Acta Biochim Biophys Sin xxxx, xx(x): xx-xx
https://cstr.cn/32197.14.abbs.2026065
https://doi.org/10.3724/abbs.2026065
Original Article

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Ginsenoside Rh2 alleviates osteoporosis by attenuating oxidative stress-induced osteoblast dysfunction via the FoxO1/β-catenin pathway

Wenqi Jin[1], Yujing Tan[1], Xuenan Chen[1], Zehan Guo[1], Jixiang Ren[2,*], and Liwei Sun[1,*]

[1]Research Center of Traditional Chinese Medicine, the Affiliated Hospital of Changchun University of Chinese Medicine, Changchun 130021, China, and [2]Center of Preventive Treatment of Diseases, the Affiliated Hospital of Changchun University of Chinese Medicine, Changchun 130021, China

*Correspondence address. E-mail: renjx@ccucm.edu.cn (J.R.); sunlw@ccucm.edu.cn (L.S.)

Received 9 November 2025 Accepted 16 March 2026 Published 24 June 2026

Abstract

The degree of oxidative stress decreases osteoblast function with age, which leads to a decline in bone compressive capacity. Ginsenoside Rh2 is a known clinical or adjuvant therapy for various tissues. In this study, we investigate the pharmacological effects of Rh2 against oxidative stress-induced osteoblasts. Osteoblasts are pretreated with Rh2 for 48 h and then exposed to hydrogen peroxide (H_2O_2), which results in significantly decreased ROS levels, increased antioxidant enzyme activity, and enhanced mitochondrial function. Functionally, Rh2 increases alkaline phosphatase (ALP) expression, together with enhanced mineralization and expression of osteogenesis-associated genes. Rh2 also promotes the nuclear translocation of FoxO1 and β-catenin, whereas it does not reverse reduced mineralization caused by decreased FoxO1 or β-catenin activity, indicating that its effect is mediated through the functional interaction between FoxO1 and β-catenin. In a mouse model of lipopolysaccharide (LPS)-induced bone loss, Rh2 administration improves trabecular microstructure, increases osteoblast numbers, and upregulates serum metabolites associated with bone formation. Immunofluorescence analysis further reveals that Rh2 promotes the nuclear co-localization of FoxO1 and β-catenin in femurs, indicating their coordinated action within this signaling axis. These findings indicate that Rh2 mitigates oxidative stress-induced osteoblast dysfunction via the FoxO1/β-catenin pathway, highlighting the pivotal role of redox balance in bone remodeling and suggesting a promising therapeutic strategy for osteoporosis.

Key words ginsenoside Rh2, osteoblasts, oxidative stress, osteogenic, FoxO1/β-catenin

Introduction

Osteoporosis is a degenerative disease characterized by progressive deterioration of bone microstructure, leading to reduced bone mass and an increased fracture risk [1]. It tends to be associated with age and thus represents a major problem as populations age and life expectancies increase. Bone remodeling involves two interconnected processes: osteoclast-mediated resorption or destruction of the mineralized bone matrix and osteoblast-mediated formation of new bone [2]. Imbalances in the differentiation of osteoblasts and osteoclasts, characterized by reduced osteoblast activity and increased osteoclast activity, result in osteoporosis. Accumulating evidence suggests an association between elevated oxidative stress and reduced osteoblast differentiation, demonstrating that higher levels of oxidative stress result in the dysfunction and apoptosis of bone cells, ultimately causing bone loss [3,4] Therefore, minimizing ROS production, scavenging free radicals, and enhancing antioxidant activity are important for preventing or delaying bone loss.

Excessive accumulation of ROS leads to oxidative stress, which damages cellular macromolecules and ultimately results in cell death [5]. Forkhead box O (FoxO) proteins are highly conserved transcription factors that regulate biological processes, including proliferation, differentiation, oxidative stress resistance, apoptosis, and inflammation, in response to various endogenous and exogenous stimuli [6,7]. FoxOs are primarily phosphorylated by PI3K/Akt signaling, resulting in their translocation from the nucleus to the cytoplasm, inhibiting FoxO-dependent transcription of target

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genes [8]. Oxidative stress induces FoxO1 expression, which upregulates antioxidant enzymes to counteract ROS [9,10]. Therefore, FoxO1 acts as a crucial cellular sensor pivotal for mitigating oxidative damage. FoxOs have been shown to modify the pathogenesis of skeletal diseases by their ability to influence both bone mass and strength [11]. Specifically, FoxO1 is a master regulator of osteoblast function and redox balance according to cell-specific knockout and molecular analysis [12,13]. The interaction of FoxO1 with Runx2 or its promoter stimulates osteoblast differentiation and mineralization nodule formation [14]. β-Catenin functions as an essential cofactor for FoxO1, and their interaction is central to transducing ROS-induced downstream signaling [15]. This leads to altered transcription of target genes and the antagonization of Wnt/TCF pathways in osteoblast precursors under stress conditions [16]. Consequently, pharmacological modulation of FoxO1/β-catenin signaling may represent a promising therapeutic strategy for osteoporosis by preserving osteoblast function in an adverse redox environment.

Ginseng (*Panax ginseng* C.A. Meyer) is a traditional Chinese medicinal herb that contains ginsenosides as its principal bioactive constituents, which are widely utilized in clinical and adjuvant treatments for various diseases. Ginsenosides have beneficial effects through multiple molecular mechanisms, including anti-apoptotic, anti-oxidative, anti-aging, anti-cancer, and functional restoration activities [17–19]. Notably, recent studies have documented that ginsenosides such as Rh2 inhibit osteoclastogenesis while promoting osteoblast differentiation and mineralization [20]. Nevertheless, the specific mechanisms by which Rh2 regulates osteoblast function, particularly under conditions of oxidative stress, remain unclear, and current evidence is still limited.

Herein, the protective effects of Rh2 on osteoblast function under conditions of oxidative stress were investigated. Osteoblasts were exposed to H_2O_2 *in vitro* to induce oxidative stress. The effects of Rh2 on osteoblast differentiation, oxidative damage resistance, and mineralization were evaluated, with a particular focus on its regulation of the FoxO1/β-catenin signaling. To further validate the protective effects *in vivo*, we employed a lipopolysaccharide (LPS)-induced bone loss model. LPS triggers systemic inflammation that activates NADPH oxidase in immune and bone cells, leading to a surge in ROS and subsequent oxidative stress. This state of oxidative stress impairs osteoblast function and promotes osteoclast formation, thereby recapitulating key pathological features of osteoporosis associated with oxidative stress. In this model, we evaluated the therapeutic and preventive effects of Rh2 on osteoporosis. The findings provide novel insights into the mechanism by which Rh2 protects osteoblasts from oxidative stress-induced damage.

Materials and Methods

Materials

Ginsenoside Rh2 (≥ 97%), H_2O_2, MTT, dimethyl sulfoxide (DMSO), AS1842856, and ICG001 were purchased from Merck (Darmstadt, Germany), while fetal bovine serum (FBS) and α-MEM were purchased from Gibco (Grand Island, USA). The Annexin VFITC/PI Apoptosis Detection Kit, ROS Kit, and JC-1 Kit were from BD Biosciences (San Diego, USA). Antibodies against cleaved caspase-3 (9664), Cytochrome C (Cyt C; 4280), p-GSK-3β (9323), GSK-3β (9315), Histone H3 (3638), and GAPDH (2118) were purchased from Cell Signaling Technology (Danvers, USA). Anti-collagen I (Col I; ab260043), osteopontin (OPN; ab307994), Runx2 (ab76956), osterix (Osx; ab209484), Bcl-2 (ab196495), and Bax (ab32503) were purchased from Abcam (Cambridge, USA). All other reagents were of analytical grade.

Ethical declaration

All animal protocols were carried out following the guidelines for the care of experimental animals indicated by the Animal Ethics Committee of Changchun University of Chinese Medicine and in accordance with the NIH Guide for taking care and use of laboratory animals. In addition, all experimental procedures adhered to the guidelines and regulations of the Animal Ethics Committee of Changchun University of Chinese Medicine (approval No. 2022486, 9/13/2022), ensuring compliance with national and international ethical standards.

Cell culture and treatment

Bone marrow mesenchymal stem cells (BMSCs) were obtained from the tibias and femurs of six 4-week-old male SD rats (Changchun Yisi Animal Feeding Center, Changchun, China). In brief, rats were euthanized by intraperitoneal injection of pentobarbital at a dose of 60 mg/kg. The femur and tibia were dissected aseptically, and α-MEM was drawn into a syringe to repeatedly flush the bone marrow cavity. The collected bone marrow cells were centrifuged at 400 *g* for 5 min, and the supernatant washing buffer was discarded. The resultant bone marrow cell suspension was mixed with complete α-MEM with 10% FBS and 1% PS and filtered through a 70-μm filter to remove possible bone fragments. Cells were cultured at 37°C in an incubator with 5% CO_2. These cells were identified as BMSCs based on flow cytometric analysis of their positive expression of the mesenchymal stem cell marker CD90 and negative expression of the hematopoietic marker CD45. Clones were grown until confluent and then treated with trypsin-EDTA (Gibco) for passage, with cells from the second to fifth passages used for experiments. Upon reaching confluence, cells were treated with 10 mM β-glycerophosphate and 50 μg/mL ascorbate (Sigma-Aldrich, St Louis, USA) to induce osteoblast differentiation for 18 days, following established protocols [21].

Cell viability assay

BMSCs in 96-well plates (2×10^4 cells/well) were differentiated into osteoblasts for 10 days. Cells were then treated with Rh2 at graded concentrations (0, 1.25, 2.5, 5, 10, 20, and 40 μM) for 48 h. The spent media were aspirated, and the cells were incubated with or without H_2O_2 (200 μM) for 6 h. Following the inclusion of 0.5 mg/mL MTT in PBS at 37°C for 4 h, the precipitates were solubilized in DMSO (150 μL per well), and absorbances at 570 nm were read in a microplate reader (5082; TECANA, Magellan, Austria). The viability was calculated as the number of viable cells in relation to the vehicle control.

Flow cytometric analysis

Prior to harvest, cells were pretreated with Rh2 (2.5, 5, or 10 μM) for 48 h and then exposed to H_2O_2 (200 μM) for 6 h. The collected cells were subsequently incubated with Annexin V-FITC (5 μL) in binding buffer for 15 min, followed by PI (10 μL) treatment for 5 min. The numbers of apoptotic cells were assessed with a FACScan flow cytometer (BD Bioscience, San Jose, USA), recording 10,000 events. The intracellular ROS level was determined using the ROS assay kit, and the mitochondrial membrane potential (MMP)

was assessed using the JC-1 kit following loading with 5 μM JC-1 for 20 min. Both ROS levels and MMP were measured on the FACScan flow cytometer and reported as mean fluorescence intensity (MFI).

Fluorescence microscopy

Cells were incubated with Rh2 (2.5, 5, or 10 μM) and/or H_2O_2 (200 μM), followed by the addition of fresh media with 50 nM MitoTracker Green and incubation at 37°C for 30 min. Morphological assessment of the mitochondria was undertaken using a cell imaging multifunctional test system (Cytation 5; BioTek, Winooski, USA), quantification of the fluorescence intensities with ImageJ (NIH, Bethesda, USA) and presentation as the percentage of the relative fluorescence intensity.

Antioxidant enzyme activity measurement

After two rinses in PBS, cells were lysed in 0.5 mL of 0.1 M HCl containing 0.1% Triton X-100 (Solarbio, Beijing, China) on ice for 30 min. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured using commercial kits (Beyotime, Beijing, China) according to the manufacturer's instructions.

ALP activity assay

ALP activity was measured to investigate whether Rh2 mitigates H_2O_2-induced osteoblast function. After pretreatment with different concentrations of Rh2 for 48 h, cells were induced to differentiate for 7 to 10 days, and then exposed to 200 μM H_2O_2 for 6 h. After fixation with 4% paraformaldehyde (PFA; Solarbio) and washing, cells were incubated with BCIP/NBT as specified in the ALP assay kit (Beyotime) for 30 min in the dark. ALP activity is shown as a percentage of activity relative to that of the control.

Calcified nodule staining assay

Cells were grown for 18 days and stained with 2% alizarin red solution (Sigma-Aldrich) for 30 min to assess extracellular mineralization. Stained cells were rinsed thrice with distilled water, and calcium nodules, stained bright red, were visualized under a light microscope (TH4-200; Olympus, Tokyo, Japan). The stain was released from the cells by incubation with 100 mM cetylpyridinium chloride for 1 h, and the absorbance at 570 nm was read with a microplate reader (Bio-Rad, Hercules, USA), as described previously [21]. Results were expressed in relation to the total protein content of each well. For the pathway inhibition experiment, before Rh2 treatment, the cells were treated with or without the FoxO1/β-catenin inhibitor AS1842856 (2 μM)/ICG001 (20 μM) for 2 h.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol (TIANGEN, Beijing, China) and evaluated on a NanoDrop 2000/c spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Two micrograms of the RNA were reverse-transcribed to cDNA using the PrimeScript RT reagent (TaKaRa, Dalian, China). qPCR was performed using SYBR Green PCR Master Mix (Bio-Rad) in a Bio-Rad CFX96 System. The reaction conditions were 40 cycles of 95°C for 5 min, followed by 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to *β-actin* as an internal control. The primer sequences are given in Table 1.

siRNA transfection

Osteoblasts were cultured in 6-well plates until cells reached 80% confluence. Then, cells were transfected with either 30 nM *FoxO1*-specific siRNA or negative control siRNA (NC-siRNA; AM4611; Thermo Fisher Scientific) for 24 h in Opti-MEM™ medium (Gibco) using Lipofectamine™ 3000 reagent (Invitrogen, Waltham, USA) according to the manufacturer's instructions. The nucleotide sequences of the *FoxO1* siRNA are as follows: sense, 5′-CCAGGCAC CUCAUAACAAA-3′ and antisense, 5′-UUUGUUAUGAGGUGC CUGG-3′. The sequences of the NC-siRNA are sense, 5′-UUCUCCGA ACGUGUCACGU-3′, and antisense, 5′-ACGUGACACGUUCGGAG AA-3′. After transfection, the efficiency was evaluated using reverse transcription-quantitative PCR (RT-qPCR) and assessment of osteogenic differentiation.

Western blot analysis

Cells were lysed with RIPA buffer (Beyotime) containing protease inhibitors (Sigma-Aldrich) for 30 min on ice. Protein concentration was measured by the BCA reagent (Beyotime), and proteins (40 μg per lane) were separated by 8%–15% SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Marlborough, USA). After blocking in 5% skim milk for 1 h, the membranes were probed with primary antibodies against cleaved caspase-3 (1:1000), Cyt C (1:1000), Bax (1:2000), Bcl-2 (1:1000), Runx2 (1:2000), Osx (1:1000), OPN (1:1000), Col I (1:1000), FoxO1 (1:1000), β-catenin (1:1000), p-GSK-3β (1:1000), GSK-3β (1:1000), H3 (1:1000) or GAPDH (1:1000) overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000; Proteintech, Wuhan, China) for 1 h and visualized using a chemiluminescent imaging system (FluorChem H2; ProteinSimple, San Jose, USA).

Animal model establishment

Male 6-week-old C57BL/6 mice were procured from Changchun Yisi Animal Feeding Center. The mice were housed in specific pathogen-free (SPF) facilities under a 12/12 h light/dark cycle with standard controlled conditions (temperature, 24 ± 2°C; humidity, 55%) and free access to food and water. After one week of acclimatization, the mice were randomly assigned to three groups (n = 6/group) using a random number table. The groups were as following: control

Table 1. Sequence of the primers used in this study

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
ALP	ACCTCCTCACACCTACCAGTCTC	CCCACACTCCACCCGATGAAC
Col1A1	CCTGCCACCTGTGCCTATGTC	AGGAGGAGGAAGAGGAAGGAGAAG
OPN	GACGACGATGACGACGGAGAC	TGTGTGCTGGCAGTGAAGGAC
Runx2	CCTTCCACTTCCGCCACACTC	CCATCGCAACTACCGCTCTAGG
Osx	CCACTGAGTAGCGGCAAGGAC	CTGGCACGAAGATTACTGGCAATG
β-actin	TGGCACCCAGCACAATGAA	GTCATAGTCCGCCTAGAAGCA

(treated with PBS only), LPS (treated with LPS only), and LPS + Rh2 groups. LPS (10 mg/kg body weight) was injected intraperitoneally on days 1 and 4 to induce bone loss in the mice. Rh2 (100 mg/kg/day) or PBS was administered intragastrically one day before the initial LPS injection and was continued every other day for 8 days. All mice were euthanized by CO_2 inhalation, and blood and tissue samples were collected for analysis. The serum levels of carboxy-terminal cross-linked telopeptide of type 1 collagen (CTX-1) and bone alkaline phosphatase (BALP) were determined with commercial kits (Jiancheng Biotech, Nanjing, China) following the provided directions.

Histochemical analysis

The femurs of mice were dissected, fixed in 4% PFA, and decalcified with 12% EDTA (pH 7.2). The softened bone was removed, dehydrated in ethanol, and embedded in wax, after which the trimmed wax block was placed in a microtome for slicing into 4-μm-thick sections. The sections were stained with hematoxylin and eosin (HE) and toluidine blue (TB) to examine bone histomorphometry. The bone volume fraction and number of osteoblasts per bone surface (N.Ob/BS) were calculated using ImageJ software.

Immunofluorescence staining

Sections were incubated with primary antibodies against FoxO1 (1:100; 2880; Cell Signaling Technology) and β-catenin (1:500; 54025; Cell Signaling Technology) overnight at 4°C. Following washes with PBS, sections were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated secondary antibodies (Proteintech). The nuclei were counterstained with DAPI for 5 min. Images were captured using a confocal laser scanning microscope (Nikon, Tokyo, Japan) and analyzed with ImageJ software for colocalization assessment.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) from three separate experiments. One-way ANOVA followed by Tukey *post hoc* tests were employed to assess the significance of differences using Prism 8.0 (GraphPad, San Diego, USA). Statistical significance thresholds were established at $P < 0.05$.

Results

Ginsenoside Rh2 increases the viability of H_2O_2-induced osteoblasts

First, we evaluated the osteoblast viability after Rh2 treatment, thus determining its effective concentration range. MTT assay results showed that Rh2 concentrations below 20 μM were noncytotoxic (Figure 1A). Exposure to 200 μM H_2O_2 for 6 h significantly lowered viability, and this was selected as the model for oxidative damage. Pretreatment with Rh2 (2.5, 5, and 10 μM) for 48 h significantly reversed the H_2O_2-induced cell viability inhibition in a dose-dependent manner. Notably, the protective effect of 10 μM was comparable to that of 20 μM, with no additional benefit observed at the higher concentration (Figure 1B). We finally determined the range of 2.5–10 μM for subsequent mechanistic research. These findings indicate that Rh2 treatment increased cell viability after H_2O_2-induced injury in osteoblasts.

Ginsenoside Rh2 attenuates H_2O_2-induced oxidative stress in osteoblasts

Highly reactive oxygen radicals derived from mitochondrial transport chains are responsible for ROS generation, leading to most oxidative stress in cells. The effects of Rh2 pretreatment for 48 h on ROS level, mitochondrial function, and antioxidant levels were thus evaluated. As shown in Figure 2A, after H_2O_2 exposure, there was a marked increase in ROS. However, these levels were reduced after pretreatment with different Rh2 concentrations, with optimal inhibition observed at 10 μM Rh2 ($P < 0.01$). The red/green JC-1 fluorescence ratio represents the ΔΨm, with lower values indicating mitochondrial depolarization, leading to apoptosis. Flow cytometry showed that Rh2 pretreatment restored MMP in H_2O_2-treated osteoblasts ($P < 0.01$) (Figure 2B). The effects of Rh2 on oxidative stress causing mitochondrial compromise, seen as altered mitochondrial density, were then assessed. Rh2 was effective in preventing H_2O_2-mediated reductions in mitochondrial fluorescence ($P < 0.001$) (Figure 2C). Rh2 treatment recover the activities of SOD, CAT, and GPx (Figure 2D). Thus, these findings indicate that Rh2 is effective in reducing ROS accumulation and mitochondrial damage, increasing the contents of antioxidants to protect osteoblasts from oxidative stress induced by H_2O_2.

Ginsenoside Rh2 prevents H_2O_2-induced reduction of osteoblast differentiation and mineralization

To address the role of Rh2 in BMSC-derived osteogenesis, changes in the early-stage differentiation and late-stage mineralization of osteoblasts were observed in osteogenic induction medium. H_2O_2 exposure reduced ALP expression and mineralization in osteoblasts on day 7 and day 18, respectively, compared to controls ($P < 0.001$). Pretreatment with Rh2 for 48 h restored ALP-positive cell

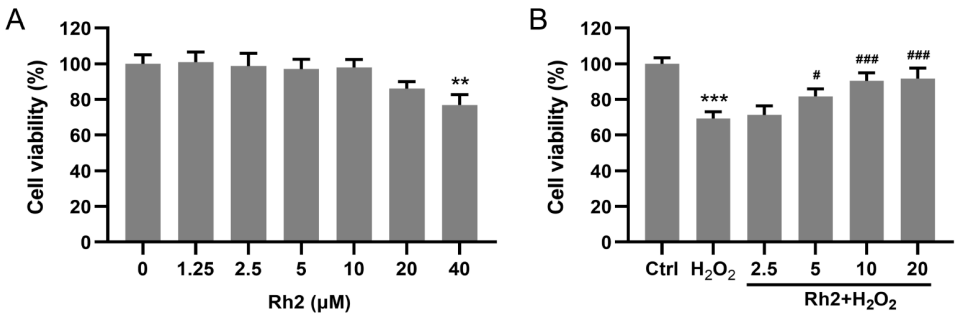

Figure 1. Effects of Rh2 on cell viability in H_2O_2-exposed osteoblasts (A) Osteoblasts were treated with various concentrations of Rh2 for 48 h, and then cell viability was determined. (B) The cells were treated with or without Rh2 at the indicated concentrations for 48 h in the presence of H_2O_2. Values are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs Ctrl group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs H_2O_2 group.

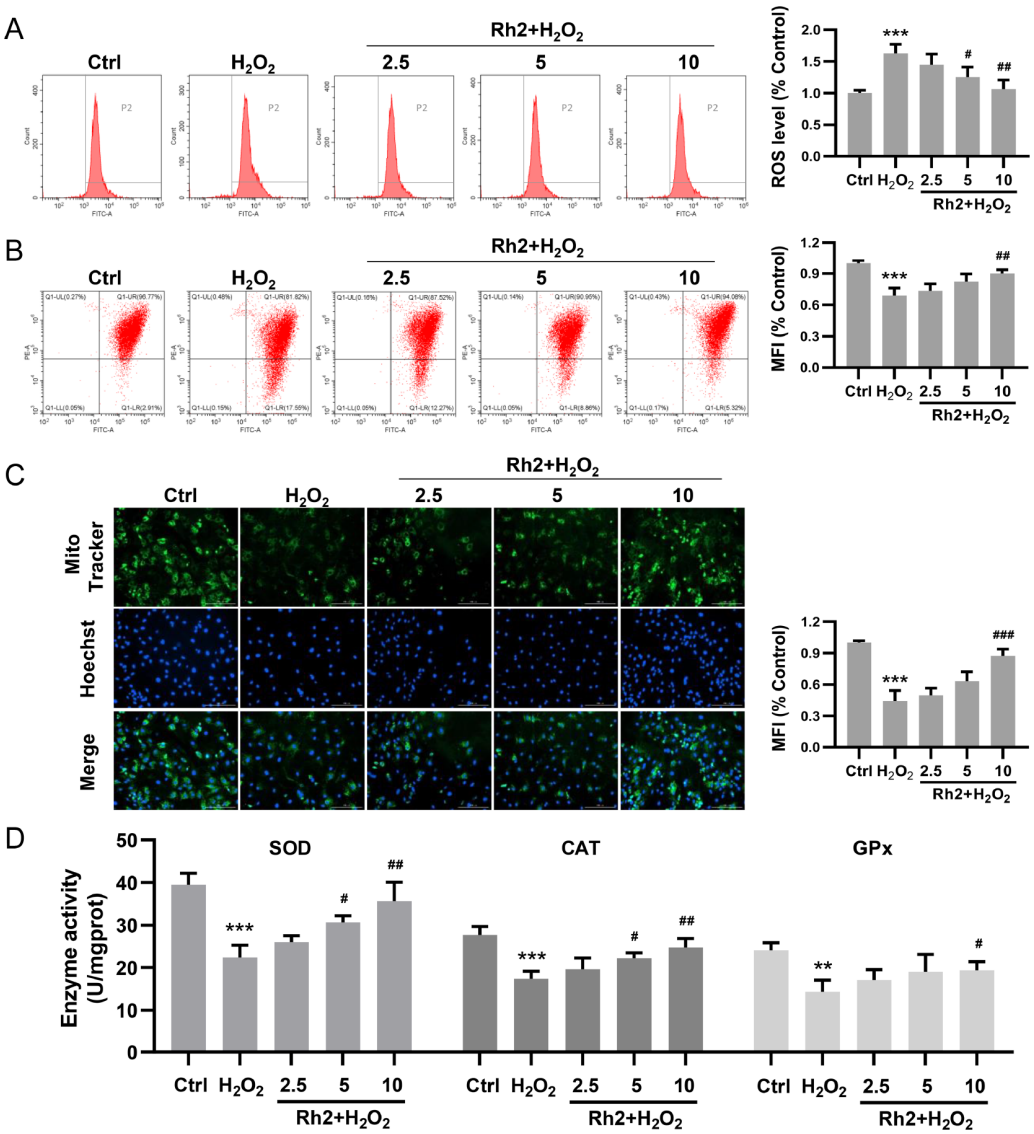

Figure 2. Effects of Rh2 on ROS generation, mitochondrial damage and antioxidant enzyme activity in H_2O_2-exposed osteoblasts (A) Intracellular ROS levels in osteoblasts treated with or without Rh2 for 48 h before treatment with 200 μM H_2O_2 were detected by flow cytometry. (B) Mitochondria membrane potential (MMP) analysis by JC-1 intake method. (C) Representative images of mitochondrial staining generated by MitoTracker Green. Magnification, 200×. (D) Activities of SOD, CAT, and GPx in H_2O_2-exposed osteoblasts treated with Rh2. Values are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs H_2O_2 group.

populations and prevented mineralization loss under oxidative stress (Figure 3A). Consistent with the staining results, Rh2 was found to prevent reductions in ALP activity and extracellular matrix mineralization (Figure 3B,C). These results indicate that the addition of Rh2 prevents oxidative stress-induced reductions in osteoblast differentiation.

Ginsenoside Rh2 counteracts H_2O_2-induced down-regulation of osteoblastogenic markers

To investigate the effect of Rh2 on osteogenic dysfunction after H_2O_2 exposure, the biological effects of Rh2 on the mRNA and protein expression of osteoblast markers and whether osteogenic disruption is associated with oxidative stress were examined. The data showed that H_2O_2 adversely affected osteoblast differentiation, as seen in the downregulation of the osteoblast markers ALP, Runx2, Osx, Col1A1, and OPN ($P < 0.01$). However, pretreatment with Rh2 reversed these effects (Figure 4A–E). Western blot analysis showed that H_2O_2 reduced Col I, OPN, Runx2, and Osx protein levels, while Rh2 pretreatment prevented this reduction ($P < 0.05$; Figure 4F,G). These results suggest that Rh2 counteracts the oxidative damage-induced reduction in osteoblast differentiation by enhancing the expression of the transcription factor Runx2 and its encoded bone differentiation markers.

Ginsenoside Rh2 attenuates H_2O_2-induced apoptosis in osteoblasts

Cells were treated with graded concentrations of Rh2 prior to H_2O_2 exposure to evaluate its protective effect against oxidative injury.

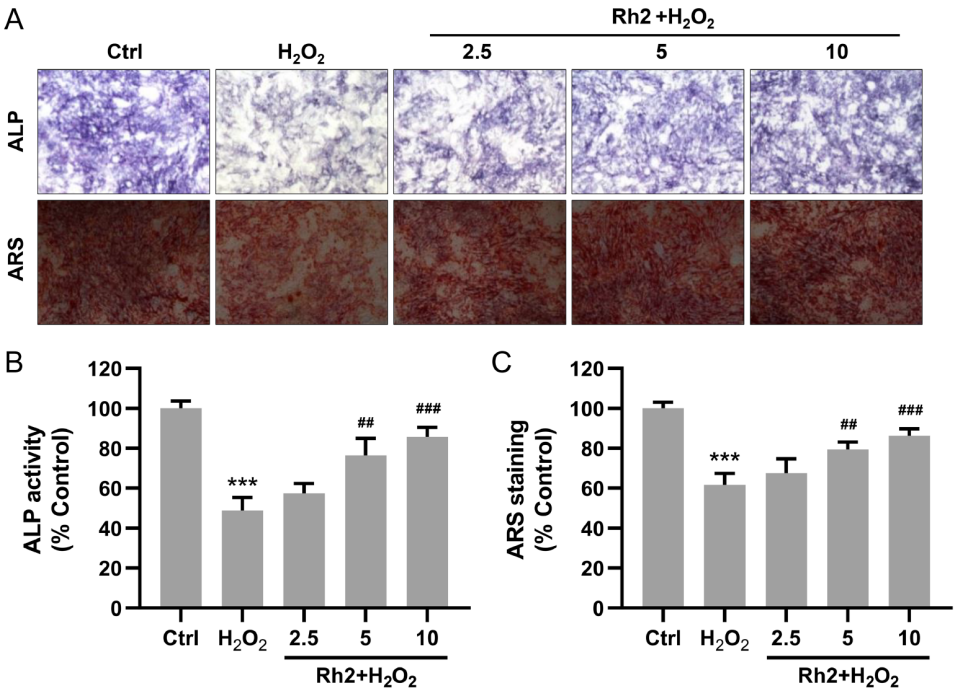

Figure 3. Rh2 alleviates H_2O_2-induced inhibition of ALP expression and mineralization in osteoblasts (A) Positive cells seen on a 7-day-old culture after ALP staining and on an 18-day-old culture after alizarin red S staining. Magnification, 200×. (B) ALP activity in the cell lysates was digitally graded on day 7. (C) Quantitative analysis of mineralized nodules (Ca^{2+} deposition) on day 18. Values are presented as the mean ± SD from three independent experiments. $^{***}P < 0.001$ vs Ctrl group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs H_2O_2 group.

Flow cytometry analysis revealed that Rh2 dose-dependently reduced apoptosis, with apoptosis rate decreasing to 19.38%, 16.09%, and 12.09% with increasing concentrations, respectively (Figure 5A). The mechanism by which Rh2 influences apoptosis was evaluated using western blotting, assessing the translocation of mitochondrial Cyt C to the cytosol, indicative of the cell's commitment to apoptosis. As shown in Figure 5B–D, H_2O_2 treatment elevated Cyt C levels, which were attenuated by Rh2 in a dose-dependent manner ($P < 0.001$). Rh2 treatment increased the Bcl-2/Bax ratio ($P < 0.001$) and decreased cleaved caspase-3 expression ($P < 0.05$) compared to H_2O_2-treated controls. These results demonstrate that Rh2 protects osteoblasts from oxidative apoptosis by restraining mitochondrial apoptotic effectors.

Ginsenoside Rh2 ameliorates oxidative stress damage via the upregulation of the FoxO1/β-catenin pathway

The FoxO1 transcription factor regulates cellular resistance to oxidative stress in a manner dependent on its co-activator β-catenin, which is also associated with the proliferation and differentiation of osteoblasts. Here, to clarify the molecular mechanism underlying the energy efficiency of Rh2, the FoxO1/β-catenin defense pathway was investigated. Exposure to H_2O_2 decreased nuclear FoxO1 levels while increasing its cytoplasmic accumulation, while the expression of β-catenin was reduced in both the nucleus and cytoplasm in comparison with the controls. In contrast, Rh2 administration significantly enhanced the nuclear translocation of both FoxO1 and β-catenin under oxidative stress ($P < 0.05$; Figure 6A,B). Treatment with the FoxO1 inhibitor AS1842856 and the β-catenin inhibitor ICG001 markedly reduced the number of calcium nodules in osteoblasts ($P < 0.001$); while Rh2 failed to restore mineralization under these inhibitory conditions ($P > 0.05$; Figure 6C). Moreover, osteoblasts were transfected with *FoxO1* siRNA to suppress FoxO1 expression. Evaluation of the silencing efficiency showed that compared with the si-control group, cells treated with *FoxO1* siRNA had lower FoxO1 expression (Figure 6D). The ability of Rh2 to increase osteogenic differentiation, as shown by ALP activity, was also reduced by the downregulation of FoxO1 (Figure 6E). These findings may further indicate that Rh2 partially reduces oxidative stress-induced osteoblast dysfunction by activating the FoxO1/β-catenin pathway.

Ginsenoside Rh2 attenuates LPS-induced osteopenia by activating FoxO1/β-catenin pathway

To identify the pharmacodynamic effect of Rh2 in osteoporosis, an LPS-induced bone loss mouse model for *in vivo* experiments was adopted. Mice were peritoneally injected with LPS with or without Rh2 treatment. As shown in Figure 7A, HE staining of femoral sections showed extremely damaged, sparse, disorganized trabeculae in LPS-treated mice compared to controls. Rh2 treatment improved trabecular structure. To investigate whether Rh2 affects histomorphometry in mice with bone loss, TB staining was performed. The strip cells indicated by the red arrow were osteoblasts. The osteoblasts per unit area were quantified, and the results showed that the number of osteoblasts/bone surface (N.Ob/BS) in the Rh2-treated group was much greater than that in the LPS-induced group ($P < 0.05$; Figure 7B). Then, the levels of the bone metabolism markers BALP and CTX-1 were detected in the serum of mice. Rh2 administration increased BALP level and decreased CTX-1 level in serum (Figure 7C,D), collectively indicating a shift in bone metabolism toward enhanced formation, reflecting a net anabolic bone state. The expressions of FoxO1 and β-catenin were further detected by immunofluorescence co-localization in bone

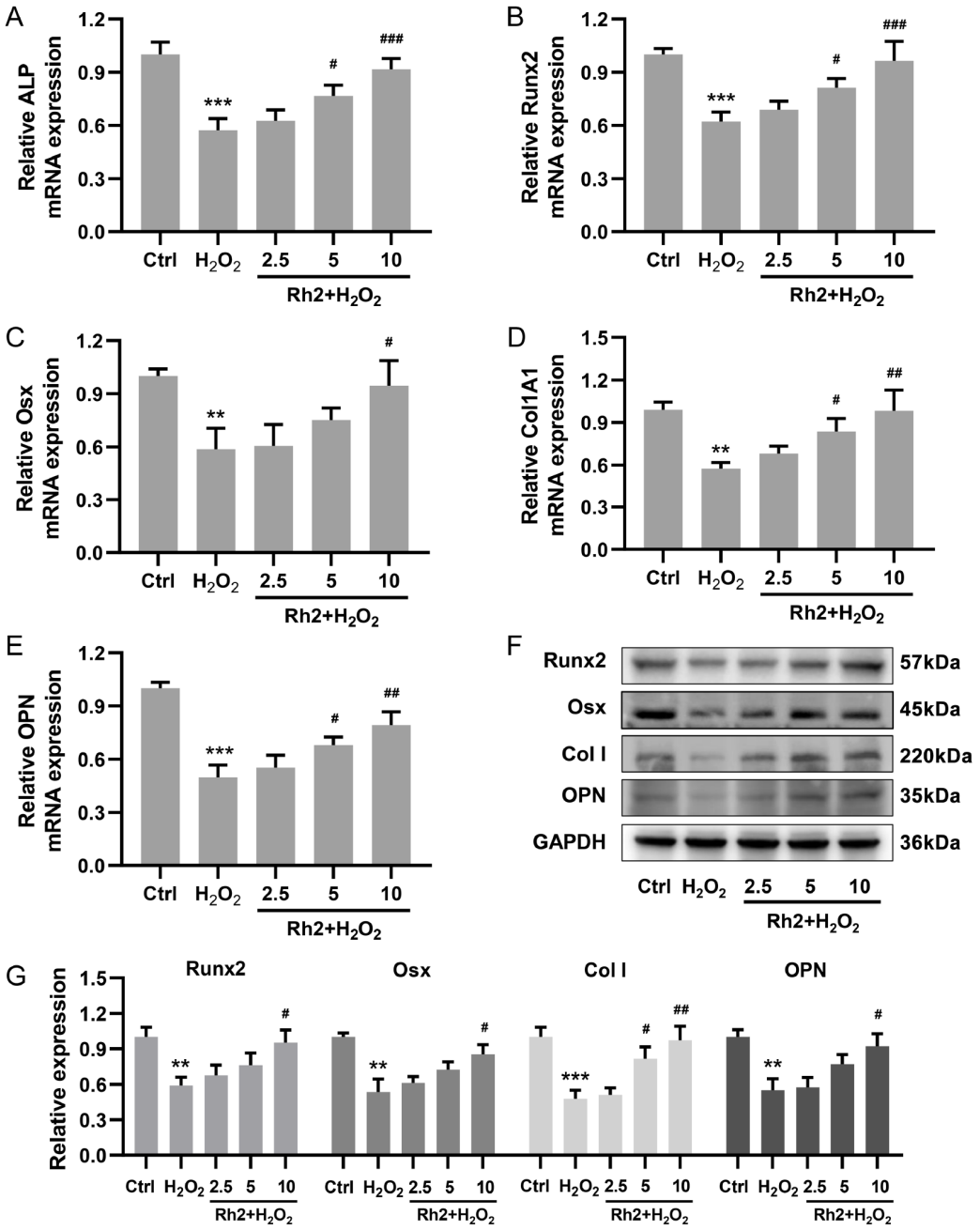

Figure 4. Rh2 enhances osteogenic differentiation and counteracts H_2O_2-induced osteoblast dysfunction Cells were pretreated with the indicated concentrations of Rh2 for 48 h, followed by treatment with H_2O_2 for 6 h. (A–E) The mRNA expression levels of ALP, Col1A1, OPN, Runx2, and Osx were assessed by qRT-PCR and normalized to *β-actin*. (F) The protein expression levels of osteoblast-specific markers were detected by western blot analysis. (G) Quantification of bands relative to GAPDH using ImageJ software. Values are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs H_2O_2 group.

tissue. In LPS-induced mice, FoxO1 showed increased translocation from the nucleus to the cytoplasm, while nuclear expression of β-catenin was markedly reduced without obvious changes in the cytoplasm. Rh2 treatment effectively reversed the FoxO1 cytoplasmic shift and enhanced β-catenin nuclear accumulation ($P < 0.05$; Figure 7E). The co-localization and coordinated nuclear trafficking of FoxO1 and β-catenin further suggest their potential interaction within this pathway, corroborating our *in vitro* evidence that Rh2 promotes osteogenesis via the FoxO1/β-catenin pathway.

Discussion

Age-related osteoblast senescence compromises differentiation and function, contributing to progressive bone degeneration [22,23]. This dysfunction is primarily driven by oxidative stress arising from ROS generated in the bone microenvironment and osteoblast metabolism [24,25]. Maintaining balanced ROS homeostasis is crucial for osteoblast differentiation, selfrenewal, and proliferation, while elevated ROS levels suppress osteogenic gene expression [26]. Hence, the notion that marked accumulation of oxidative stress is

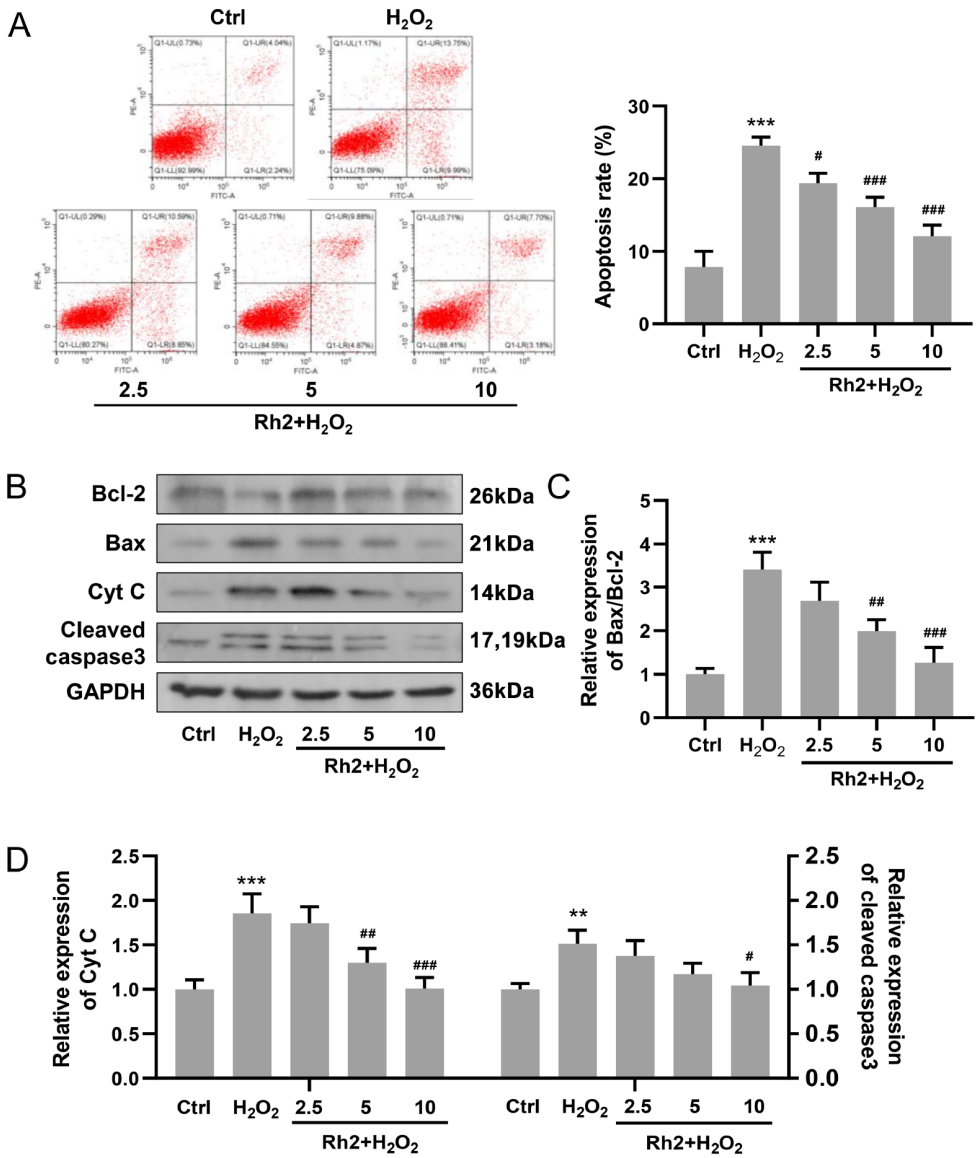

Figure 5. Rh2 protects against H_2O_2-induced apoptosis of osteoblasts Cells were untreated or pretreated with Rh2 at the indicated concentrations for 48 h before exposure to H_2O_2. (A) Flow cytometry analysis of cells labeled with Annexin V-FITC and PI. (B) Western blot analysis was performed using the indicated antibodies. (C,D) Quantitative analysis of Cyt C, cleaved caspase-3, and Bcl-2/Bax levels was performed using ImageJ software. Values are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs H_2O_2 group.

the cause of bone loss is becoming increasingly attractive. This study demonstrated that ginsenoside Rh2 is a potent antioxidant agent that can attenuate oxidative damage caused by H_2O_2 and upregulate the expression of osteoblastogenic markers. While several ginsenosides (*e.g.*, Re, Rc, Rb1, Rg1) have been reported to promote bone formation under physiological conditions [27], the efficacy of Rh2 specifically against oxidative stress-induced osteoblast dysfunction is less clear. Our findings reveal that this protective effect is mechanistically mediated by the FoxO1/β-catenin signaling pathway. This delineates a distinct mechanism for Rh2, setting it apart from other bone-active ginsenosides and addressing a key gap in the current understanding of its pharmacological action. Our results thus provide new evidence elucidating the protective effects of Rh2 against oxidative damage in osteoblasts and identify key signaling molecules that may serve as potential targets for intervention in osteoporosis.

ROS are natural by-products of oxidative metabolism and are primarily generated by electron leakage from the mitochondrial electron transport chain [28]. Studies have shown that the antioxidant N-acetylcysteine (NAC) can counteract ROS-mediated suppression of osteoblast function and restore antioxidant defense responses [29,30]. Mitochondria are also essential for maintaining the synthesis and biological function of metabolic factors, including BMPs, Wnt, and parathyroid hormone (PTH), in response to osteoblast differentiation [4]. In this study, Rh2 administration reversed H_2O_2-induced mitochondrial dysfunction, restoring membrane potential and mitochondrial density and thereby preserving osteoblast activity and function. Moreover, the activity of antiox-

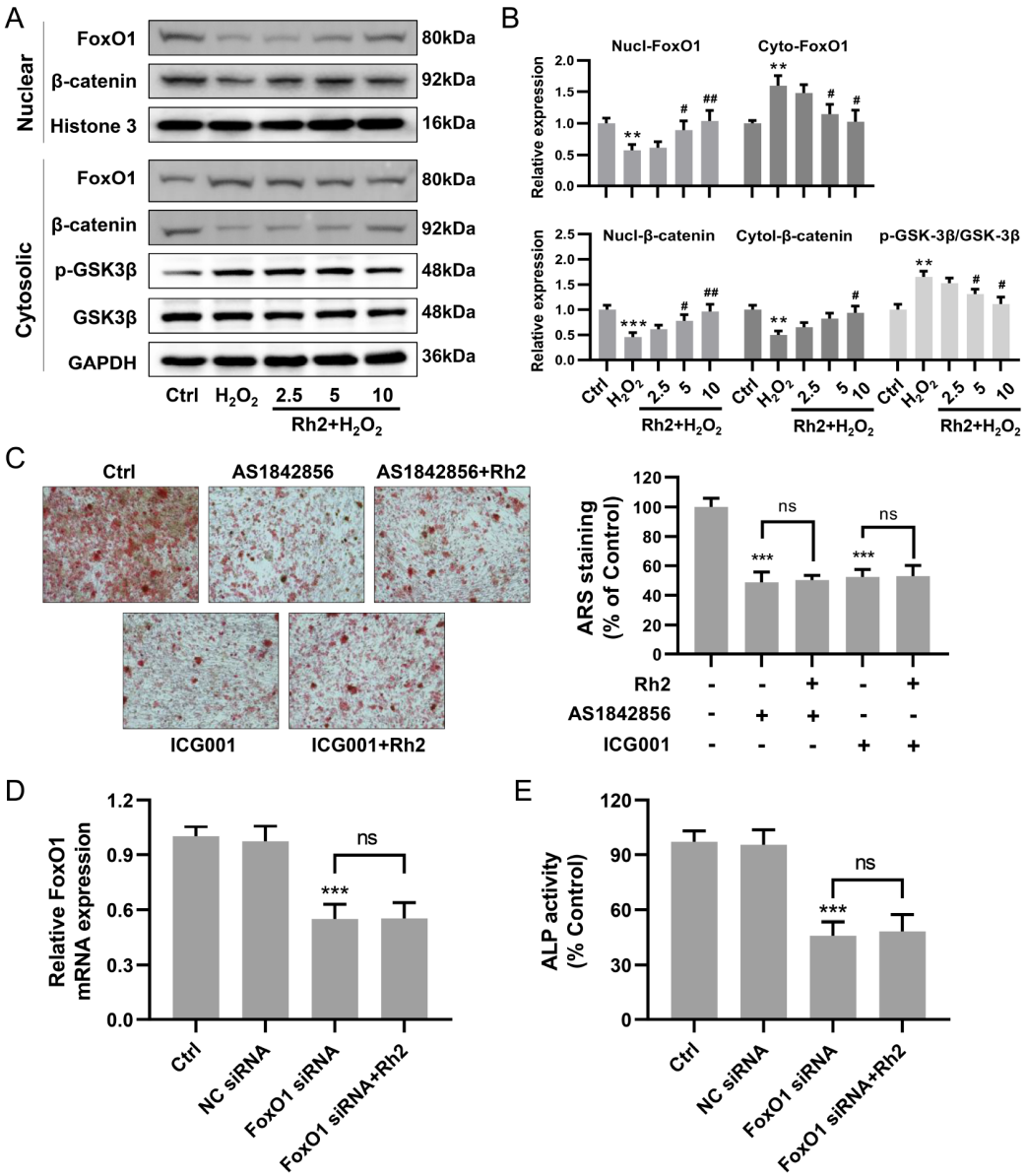

Figure 6. Rh2 attenuates the damage of osteoblasts to oxidative stress response to FoxO1/β-catenin pathway Cells were untreated or pretreated with Rh2 at the indicated concentrations for 48 h before exposure to H_2O_2. (A) The protein expression from whole cell lysates and nuclear fractions was immunoblotted for FoxO1, β-catenin, and GSK-3β. Histone 3 and GAPDH served as nuclear and cytoplasmic loading controls, respectively. (B) Quantitative analysis of relative band intensities of protein expression. (C) The relative changes in mineralized nodules were stained with alizarin red S after treatment with AS1842856 or ICG001 alone or in combination with Rh2. Magnification, 200×. (D,E) Relative mRNA expression levels were measured using RT-qPCR in *Foxo1* siRNA-transfected osteoblasts, while the effects of Rh2 on osteogenic differentiation were assessed by measuring ALP activity. Values are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs Ctrl group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs H_2O_2 group. ns, not significant.

idant defense enzymes was also improved, and they may antagonize the ROS generated by elevated oxygen consumption, thereby contributing to osteoblast differentiation. High ROS level can impair mitochondrial function by opening mitochondrial permeability transition pores, resulting in dysfunction caused by the presence of cytoplasmic Cyt C, where it interacts with caspase 9, resulting in caspase 3 activation and the triggering of apoptosis [31]. Rh2 pretreatment significantly reduced Cyt C expression and the Bax/Bcl-2 ratio, demonstrating that Rh2 protects osteoblasts from caspase-3 cleavage and subsequent cell death caused by the oxidative stress-induced mitochondrial apoptotic cascade.

FoxO1 serves as a critical regulator of redox homeostasis, dynamically responding to oxidative and other cellular stresses. It upregulates downstream antioxidant enzymes such as SOD and CAT, which catalyze the conversion of O_2^- into H_2O_2 and subsequently neutralize H_2O_2 [7,32]. Promoting endogenous initiation of antioxidant mechanisms breaks down the persistent increase in ROS after oxidative stress. Furthermore, within the FoxO family, FoxO1 is known to promote osteogenesis, which results from controlling bone mass and maintaining skeletal homeostasis. *FoxO1*

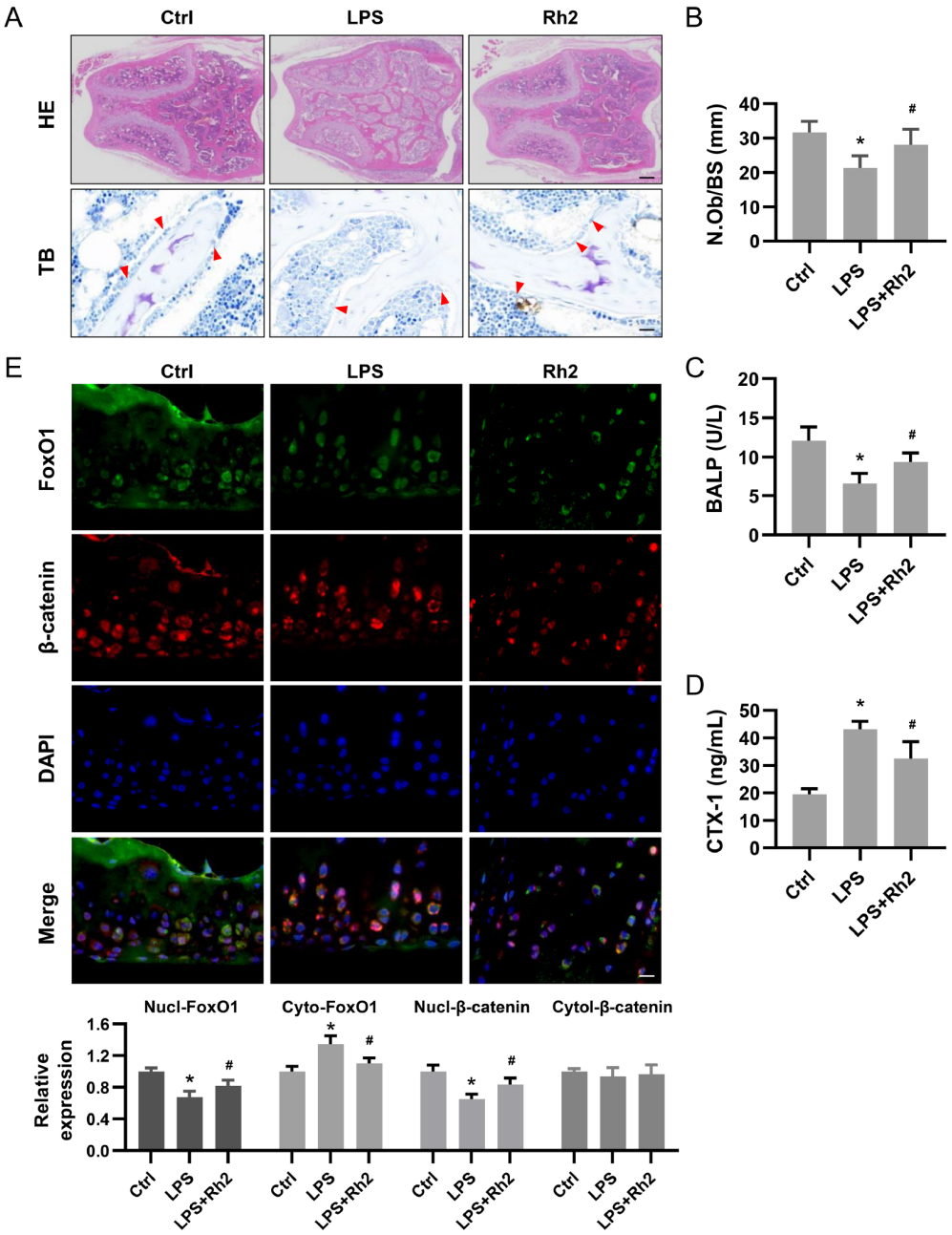

Figure 7. Rh2 promotes bone formation via the FoxO1/β-catenin pathway in LPS-induced bone loss mice (A) Representative HE- and TB-stained images of femurs from each group. Scale bar: 200 μm (HE) and 50 μm (TB). (B) Number of osteoblasts (N.Ob) per square millimeter of the trabecular bone surface (BS). (C,D) Serum levels of BALP and CTX-1 ($n = 6$ per group). (E) Immunofluorescence staining for FoxO1, β-catenin (red) and DAPI (blue) in the femurs of mice. Scale bar: 20 μm. Values are presented as the mean ± SD from three independent experiments. *$P < 0.05$ vs Ctrl group; #$P < 0.05$ vs LPS group.

knockout in osteoblasts reduces cell numbers, bone formation rates, and bone volume, especially the synthesis of collagen, while increasing oxidative stress [11,33]. Moreover, impaired protein synthesis also inhibits the accumulation of glutathione, which induces oxidative stress [34]. Consequently, restoring FoxO1 function, whether through antioxidant intervention or enhanced protein availability, supports osteoblast activity. FoxO1 also interacts with Runx2, ALP, or their gene promoters to promote mineralized nodule formation [35]. Consistent with this, *in vivo FoxO1* knockdown suppresses osteogenic marker expression and compromises bone formation [9]. More importantly, our results elucidate how Rh2 modulates oxidative stress and osteoblast function via FoxO1, highlighting its potential in mitigating redox-related bone loss.

To activate the oxidative defense system, FoxO1 and T-cell factor (TCF) compete to bind to β-catenin and activate transcriptional programs to defend against oxidative stress or osteoblast differentiation [36]. Exposure of cells to H_2O_2 has been shown to activate ROS-dependent pathways rather than β-catenin/TCF-mediated transcription, together with the phosphorylation of FoxO1, provid-

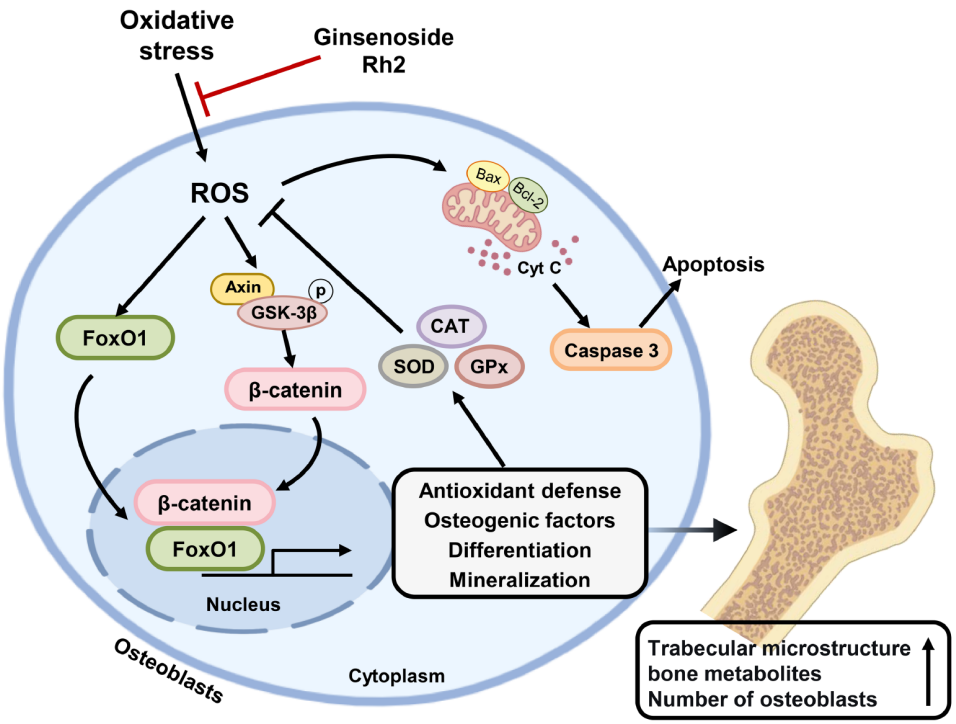

Figure 8. Schematic diagram of the mechanism by which ginsenoside Rh2 protects against osteoblast dysfunction induced by oxidative stress

ing evidence for the involvement of the FoxO1/β-catenin axis in osteoblast apoptosis and dysregulated bone formation [37]. Similarly, β-catenin enhances the transcriptional activity of Runx2 and upregulates the transcriptions of downstream osteogenic genes [38]. In the current study, we found that Rh2 is essential for the mechanism by which ROS inhibit the activity and function of osteoblasts through the FoxO1/β-catenin pathway (Figure 8), highlighting its potential as a therapeutic agent for preventing or treating osteoporosis and other age-related diseases.

Nevertheless, our research has several limitations. Bone homeostasis relies on the dynamic balance between osteoblast and osteoclast functions. However, this research has not yet revealed the effects of Rh2 on osteoclasts or its broader regulatory role in overall bone remodeling. Future studies should systematically investigate Rh2's impact on osteoblast-osteoclast coupling and bone turnover balance using models such as osteoclast differentiation assays, co-culture systems, and osteoporotic animal models. Moreover, while exogenous H_2O_2 was used here to simulate oxidative damage, osteoporosis (*e.g.*, postmenopausal, age-related) involves more complex oxidative stress sources. The signaling pathways and biological effects of ROS may vary depending on their origin. Therefore, multiple inducers should be employed to validate the universality and mechanistic specificity of Rh2's antioxidant effects, enabling more accurate simulation of different pathological states and a better assessment of its therapeutic potential.

In summary, our results demonstrate that Rh2 administration attenuates oxidative stress-induced osteoblast apoptosis and mitochondrial dysfunction and upregulates the expression of osteoblastogenic markers. Furthermore, these findings provide additional evidence that Rh2 may improve osteoblast activity by regulating the FoxO1/β-catenin signaling pathway, providing additional therapies for the prevention and/or treatment of osteoporosis and even age-related diseases.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Wenqi Jin: Conceptualization, methodology, investigation, project administration, writing—original draft, writing—review and editing. Yujing Tan: Validation, data curation, formal analysis. Xuenan Chen: Investigation, visualization. Zehan Guo: Methodology, formal analysis. Jixiang Ren and Liwei Sun: Conceptualization, supervision, writing—review and editing. All authors reviewed the manuscript.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 82104902) and the Science and Technology Development Plan of Jilin Province (No. YDZJ202301ZYTS337).

Conflict of Interest

The authors declare that they have no conflict of interest.

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