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Zoledronic acid accelerates osteogenesis of bone marrow mesenchymal stem cells by attenuating oxidative stress *via* the SIRT3/SOD2 pathway and thus alleviates osteoporosis

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Abstract. – OBJECTIVE: The aim of this study was to clarify the potential effect of zoledronic acid on alleviating oxidative stress and promoting bone marrow mesenchymal stem cells (BMSCs) osteogenesis through the SIRT3/SOD2 pathway, thus alleviating the progression of osteoporosis.

MATERIALS AND METHODS: Relative expression levels of osteogenesis-related genes (ALP, RUNX2, and Bglap) were determined. Meanwhile, ALP activity and capacity of mineralization in BMSCs treated with different doses of zoledronic acid were measured. Subsequently, viability and ROS level in H2O2-induced BMSCs influenced by zoledronic acid treatment were assessed. The regulatory effect of zoledronic acid on the SIRT3/SOD2 pathway was detected by Western blot. Furthermore, the involvement of the SIRT3/SOD2 pathway in zoledronic acid-mediated BMSCs osteogenesis was evaluated.

RESULTS: Zoledronic acid treatment significantly up-regulated the levels of ALP, RUNX2, and Bglap. Meanwhile, it improved ALP activity and capacity of mineralization in BMSCs dose-dependently. H_2O_2 induction markedly suppressed viability and enhanced ROS level in BMSCs, which were reversed by zoledronic acid treatment. Besides, zoledronic acid protected H2O2-induced SIRT3 down-regulation and AC-SOD2/SOD2 up-regulation in BMSCs. In addition, silence of SIRT3 reversed the protective effects of zoledronic acid on osteogenesis of BMSCs.

CONCLUSIONS: Zoledronic acid alleviates the progression of osteoporosis. Meanwhile, it accelerates BMSCs osteogenesis by inhibiting oxidative stress via the SIRT3/SOD2 pathway.

Key Words:
Osteoporosis, Zoledronic acid, SIRT3/SOD2 pathway, Osteogenesis.

Introduction

Osteoporosis is a type of systematic metabolism bone disease. It is characterized by bone volume decline, microstructural changes in bone tissues, and increased bone fragility. Osteoporosis easily results in bone fractures, especially hip fractures. It is known to all that hip fracture is a vital reason for disability and death in middle-aged and elderly people[1]. Current anti-osteoporosis drugs are developed targeting on the prevention of bone absorption[2]. Moreover, the stimulation of osteogenesis has been a research hot topic in the prevention of osteoporosis.

As a new generation of high-efficiency nitrogen-containing bisphosphonates, the American Society of Clinical Oncology (ASCO) has recommended zoledronic acid as a first-line drug for bone metastases. Zoledronic acid has been observed to alleviate osteoporosis and relieve pain and discomfort. Meanwhile, it inhibits osteoclast activity, induces osteoclast apoptosis, and elevates calcium absorption, thereby increasing bone density and achieving anti-osteoporosis outcome[3]. In addition, zoledronic acid can also reduce the release of inflammatory mediators, prostaglandins, lactic acid, and other pain-causing factors[4]. Currently, it has been reported that zoledronic acid alleviates the progression of osteoporosis by stimulating osteogenesis of bone marrow mesenchymal stem cells (BMSCs)[5].

SIRT3 is an important mitochondrial protein, which inhibits oxidative stress and reduces the production of reactive oxygen species (ROS) by stabilizing mitochondrial membrane potential and maintaining the normal function of mitochondria[6]. SIRT3 is highly expressed in human oral cancer

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cells. Alhazzazi et al[7] have found that down-regulation of SIRT3 inhibits tumor cell growth and proliferation, and enhances the sensitivity to radiotherapy and chemotherapy. SOD2 is mainly localized in the mitochondrial matrix. It is activated by inflammatory factors, tumor necrosis factor, and oxidative stress[8]. Recent studies have demonstrated that SOD2 is highly expressed in some types of cancers. In tongue squamous cell carcinoma, SOD2 is up-regulated by MYC regulation, further triggering metastatic phenotype of tumor cells[9]. A relevant study[10] has demonstrated that SIRT3 can directly enhance the activity of SOD2 through deacetylation to exert its antioxidant function. Moreover, it has been indicated that melatonin improves mitochondrial oxidative stress and increases bone mass surrounding prosthesis in OVX rats[11]. Therefore, the aim of this study was to explore the role of zoledronic acid in alleviating the progression of osteoporosis by suppressing oxidative stress through the SIRT3/SOD2 pathway.

Materials and Methods

Cell Culture and Osteogenesis

BMSCs were cultured in α-modified Eagle's Medium (α-MEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 1% penicillin-streptomycin. Osteogenic induction medium was applied, including α-MEM + 10% FBS + 1% penicillin-streptomycin + 10 mmol/L β-glycerophosphate + 50 μg/mL ascorbic acid. The fresh medium was replaced every 2 days. Osteogenesis of BMSCs was conducted for 6 days.

Cell Treatment

Cell suspension was prepared at a density of 5-8 $\times 10^4$ mL/L. 100 μL of cell suspension was applied into 96-well plates. Until cell adherence, medium containing zoledronic acid (0, 1, 2 μM) or H_2O_2 (0, 50, 100, 400, 800 μM) was applied for cell culture.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract cellular RNA, followed by purification by DNase I treatment. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The amplification of cDNA was achieved by real-time quantitative PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Primers used in this study were as follows: RUNX2, forward: 5′-GGGTAAGACTGGTCATAGGACC-3′, reverse: 5′-CCCAGTATGAGAGTAGGTGTCC-3′; ALP, forward: 5′-ACCACCACGAGAGTGAACCA -3′, reverse: 5′-CGTTGTCTGAGTACCAG TCCC-3′; Bglap, forward: 5′-AAAGCC TGGTGATGCAGAGT-3′, reverse: 5′-CTAGACTGGGCCGTAGAAGC-3′; GAPDH, forward: 5′-TTCTT TTGCGTCGCCAGCCGA-3′, reverse: 5′-GTCA CCACCCGCCCAATACGA-3′.

Western Blot

Cells were lysed and the total protein was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The concentration of protein samples was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, protein samples were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibodies for 2 h. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

ALP Activity Determination

After osteogenesis in BMSCs, the cells were washed with pre-cold phosphate-buffered saline (PBS) for three times. Then, cells were lysed in pre-cold 1% Triton X-100 on ice for 30 min. Cell lysate was subjected to ALP activity determination. Absorbance at 405 nm was normalized to that of the total protein concentration.

Alizarin Red Staining (ARS)

BMSCs were collected, re-suspended in low-glucose DMEM, and cultured overnight. On the next day, complete DMEM was replaced. Until the formation of visible mineralized nodules, the cells were incubated in 95% ethanol for 10 min and 0.1% ARS-Tris-HCL solution (pH4.3) at 37°C for 30 min. Nodules were finally observed and captured using an inverted microscope.

ROS Determination

Diluted DCFH-DA (2', 7'-dichlorofluorescin diacetate) probe was applied in cells and cul-

tured in the dark for 20 min. Subsequently, the cells were washed with serum-free medium three times. Absorbance at 485 and 505 nm was determined, respectively.

Cell Proliferation Assay

A total of 2.0×10^3 cells per well were inoculated into 96-well plates. Cell viability was determined at appointed time points in accordance with cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 490 nm was recorded, and the viability curve was plotted.

Cell Transfection

Cell density was adjusted to 1×10^6 ml/L, and the cell suspension was inoculated into 24-well plates with 100 µL per well. 50 nmol/L transfection vector and HiperFect transfection reagent were diluted in 100 µL of serum-free medium. Transfection efficacy was determined at 6 h. Sequences of si-SIRT3 were: sense: 5'-GCCCAATGTCACTCACTACTT-3' and anti-sense: 5'-AGACAGCTCCAACACGTTTAC-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. Student's *t*-test was applied to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Zoledronic Acid Accelerated BMSCs Osteogenesis

After treatment of 0, 1, and 2 µM zoledronic acid, the expression levels of osteogenesis-related genes ALP, RUNX2, and Bglap were dose-dependently up-regulated in BMSCs (Figure 1A). Similarly, the protein expression level of RUNX2 was gradually upregulated by zoledronic acid treatment (Figure 1B). In addition, ALP activity (Figure 1C) and capacity of mineralization (Figure 1D) in BMSCs enhanced alongside with treatment of increased doses of zoledronic acid. These results indicated that zole-

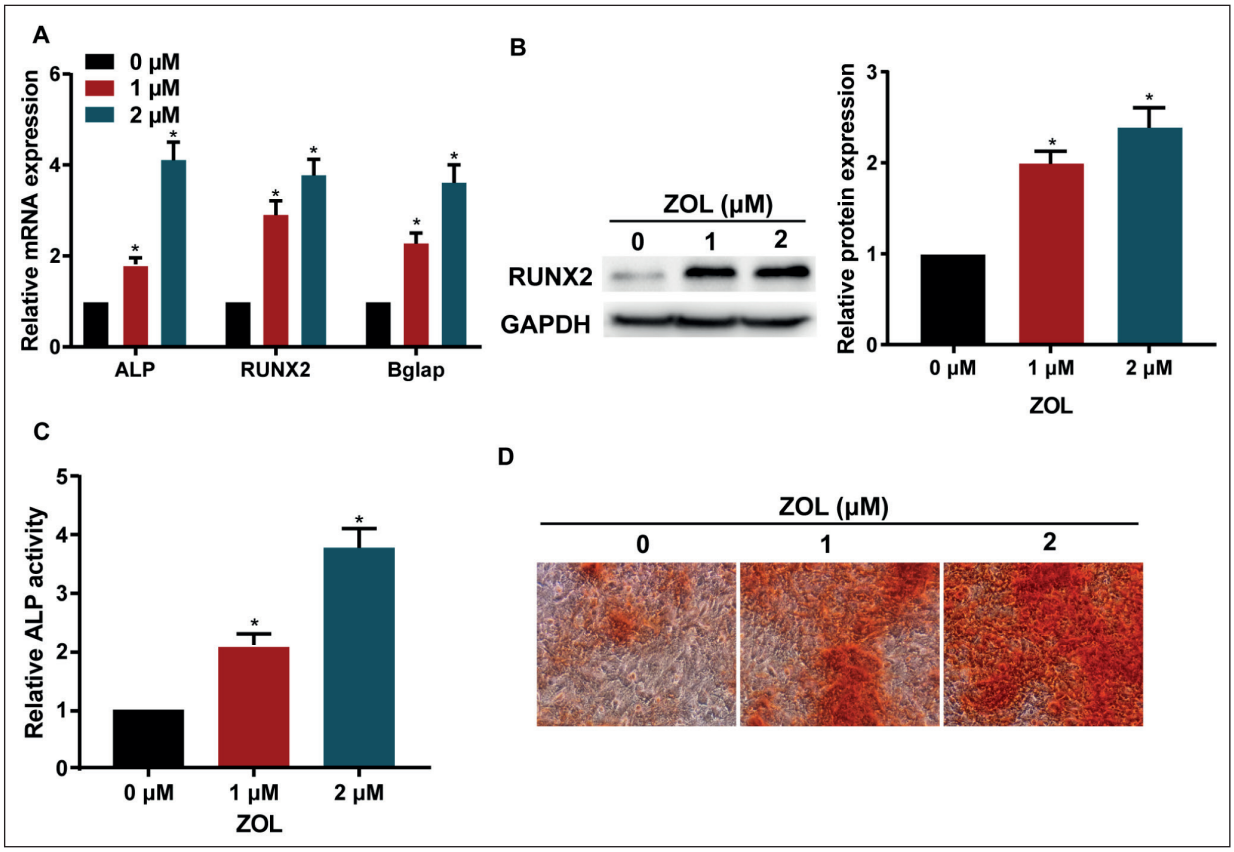

Figure 1. Zoledronic acid accelerated BMSCs osteogenesis. BMSCs were treated with 0, 1 or 2 µM zoledronic acid. **A,** Relative levels of ALP, RUNX2, and Bglap. **B,** Protein level of RUNX2. **C,** ALP activity. **D,** Capacity of mineralization (×200).

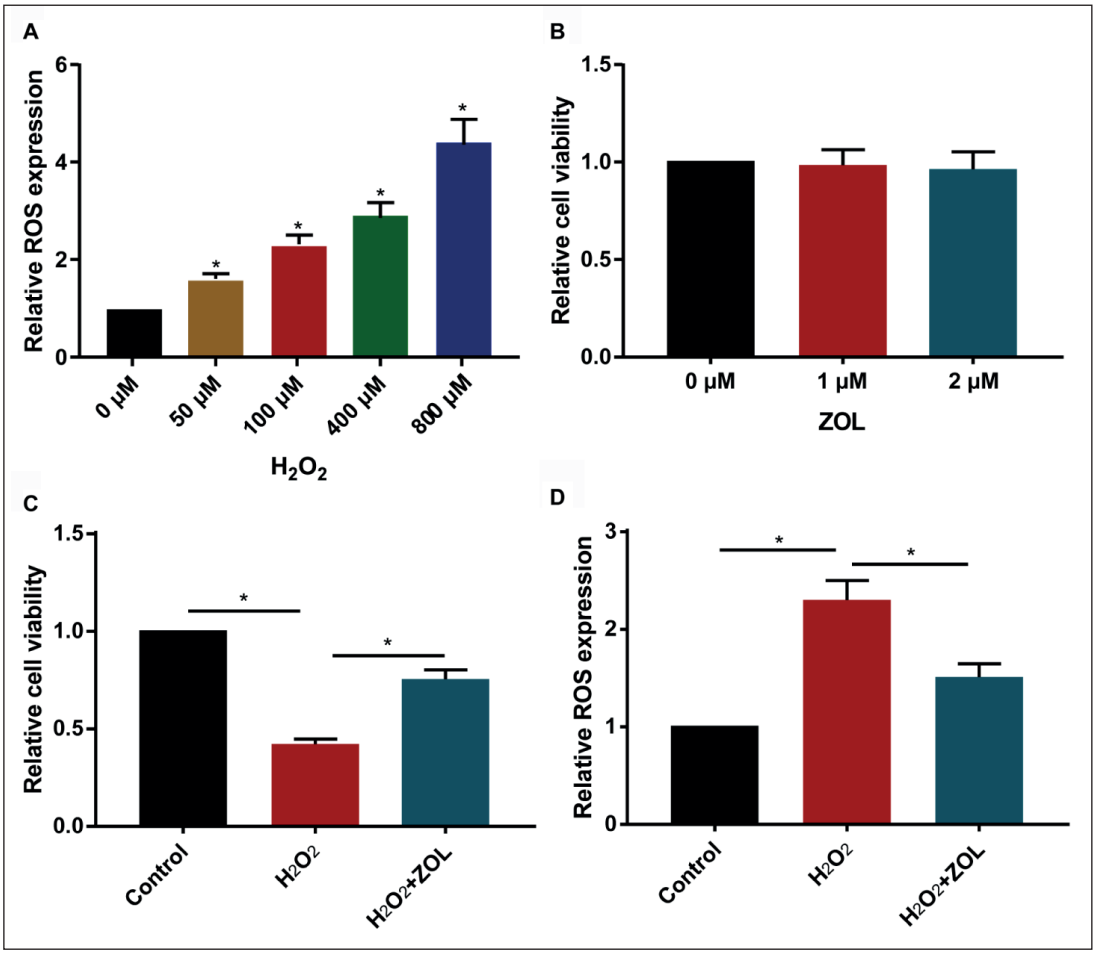

Figure 2. Zoledronic acid alleviated oxidative stress. **A,** ROS expression in BMSCs treated with 0, 50, 100, 400 or 800 μM H_2O_2. **B,** Cell viability in BMSCs treated with 0, 1 or 2 μM zoledronic acid. **C,** Cell viability in BMSCs treated with control, H_2O_2 or H_2O_2 + zoledronic acid. **D,** ROS expression in BMSCs treated with control, H_2O_2 or H_2O_2 + zoledronic acid.

dronic acid was capable of accelerating osteogenesis of BMSCs.

Zoledronic Acid Alleviated Oxidative Stress

Previous studies have speculated that oxidative stress may be responsible for influencing BMSCs osteogenesis regulated by zoledronic acid. With the treatment of increased doses of H_2O_2 (0, 50, 100, 400, and 800 μM), ROS level in BMSCs elevated in a dose-dependent manner (Figure 2A). No significant changes were observed in the viability of BMSCs after treatment of either 1 μM or 2 μM zoledronic acid (Figure 2B). Notably, H_2O_2 induction reduced cell viability and enhanced ROS level, which could be partially reversed by zoledronic acid treatment (Figure 2C, 2D). Therefore, zoledronic acid was proved to suppress oxidative stress in BMSCs.

Zoledronic Acid Regulated the SIRT3/SOD2 Pathway

To clarify the regulatory effect of zoledronic acid on the SIRT3/SOD2 pathway, the protein levels of SIRT3, acetylated SOD2 (AC-SOD2) and SOD2 in BMSCs were detected by Western blot. H_2O_2 induction markedly down-regulated SIRT3 while up-regulated AC-SOD2/SOD2 in BMSCs, which were reversed after treatment of zoledronic acid (Figure 3).

Zoledronic Acid Stimulated BMSCs Osteogenesis Through the SIRT3/SOD2 Pathway

Based on the previous findings, we speculated that the SIRT3/SOD2 pathway was responsible for BMSCs osteogenesis regulated by zoledronic acid. QRT-PCR data revealed that zoledronic acid treatment reversed the down-regulation of ALP,

RUNX2, and Bglap in H_2O_2-induced BMSCs. This was further blocked by transfection of si-SIRT3 (Figure 4A). A similar trend was observed in the protein level of RUNX2 (Figure 4B). After silence of SIRT3, ALP activity (Figure 4C) and capacity of mineralization (Figure 4D) protected by zoledronic acid in H_2O_2-induced BMSCs were significantly reduced. Collectively, zoledronic acid promoted BMSCs osteogenesis through the SIRT3/SOD2 pathway.

Discussion

Osteoporosis and relevant bone fractures are common in middle-aged and elderly people, posing a great burden on the whole society. Bone regeneration technologies based on stem cells are important for the treatment of osteoporosis and bone impair. Precise regulation of self-renewal and directional differentiation of BMSCs are key events for bone regeneration. Therefore, it is necessary to clarify an ideal microenvironment for directional differentiation of BMSCs[12].

Zoledronic acid is a third-generation bisphosphonate, which is considered the most potent drug for clinical inhibition of bone resorption. Lymphocytes in the bone marrow are key immune cells responsible for regulating bone remodeling. Meanwhile, reconstructed bone tissue also affects the immune system[13]. Steller et al[14] have shown that the inhibitory effect of zoledronic acid on bone resorption is closely linked to lymphocyte regulation on bone remodeling. In this experiment, the effect of H_2O_2-induced cell viability declines and ROS excessive production in BMSCs were reversed by zoledronic acid treatment. In addition, down-regulated SIRT3 and up-regulated acetylated SOD2 in H_2O_2-induced BMSCs could be partially reversed by zoledronic acid treatment as well. All these findings suggested that zoledronic acid stimulated the osteogenesis of BMSCs and alleviated oxidative stress.

Bone development initiates from mesenchymal stem cells, followed by evolution to progenitor cells, pre-osteoblasts, and osteoblasts. Finally, most osteoblasts undergo apoptosis. Only a small number of osteoblasts are embedded by collagen secreted, further differentiating into bone cells[15]. This process is directly controlled by its microenvironment, as well as multiple signaling pathways. Wnt and Notch pathways have been confirmed to exert important roles in osteogenesis metabolism[16]. Boyden et al[17] reported that suppression or elevation of Lrp5 activity leads to osteoporosis or high bone mineral density syndrome, respectively. The Notch pathway promotes the differentiation of BMSCs into osteoblasts. During the process of fracture healing, receptors, and ligands of the Notch pathway are significantly upregulated[18,19]. Gao et al[20] have demonstrated that the SIRT3/SOD2 pathway maintains osteoblast differentiation and bone formation by regulating mitochondrial stress. Our findings showed that H_2O_2 treatment induced down-regulation of osteogenesis-related genes. Meanwhile, H_2O_2 treatment resulted in the decline of ALP activity and capacity of mineralization in BMSCs. This could

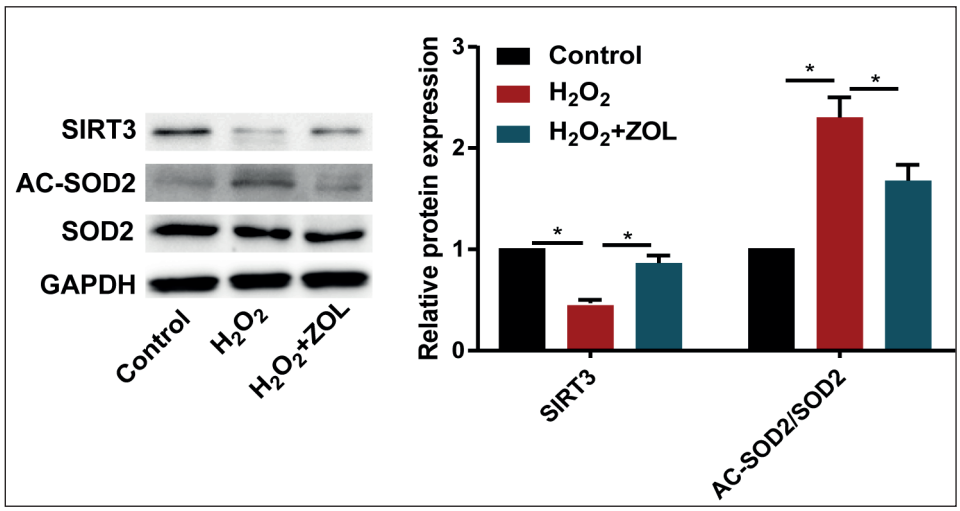

Figure 3. Zoledronic acid regulated the SIRT3/SOD2 pathway. The protein levels of SIRT3, AC-SOD2, and SOD2 in BMSCs treated with control, H_2O_2 or H_2O_2 + zoledronic acid.

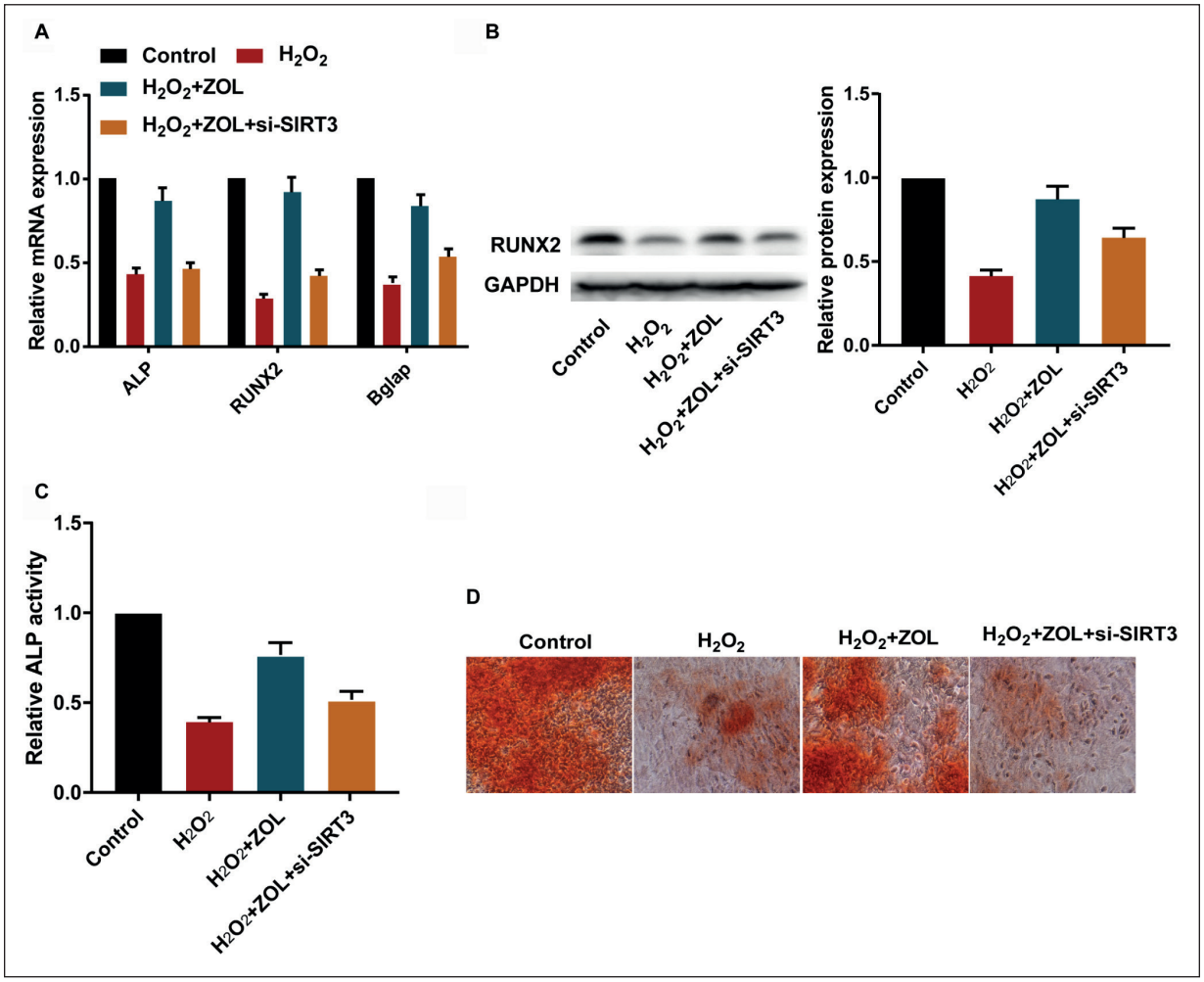

Figure 4. Zoledronic acid stimulated BMSCs osteogenesis through the SIRT3/SOD2 pathway. BMSCs were treated with control, H_2O_2, H_2O_2 + zoledronic acid or H_2O_2 + zoledronic acid + si-SIRT3 transfection. **A,** Relative levels of ALP, RUNX2, and Bglap. **B,** Protein level of RUNX2. **C,** ALP activity. **D,** Capacity of mineralization (×200).

be reversed by zoledronic acid treatment. However, the regulatory effects of zoledronic acid on BMSCs were blocked by silence of SIRT3.

To sum up, SIRT3/SOD2 was responsible for zoledronic acid to prevent oxidative stress and accelerate BMSCs osteogenesis. All these findings indicated that Zoledronic acid was beneficial to clinical prevention and treatment of osteoporosis.

Conclusions

We showed that Zoledronic acid alleviated the progression of osteoporosis. In addition, it accelerated BMSCs osteogenesis by inhibiting oxidative stress *via* the SIRT3/SOD2 pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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