed by dehydration through an ethanol series (70, 80, 95, and 100%) and xylene clearing. Neutral balsam was added, and coverslips were applied. The localization and expression intensity of TRPV1 were observed under an optical microscope (Leica, Germany).

Cell viability

BMSCs were plated in 96-well plates (1×10^5 cells/mL, 100 μ L/well) for 24 h. To induce oxidative stress, cells were co-treated with 200 μ M H₂O₂ (Sigma, USA) and ascending concentrations of capsaicin (1–100 μ M; Sigma) for 4 h, followed by H₂O₂ removal and subsequent 24 h incubation with capsaicin-containing complete medium. Cell viability was assessed using CCK-8 assay (Dojindo, Japan) by adding 10 μ L reagent mixed into 90 μ L α -MEM to each well, ensuring the total reaction volume remained at 100 μ L. After 2 h light-protected incubation, absorbance was measured at 450 nm using a microplate reader (Bio-Rad, USA).

Analysis of apoptosis

BMSCs cultured in 6-well plates (1×10^5 cells/mL, 2 mL/well) were treated with 200 μ M H₂O₂ and 10 μ M capsaicin for 4 h. For mRNA analysis, apoptosis-related genes (Bax, Bcl-2) were quantified by RT-qPCR. GAPDH served as an endogenous control, and relative gene expression was calculated via the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 1.

Intracellular ROS determination

BMSCs were plated in 12-well plates (1×10^5 cells/mL, 1 mL/well) for 24 h. The cells were then incubated with serum-free medium containing 10 μ M

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, China) for 20 min at 37°C. Following probe loading, cells were treated with 200 μ M H₂O₂ and different concentrations of capsaicin (1–10 μ M) for 4 h. Intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (ROS Assay Kit; Beyotime) and visualized via fluorescence microscopy (Leica). ROS fluorescence was quantified as mean intensity per field of view (ImageJ) under consistent imaging parameters. While not normalized to cell number, parallel CCK-8 assays confirmed viability differences were accounted for in data interpretation.

Osteogenic differentiation *in vitro*

The osteogenic medium (OM) was used to induce osteogenic differentiation of BMSCs. The medium contained 10% FBS, 1% penicillin/streptomycin, 50 μ g/mL ascorbic acid (Sigma), 10 mM sodium β -glycerophosphate (Sigma), and 10 nM dexamethasone (Sigma). The OM was used in subsequent experiments for induction for 7 days (ALP staining, real-time PCR, and Western blot) and 21 days (Alizarin Red S staining).

Alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining analysis

BMSCs cultured in 24-well plates (1×10^5 cells/mL, 0.5 mL/well) were treated with 200 μ M H₂O₂ and capsaicin (1–10 μ M) for 4 h, followed by differentiation in OM with corresponding capsaicin concentrations. BMSCs were cultured in OM for 7 days (ALP staining) and 21 days (ARS staining), then fixed with 4% paraformaldehyde (Servicebio) for 15 min at RT and washed three times with PBS. ALP staining was performed using an ALP staining kit (Beyotime), while mineralization was assessed by Alizarin red S (ARS) staining (Beyotime), with both staining procedures lasting 15–30 min at RT followed by three washes with distilled water. Stained cells were then imaged under a bright-field microscope (Leica) for qualitative analysis.

Analysis of protein and mRNA expression level

BMSCs cultured in 6-well plates (1×10^5 cells/mL, 2 mL/well) were treated with 200 μ M H₂O₂ and capsaicin

Table 1 Nucleotide sequences of the primers used for RT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Bax	CATGAAGACAGGGCCTTTTG	TCAGTCTCTTGGTGGATGCGTC
Bcl-2	GATGACCGAGTACCTGAACCG	CAGAGACAGCCAGGAGAAATC
Alp	AACGTGGCCAAGAATCATCA	TGTCCATCTCCAGCCGTGTC
Ocn	TG AGGACCCTCTCTCTGCTC	GGGCTCCAAGTCCATTGTT
Runx2	GCACCCAGCCCAATAGA	TTGGAGCAAGGAGAACCC
Beclin1	CAGTGTTGTTGCTCCATGCT	CACCTGCTCCAGTGTCTTCA
P62	CAAGATGGAGCCGGAGAATA	GCACCACTCTCTTCTGGAG
Lc3b	CCTGTCTGGATAAGACCAAG	GGATACACTACCATGTGTGTG
GAPDH	CAGGGCTGCCTTCTCTGT	TCCCGTTGATGACCAGCTTC

(1–10 μ M) for 4 h, followed by differentiation in OM with corresponding capsaicin concentrations. Total protein was extracted from cells cultured in OM at day 0 (pre-induction) and day 7 (post-induction) using RIPA lysis buffer (BioSharp) containing protease/phosphatase inhibitors (Beyotime), with protein concentrations quantified using a BCA assay kit (Beyotime). For Western blot (WB) analysis, proteins were probed overnight at 4°C with antibodies to GAPDH (1:5,000; Abcam), RUNX2, SP7, and ALP (1:1,000; ABclonal, China). After three 10 min TBST washes, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; ABclonal) for 1 h at RT. Protein signals were detected using ECL reagent (Millipore, USA) and imaged with an Amersham ImageQuant 800 system (Cytiva, USA), with band intensities analyzed using ImageJ software.

For mRNA analysis, osteogenesis-related genes (OCN, RUNX2, ALP) were quantified by RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen, USA), reverse-transcribed into cDNA with PrimeScript™ RT Master Mix (TaKaRa, Japan), and amplified using Hieff® qPCR SYBR Green Master Mix (Yeasen, China) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, China). GAPDH served as an endogenous control, and relative gene expression was calculated via the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 1.

Intracellular Ca^{2+} dynamics analysis

BMSCs were cultured in 96-well black plates (1×10^5 cells/mL, 100 μ L/well) and treated for 4 h under four conditions: serum-free α -MEM (control), 200 μ M H_2O_2 alone, 200 μ M H_2O_2 with 10 μ M capsaicin, and 200 μ M H_2O_2 with 1 μ M ionomycin (positive control). Cells were then loaded with 4 μ M Fluo-4 AM (Beyotime) in PBS at 37°C for 30 min, washed twice with PBS, and de-esterified for 15 min at RT. Intracellular Ca^{2+} flux was measured by real-time fluorescence on a microplate reader (excitation at 488 nm, emission at 516 nm) with a 120 s baseline (F_0). Cells were then stimulated with 10 μ M capsaicin or 1 μ M ionomycin (Beyotime). Real-time fluorescence was monitored for 180 s post-stimulation using a microplate reader (Bio-Rad). Peak Ca^{2+} responses were calculated as $\Delta F/F_0 = (F - F_0)/F_0$, with peak amplitude defined as the maximal $\Delta F/F_0$ during the first 180 s.

Accessibility of autophagy

BMSCs cultured in 6-well plates (1×10^5 cells/mL, 2 mL/well) were treated with 200 μ M H_2O_2 and 10 μ M capsaicin for 4 h. Autophagy-related protein expression was analyzed by WB using antibodies against ATG5 (1:1,000; Abmart), P62 (1:1,000; Abmart), LC3B (1:1,000; Abmart), and GAPDH (1:5,000; Abcam) as the loading control. Parallel RT-PCR analysis was performed for autophagy-related genes (*Beclin1*, *P62*, and *Lc3b*) using

the methodology described earlier, with primer sequences listed in Table 1. To assess PI3K/AKT/mTOR pathway involvement, cells were co-treated with capsaicin and 2 μ M PI3K agonist 740Y-P (APExBIO, USA), followed by evaluation of protein and mRNA expression levels of the aforementioned autophagy markers.

Pathway analysis

BMSCs cultured in 6-well plates (1×10^5 cells/mL, 2 mL/well) were treated with 200 μ M H_2O_2 and 10 μ M capsaicin for 4 h. The activation status of the oxidative stress-related PI3K/AKT/mTOR pathway was evaluated by WB analysis using the following antibodies: phospho-PI3K (P-PI3K, 1:1,000; Abmart), total PI3K (1:1,000), phospho-AKT (P-AKT, 1:1,000), total AKT (1:1,000), phospho-mTOR (P-mTOR, 1:1,000), and total mTOR (1:1,000) (all from Abmart). To further investigate the pathway regulation, parallel experiments were conducted with co-treatment of capsaicin and 2 μ M PI3K agonist 740Y-P (APExBIO), followed by analysis of the corresponding protein expression changes in the PI3K/AKT/mTOR pathway.

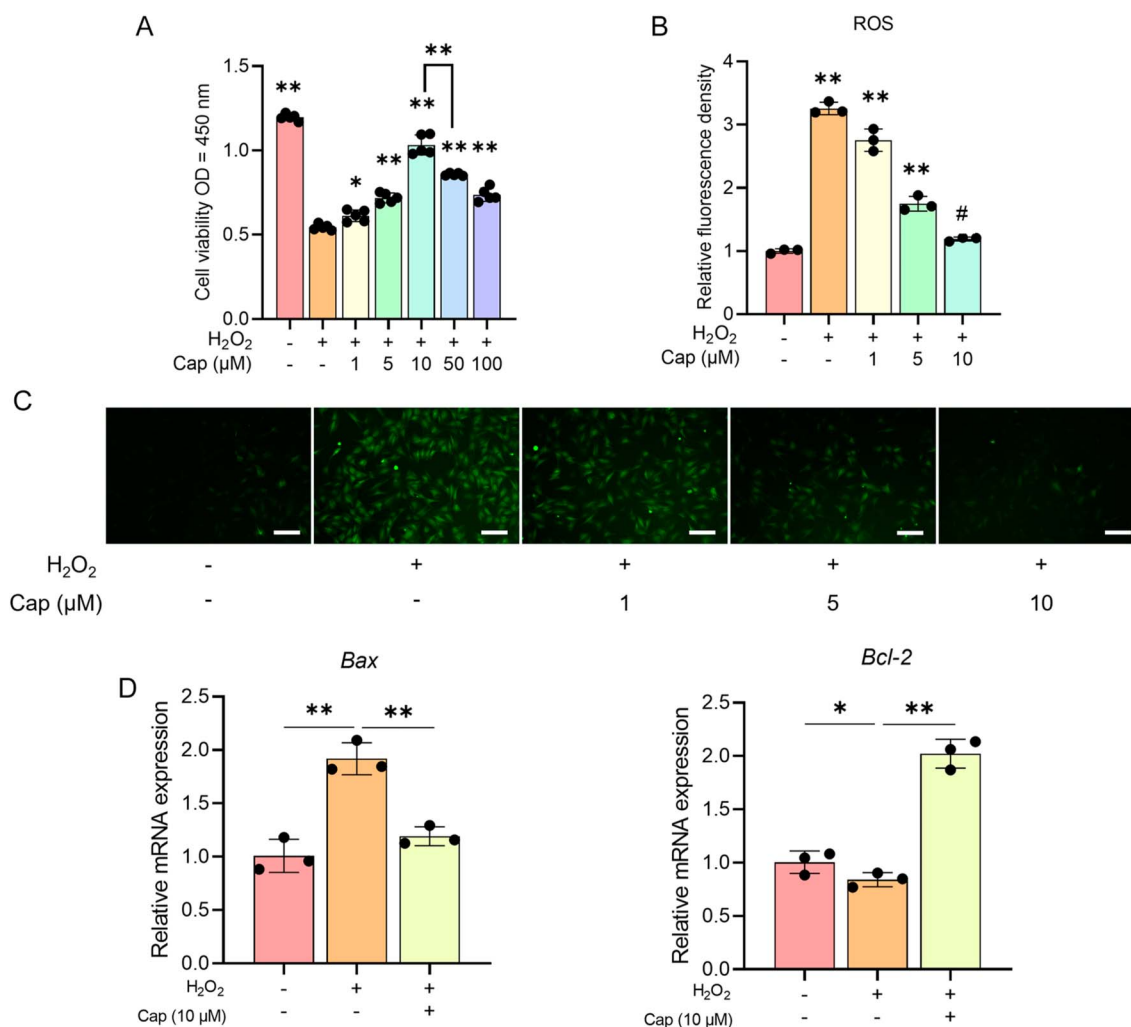
Statistical analysis

All experiments were independently repeated at least three times. Data are expressed as mean \pm standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA followed by the Bonferroni *post hoc* test for multiple comparisons, with $P < 0.05$ considered statistically significant.

Results

Capsaicin reduces oxidative stress and apoptosis in H_2O_2 -treated BMSCs

To evaluate the protective effect of capsaicin on BMSCs against oxidative stress, the viability of BMSCs under H_2O_2 -induced stress was first evaluated using the CCK-8 assay. Exposure of BMSCs to 200 μ M H_2O_2 significantly reduced cell viability (vs untreated control group Fig. 2A). Treatment with 1–100 μ M capsaicin protected BMSCs from oxidative stress, as evidenced by significantly higher viability across all concentrations compared to the H_2O_2 group, and the most significant effect was observed at a capsaicin concentration of 10 μ M (Fig. 2A). To examine capsaicin's antioxidant property, we measured intracellular ROS levels using DCFH-DA probes. H_2O_2 treatment induced significant ROS accumulation in BMSCs, confirming oxidative damage. Capsaicin treatment (1–10 μ M) dose-dependently reduced intracellular ROS levels (Fig. 2B and C), and it also reduced *Bax* mRNA and increased *Bcl-2* mRNA under oxidative stress, indicating its anti-apoptotic effect (Fig. 2D). These results demonstrate that capsaicin protects cells by reducing oxidative stress in H_2O_2 -treated BMSCs.

**Figure 2**

Capsaicin (cap) reduces oxidative stress and apoptosis in H₂O₂-treated BMSCs. (A) The cell viability of BMSCs stimulated by H₂O₂ treated with cap (1–100 μM) was detected by CCK-8 assay (**P* < 0.05, ***P* < 0.01 vs H₂O₂ group). (B) Quantitative analysis of ROS fluorescence intensity (***P* < 0.01, #*P* > 0.05 vs untreated control group). (C) Representative fluorescence images of intracellular ROS levels detected using the DCFH-DA probe (scale bar: 100 μm). (D) RT-PCR analysis of apoptotic marker mRNA expression (**P* < 0.05, ***P* < 0.01 vs H₂O₂ group). A full color version of this figure is available at

<https://doi.org/10.1530/JME-25-0063>.

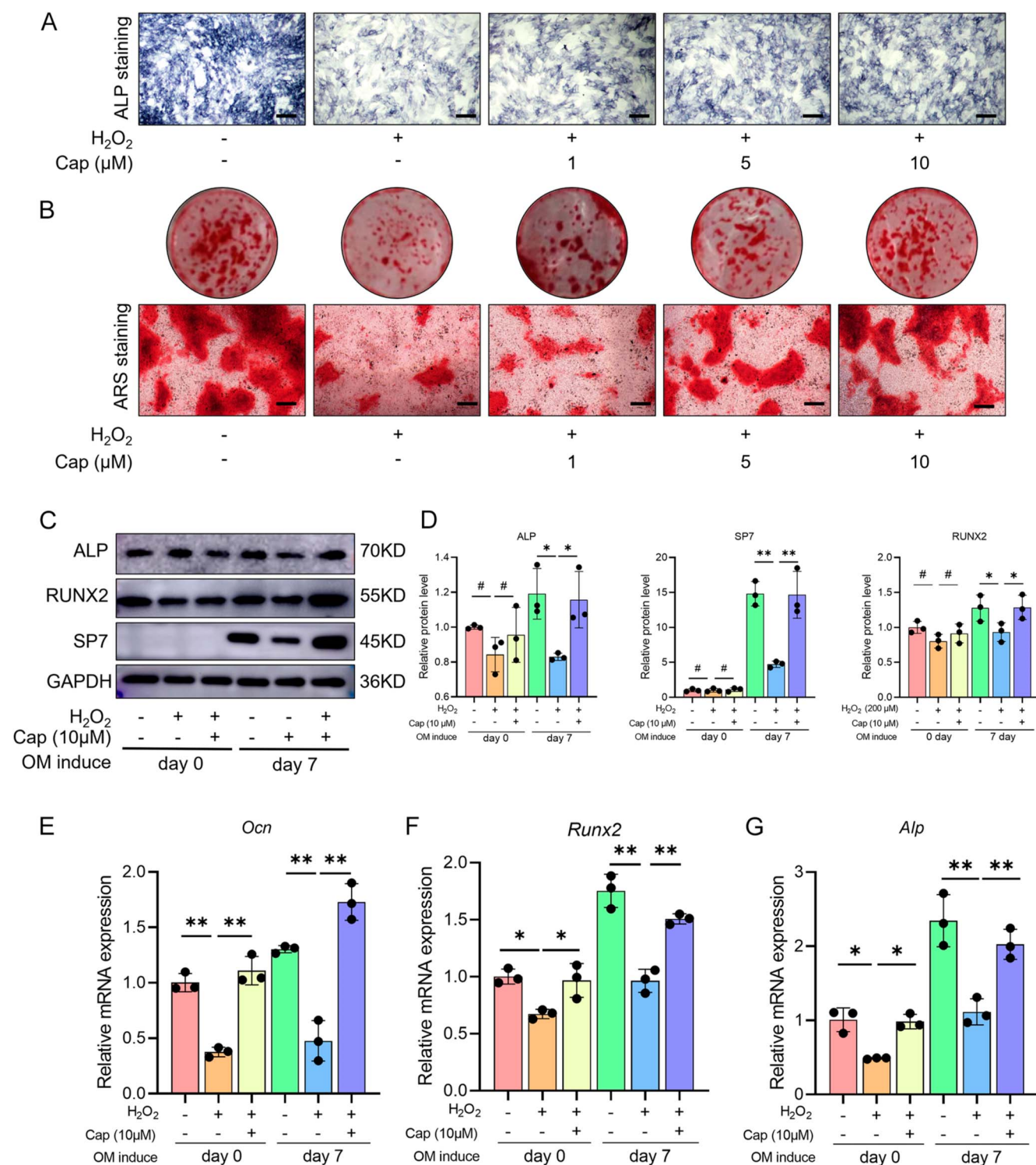
Capsaicin restores osteogenic differentiation in H₂O₂-treated BMSCs

To determine whether capsaicin can counteract the damage of H₂O₂ to the osteogenic ability of BMSCs, we conducted analysis at the phenotypic, protein, and transcriptional levels. ALP/ARS staining revealed a dose-dependent enhancement of osteogenic capacity in H₂O₂-treated BMSCs, with 10 μM capsaicin eliciting maximal alkaline phosphatase activity and mineralization (vs H₂O₂ group; Figs 3A and B and Supplementary Fig. S1A (see section on [Supplementary materials](#) given at the end of the article)). Furthermore, evidence showed that the expression of osteogenesis-related markers increased at both protein levels

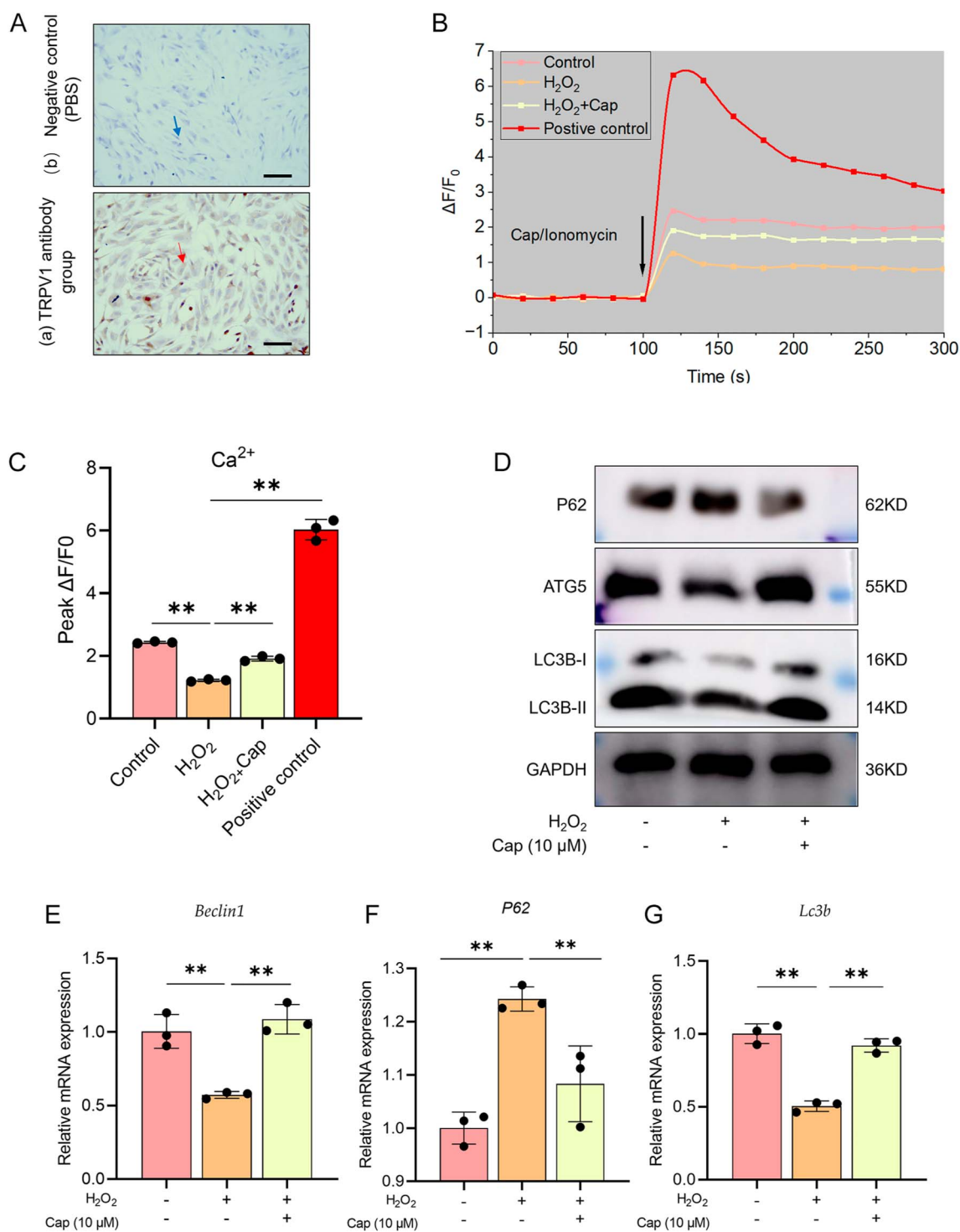
(ALP, RUNX2, SP7) and mRNA levels (*Ocn*, *Alp*, *Runx2*) compared to the H₂O₂ group (Fig. 3C, D, E, F, G). Validation with β-actin loading control yielded identical results for ALP (Supplementary Fig. S1B).

Capsaicin induces Ca²⁺ influx and autophagy in H₂O₂-treated BMSCs

Immunohistochemistry (IHC) analysis revealed TRPV1 expression in BMSCs: specific immunoreactivity was detected in antibody-treated cells, whereas no specific signal was observed in PBS-negative control (Fig. 4A), suggesting that TRPV1 may be a putative target of capsaicin in these cells. Fluo-4 assays demonstrated

**Figure 3**

Capsaicin (cap) restores osteogenic differentiation in H₂O₂-treated BMSCs. (A) Cap-treated BMSCs were ALP-stained after 7 days (scale bar: 100 μm). (B) Cap-treated BMSCs were ARS-stained after 21 days (scale bar: 100 μm). (C) Western blot analysis of osteoblast protein expression in BMSCs. (D) Quantitative analysis of osteogenic protein levels. (E, F, G) RT-PCR analysis of osteogenic marker mRNA expression ([#]*P* > 0.05, **P* < 0.05, ***P* < 0.01 vs H₂O₂ group). A full color version of this figure is available at <https://doi.org/10.1530/JME-25-0063>.

**Figure 4**

Capsaicin (cap) restores Ca^{2+} homeostasis and promotes autophagy activation in H_2O_2 -treated BMSCs. (A) IHC detection of TRPV1 in BMSCs. (a) TRPV1 antibody group: specific immunoreactivity (brown staining, red arrows). (b) Negative control (PBS): background staining (blue arrow) with no specific signal (scale bar: 100 μ m). (B) Representative Fluo-4 traces: capsaicin-induced Ca^{2+} influx (arrows = stimulus timing). (C) Quantification of peak Ca^{2+} response ($\Delta F/F_0$) (** $P < 0.01$ vs H_2O_2 group). (D) Western blot analysis of autophagy protein levels in BMSCs. (E, F, G) RT-PCR analysis of the mRNA level of autophagy genes (** $P < 0.01$ vs H_2O_2 group). A full color version of this figure is available at <https://doi.org/10.1530/JME-25-0063>.

that H_2O_2 significantly impaired agonist-evoked Ca^{2+} influx (Fig. 4B and C). However, capsaicin effectively restored Ca^{2+} responsiveness, resulting in a substantial increase in peak $\Delta F/F_0$ compared to H_2O_2 -treated cells ($P < 0.01$). In addition, ionomycin, used as a positive control, elicited robust responses in the H_2O_2 groups, thereby validating the functionality of the assay. Furthermore, capsaicin was shown to rescue autophagy impairment in BMSCs under oxidative stress conditions. Western blot and RT-PCR analyses consistently revealed capsaicin-mediated autophagy activation, as evidenced by significant alterations in key autophagy markers at both protein (ATG5, P62, LC3B-II) and mRNA (*Beclin1*, *P62*, *Lc3b*) levels (Figs 4D, E, F, G and Supplementary Fig. S2A). Collectively, these results demonstrate that capsaicin exerts protective effects against oxidative damage in BMSCs alongside coordinated changes in calcium signaling and autophagy pathways, suggesting a potential mechanistic contribution of these pathways.

Capsaicin activates autophagy through the PI3K/AKT/mTOR pathway

Western blot analysis revealed that H_2O_2 treatment significantly increased phosphorylation levels of PI3K, AKT, mTOR, P-PI3K, P-AKT, and P-mTOR, while capsaicin intervention effectively suppressed these phosphorylation events without altering total PI3K, AKT, or mTOR protein expression (Figs 5A and Supplementary Fig. S1C). Notably, co-administration of the PI3K-specific agonist 740Y-P partially reversed capsaicin-mediated inhibition of P-PI3K, P-AKT, and P-mTOR phosphorylation (Figs 5B and Supplementary Fig. S1D). To illustrate the role of the PI3K/AKT/mTOR pathway in H_2O_2 -stimulated autophagy, the PI3K agonist 740Y-P was used. Western blot and RT-PCR analyses confirmed that capsaicin activated autophagy, as evidenced by significant alterations in key autophagy-related proteins (ATG5, P62, and LC3B-II) and corresponding mRNA expression levels (*Beclin1*, *P62*, and *Lc3b*).

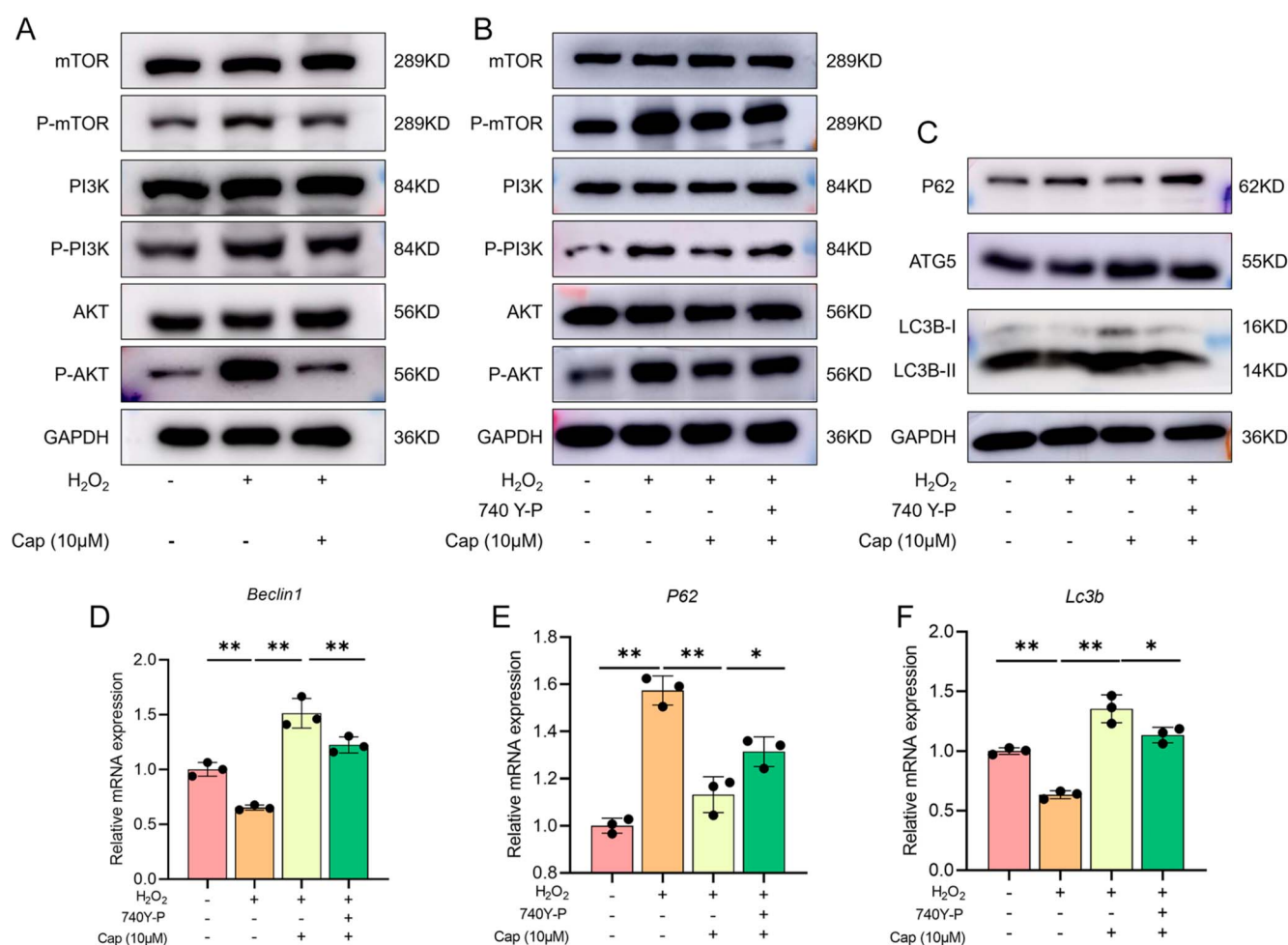


Figure 5

Capsaicin (cap) activates autophagy through the PI3K/AKT/mTOR pathway. (A) Western blot analysis of the PI3K/AKT/mTOR pathway under H_2O_2 stress. (B) Western blot analysis of the PI3K/AKT/mTOR pathway after co-treatment with 740Y-P (PI3K agonist). (C) Western blot analysis of autophagy-related proteins after co-treatment with 740Y-P. (D, E, F) RT-PCR analysis of autophagy-related mRNA after co-treatment with 740Y-P ($*P < 0.05$; $**P < 0.01$ vs H_2O_2 group). A full color version of this figure is available at <https://doi.org/10.1530/JME-25-0063>.

Importantly, the capsaicin-induced autophagy effects were significantly attenuated upon treatment with the PI3K activator 740Y-P (Figs 5C, D, E, F and Supplementary Fig. S2B), suggesting a potential involvement of the PI3K/AKT/mTOR pathway in the modulation of autophagy by capsaicin.

PI3K/AKT/mTOR pathway activation attenuates capsaicin-induced osteogenic effects

In an H₂O₂-induced oxidative stress model, capsaicin treatment markedly restored the osteogenic differentiation capacity of BMSCs by suppressing the PI3K/AKT/mTOR pathway. Specifically, a 7-day capsaicin intervention reversed H₂O₂-mediated inhibition of ALP activity (Fig. 6A), and a 21-day treatment significantly enhanced mineralization nodule formation (Fig. 6B). Upon co-treatment with the PI3K activator 740Y-P, the capsaicin-induced osteogenic effects were attenuated (Figs 6A and B, Supplementary Fig. S2C). Moreover, 740Y-P suppressed the expression of osteogenic markers ALP, RUNX2, and SP7 (Figs 6C and Supplementary Fig. S2D). Collectively, these results reveal that capsaicin alleviates oxidative stress-impaired osteogenesis in BMSCs by suppressing the PI3K/AKT/mTOR pathway.

Discussion

Osteoporosis is the most common bone disease characterized by decreased bone mineral density, structural deterioration, and increased risk of fracture, which poses a serious health threat to the aging population (NIH Consensus 2001, Brihan *et al.* 2020). Oxidative stress is a key pathological driver of this condition (Tan *et al.* 2015). In this study, capsaicin significantly reduced ROS levels and maintained the viability and osteogenic differentiation of BMSCs. Mechanistically, capsaicin protects BMSCs during oxidative stress possibly via TRPV1-mediated calcium influx and the PI3K/AKT/mTOR pathway to activate autophagy. These findings highlight its therapeutic potential for osteoporosis.

BMSCs are essential for bone regeneration, but oxidative stress significantly reduces their survival rate and osteogenic potential (Lan *et al.* 2022a). At physiological concentrations, ROS play a beneficial role, promoting cellular proliferation and differentiation (Sauer *et al.* 2001). However, excessive ROS accumulation triggers mitochondrial dysfunction and DNA oxidative damage, ultimately leading to apoptotic cell death (Liguori *et al.* 2018, Zou *et al.* 2024). Capsaicin is a natural antioxidant extracted from capsicum, which has a strong ability to scavenge ROS (Chaudhary *et al.* 2022). Studies have found that it can reduce inflammatory bone loss induced by lipopolysaccharide by inhibiting the production of prostaglandin E from osteoblasts (Kobayashi *et al.*

2012). In this study, capsaicin at 1–100 μ M significantly improved the survival rate of BMSCs under H₂O₂ damage, and BMSCs activity was highest at 10 μ M (Fig. 2A). Treatment with 10 μ M capsaicin significantly reduced ROS levels and apoptosis in BMSCs induced by 200 μ M H₂O₂ (Fig. 2B, C, D), reversed the H₂O₂-induced suppression of ALP activity and mineralized nodule formation (Figs 3A and B, Supplementary Fig. S1A), and upregulated the expression of osteogenic markers (Fig. 3C, D, E, F, G). Collectively, these results suggest that capsaicin protects the activity and osteogenic function of BMSCs via antioxidant properties.

Capsaicin can activate TRPV1 and lead to the influx of Ca²⁺ (Xu *et al.* 2020, Munjuluri *et al.* 2021). This study found that BMSCs express the TRPV1 receptors (Fig. 4A), suggesting TRPV1 as a potential molecular target for capsaicin action in these cells. Ca²⁺, as a key second messenger in cells, maintains intracellular homeostasis by regulating mitochondrial energy metabolism, endoplasmic reticulum stress responses, and enzyme activities (Iqbal *et al.* 2020, Najjar *et al.* 2021). Critically, our data reveal that H₂O₂-induced oxidative stress suppresses intracellular Ca²⁺ influx in BMSCs (Fig. 4B and C), impairing calcium signaling pathways – an effect reversed by capsaicin. Intracellular Ca²⁺ signaling has been shown to regulate autophagy, although its exact role remains ambiguous. Recent studies have demonstrated that mTOR inhibitors, such as rapamycin, enhance autophagy while remodeling the intracellular Ca²⁺-signaling machinery, highlighting the interplay between Ca²⁺ and autophagy (Decuypere *et al.* 2013). As a key pathway for cells to remove damaged components and maintain homeostasis, autophagy has been confirmed to be closely related to oxidative stress damage (Filomeni *et al.* 2015). Furthermore, autophagy activation can reduce the accumulation of ROS and promote the recovery of mitochondrial function, playing a protective role (Yun *et al.* 2020, Lin *et al.* 2021). Studies have shown that autophagy inhibition in bone cells leads to bone loss (Onal *et al.* 2013). Similar results were also found using BMSCs, indicating that adaptive autophagy can resist oxidative stress-induced apoptosis and bone loss (Wang *et al.* 2017). This study shows that capsaicin protects BMSCs under oxidative stress by activating autophagy (Figs 4D, E, F, G and S2A). Moreover, the restoration of autophagy paralleled the recovery of calcium signaling (Fig. 4), suggesting that calcium may act as the key upstream regulator for capsaicin to regulate autophagy.

The PI3K/AKT/mTOR pathway is an intracellular signaling cascade comprising phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) (Zhao *et al.* 2022b). Studies have shown that the PI3K/AKT/mTOR pathway plays an important role in osteogenesis and endochondral ossification (Valer *et al.* 2019), participating in autophagy and regulating intracellular ROS homeostasis (Yu & Cui 2016). This pathway is crucial

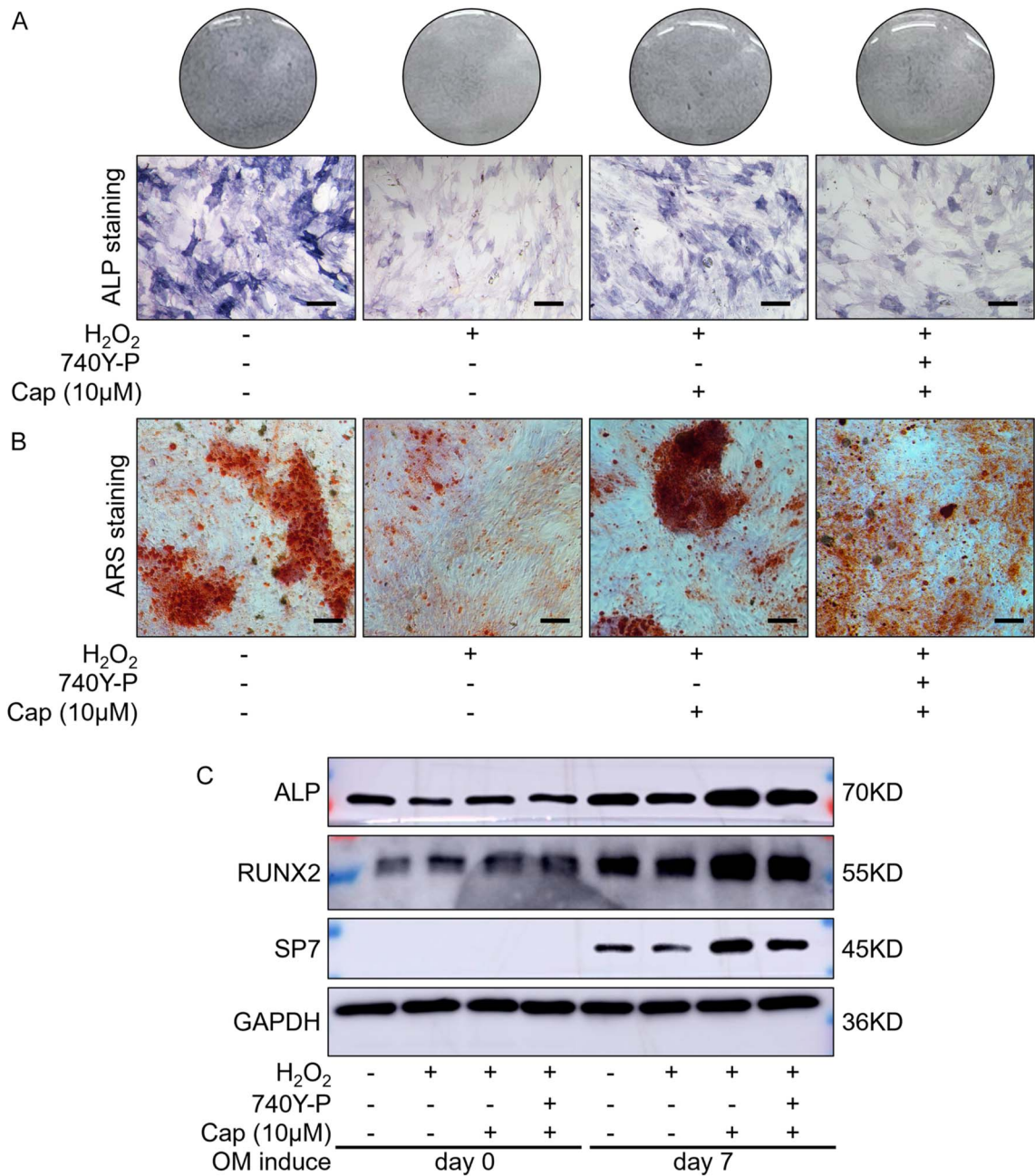
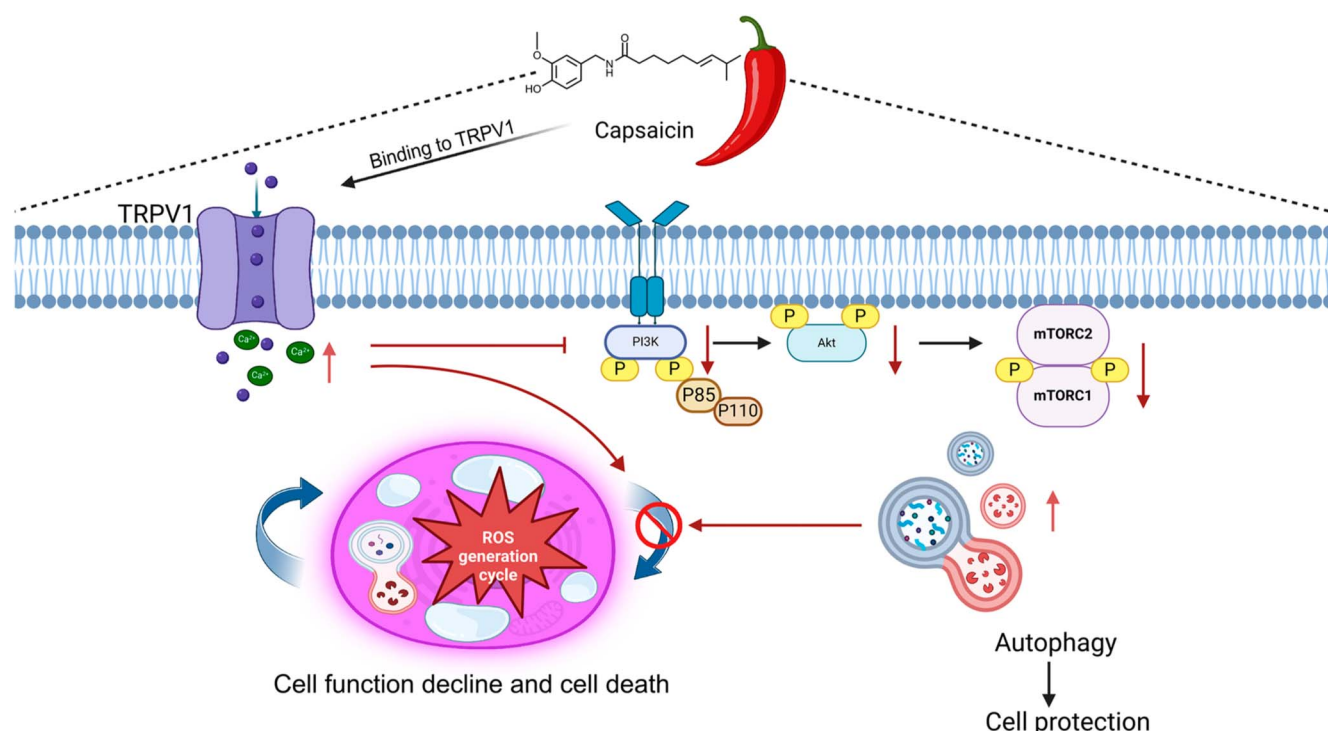


Figure 6
PI3K/AKT/mTOR pathway activation attenuates capsaicin (cap)-induced osteogenic effects. (A) BMSCs treated with cap and 740Y-P under H₂O₂-induced oxidative stress for 7 days were stained for ALP (scale bar: 100 μm). (B) BMSCs treated with cap and 740Y-P under H₂O₂-induced oxidative stress for 21 days were stained by ARS (scale bar: 100 μm). (C) Western blot analysis of osteogenesis proteins after co- treatment with 740Y-P. A full color version of this figure is available at <https://doi.org/10.1530/JME-25-0063>.

for cell survival and is generally considered to mediate the cellular response to oxidative stress (Aggarwal *et al.* 2019). Contrastingly, our results demonstrate that capsaicin treatment significantly reduced the H₂O₂-induced increase in phosphorylation levels of PI3K, AKT, and mTOR (Figs 5A and Supplementary Fig. S1C). To further verify the pathway in the function of capsaicin, the PI3K agonist 740Y-P was applied. 740Y-P partially or completely abolished capsaicin’s inhibitory effect on the PI3K/AKT/mTOR pathway (Figs 5B and Supplementary Fig. S1D).

**Figure 7**

Schematic diagram of the mechanism by which capsaicin protects the function of BMSCs under oxidative stress. Black arrows (→) indicate the progression process. The red symbol (⊥) indicates an inhibitory effect. Red downward arrows (↓) indicate a decrease, and red upward arrows (↑) indicate an increase. This figure was created using [BioRender.com](https://www.biorender.com). (Note: the specific involvement of TRPV1 is based on expression and calcium influx observations but requires functional validation). A full color version of this figure is available at <https://doi.org/10.1530/JME-25-0063>.

In fact, it has been suggested that the PI3K/AKT/mTOR pathway may act as a double-edged sword in different contexts (Yin *et al.* 2017), and an increasing number of scientists are beginning to recognize this. For instance, the benefit of inhibiting the ROS-induced DNA damage-activated PI3K/AKT/mTOR pathway was observed in *Helicobacter pylori* infection (Xie *et al.* 2018). Furthermore, more research indicates that inhibiting the PI3K/AKT/mTOR pathway also has benefits in LPS-induced kidney injury, neurodegeneration, and osteoporosis (Singh *et al.* 2017, Wang *et al.* 2020, Zhao *et al.* 2020). Therefore, based on previous reports and our research findings, and taking into account the different perspectives on the PI3K/AKT/mTOR pathway, this could be the key to explaining the contradictory issues of the PI3K/AKT/mTOR pathway in different situations. In addition, capsaicin activated autophagy, whereas co-treatment with 740Y-P suppressed this activation (Figs 5C, D, E, F and S2B). These findings are consistent with the classic negative regulation theory of autophagy (mTOR is a key negative regulator of autophagy) (Zhao *et al.* 2015). Importantly, capsaicin promoted osteogenic differentiation by activating autophagy via the PI3K/AKT/mTOR pathway, an effect reversed by the PI3K agonist 740Y-P (Figs 6A, B, C and S2C and D).

Collectively, these results indicate that capsaicin improves BMSCs' osteogenic function under oxidative stress through inhibition of the PI3K/AKT/mTOR pathway and activation of autophagy.

Although this study used only male rat BMSCs, research evidence indicates the beneficial effects of capsaicin may be gender-independent (Melekoglu *et al.* 2018, Bingül *et al.* 2024). Thus, capsaicin likely exerts a similar protective effect against oxidative stress on female BMSCs. This protective effect of capsaicin (illustrated in Fig. 7, a mechanism schematic diagram) suggests a novel therapeutic strategy for osteoporosis. Compared with traditional therapeutic drugs, the advantage of capsaicin lies in its ability to achieve long-term steady-state regulation by activating endogenous protective mechanisms (autophagy). However, it is critical to acknowledge a key limitation: while our IHC data confirmed TRPV1 expression on BMSCs and capsaicin evoked calcium influx, the absence of functional validation (e.g., using TRPV1 knockdown or knockout models) precludes definitive assignment of causal responsibility to TRPV1 for mediating the observed effects of capsaicin. Therefore, Fig. 7 depicts a hypothesized pathway where

TRPV1 signaling potentially contributes to capsaicin protecting BMSCs under oxidative stress but may not be essential for capsaicin's actions. Future studies should focus on mechanistic exploration in models that are closer to physiological and pathological conditions, particularly by employing genetic approaches such as TRPV1 knockout to directly test the involvement of this receptor in capsaicin's protective effects on BMSCs, to facilitate the translation of this natural compound into therapeutic applications.

Conclusions

This study demonstrated that BMSCs express TRPV1. Capsaicin activates protective autophagy by inhibiting the PI3K/AKT/mTOR pathway, thereby protecting the osteogenic differentiation capacity of BMSCs under oxidative stress. These findings provide a novel therapeutic approach for osteoporosis.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-25-0063>.

Declaration of interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was funded by: i) Shanghai Stomatological Hospital & School of Stomatology, Fudan University, grant numbers SSH-2024-C01 and SSH-2022-KJCX-B05; ii) Shanghai Municipal Health Commission, grant number 202140504.

Data availability

All data supporting the findings of this study are available within the article and its Supplementary Materials.

Ethics statement

The animal experiments were approved by the Animal Care and Use Committee of the Department of Laboratory Animal Science of Tongji University (ID: SYSZZH-047).

Acknowledgments

The authors would like to express their sincere gratitude to all those who contributed to this work. We are deeply grateful to Professor Qi for his invaluable guidance and support throughout this study. We would also like to extend our special thanks to Professor Wang for her expert advice and critical insights. We also thank our research team members for their dedication and collaboration, as well as our families for their unwavering encouragement during challenging times. Finally, we acknowledge the experimental platform and facilities provided by Shanghai Stomatological Hospital & School of Stomatology, Fudan University, which were essential to the completion of this research.

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