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Original Article

Curculigoside isolated from *Curculigo orchioides* prevents hydrogen peroxide-induced dysfunction and oxidative damage in calvarial osteoblasts

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Reactive oxygen species (ROS), including H_2O_2, play a critical role in the pathophysiology of osteoporosis. Therefore, agents or antioxidants that can inhibit ROS production have a high clinical value in the treatment of osteoporosis. Curculigoside (CUR), one of the main bioactive phenolic compounds isolated from the rhizome of *Curculigo orchioides* Gaertn., is reported to have potent antioxidant and anti-osteoporotic properties. However, there is no direct evidence to link the antioxidant capacity of CUR with the observed anti-osteoporotic effect, and relevant molecular mechanisms remain unclear. Therefore, we investigated the protective effects of CUR against oxidative stress in calvarial osteoblasts and discussed the related mechanisms. It was found that osteoblast viability decreased significantly after 48-h exposure to 400 μM of H_2O_2, compared with vehicle-treated cells, and the cytotoxic effect of H_2O_2 was reversed significantly when pretreated with 0.1–10 μM of CUR ($P < 0.05$). Pretreatment with 0.1–10 μM of CUR decreased ROS production and lipid peroxidation, and increased the activities of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase in osteoblasts induced by H_2O_2. In addition, H_2O_2-induced reduction of differentiation markers such as alkaline phosphatase, calcium deposition, and Runx2 level was significantly recovered in the presence of CUR. CUR also reversed H_2O_2-induced stimulation of extracellular signal-regulated kinase 1/2, and nuclear factor-κB signaling and the inhibition of p38 mitogen-activated protein kinase activation. These results provide new insights into the osteoblast-protective mechanisms of CUR through reducing the production of ROS, suggesting that CUR may be developed as a bio-safe agent for the prevention and treatment of osteoporosis and other bone-related human diseases.

Keywords curculigoside; *Curculigo orchioides* Gaertn. (Amaryllidaceae); calvarial osteoblasts; oxidative stress

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Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, leading to a consequent increase in bone fragility and fracture risk. In bone, osteoblasts are responsible for bone formation while osteoclasts participate in bone resorption. The imbalance between osteoclastic bone resorption and osteoblastic bone formation caused by increased bone resorption over bone formation can result in bone loss, leading to osteoporosis. Estrogen (one of the main risk factors) deficiency, which was strongly associated with osteoporosis [1], induces bone loss via increased generation of reactive oxygen species (ROS) [2]. Oxidative stress resulting from excessive levels of ROS has been acknowledged as a major contributor to the etiology of various degenerative diseases including osteoporosis, in which the level of oxidative stress markers is increased markedly [3,4]. Oxidative stress induced by exogenous stimuli such as hydrogen peroxide or xanthine/xanthine oxidase is able to inhibit bone cell differentiation of the pre-osteoblastic cell line (MC3T3-E1) and osteoblast cell lines that undergo osteoblastic differentiation [5,6]. Therefore, ROS may represent a critical target for the prevention of bone loss, and antioxidants could be considered as potential candidates for the treatment of osteoporosis.

Curculigo orchioides Gaertn. (Amaryllidaceae), which grows in subtropical regions of Asia, has been used as a traditional herbal medicine in China and India, and is

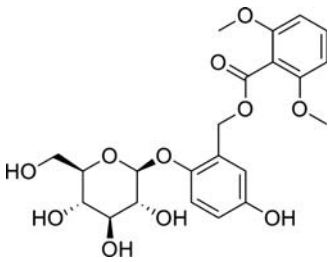

Figure 1 The chemical structure of curculigoside

believed to be a tonic for the treatment of declined physical strength. Curculigoside (CUR) shown in **Fig. 1** , one of the main bioactive phenolic compounds in the rhizome of *C. orchioides* Gaertn., has been shown to have significant antioxidant properties by scavenging superoxide radicals in the normal systems, and anti-apoptotic activities in H_2O_2-treated vascular endothelial cells [7–9]. Our previous study showed that CUR increased the proliferation and alkaline phosphatase (ALP) activity of osteoblasts, inhibited osteoclast bone resorption, osteoclast formation and tartrate-resistant acid phosphatase activity [10]. However, there is no direct evidence to link the antioxidant capacity of CUR with the observed anti-osteoporotic effect, and relevant molecular mechanisms remain unclear.

In this study, we investigated the effects of CUR on osteoblast function and oxidative modification after exposure of calvarial osteoblasts to 400 μM of H_2O_2, and found that CUR protected osteoblasts against oxidative damage and promoted osteoblastic differentiation via inhibiting extracellular signal-regulated kinase (ERK) and ERK-dependent nuclear factor-κB (NF-κB) signaling pathways and stimulating p38 signaling pathway under oxidative stress conditions, implying that it has a potential therapeutic value for the treatment of osteoporosis.

Materials and Methods

Reagents

α-Modified minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, USA). H_2O_2 was obtained from Fisher Scientific (Hudson, USA). *N*-acetyl-L-cysteine (NAC) (purity > 99%) obtained from Sigma (St Louis, USA) was used as a positive control. Kits for ROS, malondialdehyde (MDA) assay, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity measurement were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). CUR (purity > 98%) was isolated from *C. orchioides* Gaertn. as described previously [11]. Antibodies [anti-type I collagen (ab6308) and anti-Runx2 (ab76956) obtained from Abcam (Cambridge, UK); anti-osteoprotegerin (OPG) (sc11383) and anti-RANKL (sc9073) obtained from Santa Cruz (Santa Cruz, USA); anti-phospho-ERK (3371), anti-phospho-p38 (9211), and anti-phospho-I-κB (9245) obtained from Cell Signaling Technology (Beverly, USA)] were used for western blot analysis.

Cell culture

This study was approved by the Ethics Committee of the Second Military Medical University (Shanghai, China). Wistar rats aged 1 day were purchased from the Experimental Animal Center of the Second Military Medical University. Primary osteoblasts were prepared according to the literature [12]. Osteoblasts were isolated from the calvaria of newborn rats. Five calvarias were collected and washed in sterilized Hanks' balanced salt solution. Then, they were minced and subjected to sequential digestions for 30 min at 37°C in a solution of phosphate buffered saline (PBS) containing 0.25% trypsin (Gibco) and 90 min at 37°C in 3 mg/ml of collagenase II (Biochemical Corp., Twinsburg, USA). After the second digestion, cells were collected by centrifugation and re-suspended in α-MEM containing 10% FBS. Cells at passage 2 were used in the experiments.

Cell treatment

Cells were harvested when they reached about 80% confluence and randomly divided into six groups: a normal control group, a H_2O_2 group, three CUR groups (0.1, 1, and 10 μM), and a positive drug (NAC) group. The cells in the control group were incubated under the normal growth condition. The cells in the H_2O_2 group were incubated for 46 h with a medium containing 400 μM H_2O_2. In the CUR groups, the cells were pre-incubated for 2 h with different concentrations of CUR, then incubated for 46 h with 400 μM of H_2O_2 [13]. In the NAC group, the cells were pre-incubated for 2 h with 2.5 mM of NAC, and then incubated for 46 h with 400 μM of H_2O_2. Cell phenotypic observations were made using an Olympus CKX41 inverted phase-contrast microscope (Tokyo, Japan), with a digital camera system to capture images, both to monitor the differentiation status of the cultures and to record any change during treatment.

Cell viability assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously with some modifications [9]. In brief, the cells were seeded at density of 4×10^3 cells/well in 96-well plates and cultured in an incubator. At the indicated time points, the culture supernatant was removed, and cells were washed with PBS, and then 200 μl of fresh medium containing 0.5 mg/ml of MTT was added to each well. After incubation at 37°C for 4 h, the culture medium containing MTT was removed. Dimethyl sulfoxide

(150 μl) was added into each well, and the absorbance at 570 nm was measured using a spectrophotometric microplate reader (Bio-Tek, Winchester, USA).

Assay of ALP activity

The induction of ALP is an unequivocal marker for bone cell differentiation. To measure the ALP activity, cells were seeded and cultured with α-MEM containing 10% FBS, after 4 days, the cells were treated with medium containing samples for 2 days. At the end of the treatment, ALP activity was measured according to the previously described method [14]. In brief, after rinsing the cells twice with cold PBS, 100 μl of diolamine (50 mM) and 50 μl of disodium 4-nitrophenylphosphate (2.5 mM) were added, and then incubated for 30 min at 37°C. To terminate the reaction, 100 μl of NaOH (0.3 M) was added to each well. Ultraviolet absorbance of the samples and standards were measured at 405 nm. Total protein was assayed by the method of Bradford [15]. The activity of ALP was expressed as micromoles of *p*-nitrophenol liberated per nanogram protein.

Assay of calcium deposition

Osteoblast maturation was determined by evaluating cell mineralization [16,17]. The extent of mineralized nodule formation based on staining density and number of nodules was determined by alizarin red-S (AR-S) staining at 14th day. In brief, cells were incubated with osteoblasts differentiation medium containing 50 mg/ml of ascorbic acid, 10 mM of β-glycerophosphate, and 10 nM of dexamethasone for 10 days. Then, cells were continuously cultured with differentiation medium and test compounds for another 4 days. On harvesting, cells were washed with ice-cold PBS buffer, fixed in ice-cold 10% neutral formalin for 20 min, and then stained with 0.1% AR-S-Tris–HCl (PH 8.3) for 30 min. The mineralized nodules were counted under an inverted microscope. For the quantification of staining density, the staining is solubilized with 10% (*w/v*) cetylpyridinium chloride by shaking for 30 min. Optical density of the extracted stain is read at 570 nm.

Measurement of intracellular ROS

Intracellular ROS levels were examined using ROS-sensitive dye, 2′, 7′-dichlorofluorescein diacetate (Molecular Probes, Oregon, USA). Osteoblasts (3×10^5 cells/well in 6-well plates) were pre-incubated for 2 h with CUR (0.1, 1 and 10 μM), and then exposed to 400 μM of H_2O_2 for 46 h. At the end of the treatment, the supernatant was discarded and cells were washed with PBS, then 5 μM of 2′, 7′-dichlorofluorescein diacetate was added and incubated for 30 min at 37°C in the dark. The cells were washed twice with PBS, collected and analyzed for fluorescence of 2′,7′-dichlorofluorescein at 485 nm excitation and at 525 nm emission by flow cytometry.

Analysis of lipid peroxidation and antioxidant enzymes

The osteoblasts (2×10^6 cells/100 mm culture dish) were seeded and treated as described in intracellular ROS assay. At the end of the incubation, the medium in the 100 mm dish was discarded. The osteoblasts were washed with ice-cold PBS three times, collected, centrifuged at 1500 *g* for 5 min, homogenated in PBS by sonicated 10 times for 5 s interval, and centrifuged at 12,000 *g* for 10 min at 4°C. The activity of lipid peroxidation and antioxidant enzymes in the supernatant was determined according to the guidelines of the kits (Nanjing Jiancheng Bioengineering Institute). The values were expressed as units per milligrams protein and the protein concentration was determined with a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimeng, China).

Western blot analysis

The osteoblasts (2×10^6 cells/100 mm dish) were cultured for 24 h followed by incubation with various concentrations of CUR for 2 h, then treated with 400 μM of H_2O_2 for another 46 h. At the end of the incubation, the medium was removed, and the cells were washed with PBS and immediately lysed in a buffer containing 20 mM of Tris–HCl, 150 mM of NaCl, 1% Triton X-100, and protease and phosphates inhibitors. The lysates (30–40 mg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was probed with anti-type I collagen, anti-Runx2, anti-OPG, anti-RANKL, anti-phosphor-ERK, anti-phosphor-p38 MAPK, and anti-phosphor-IκBα antibody. The same membrane was stripped and re-probed. Chemiluminescent signals were detected with a Gel Doc 2000 luminescent image analyzer (Wealtec Dolphin-Doc, Sparks, USA).

Statistical analysis

The experiments were repeated three times in six replicate samples. Data were expressed as the mean ± standard deviation. One-way analysis of variance followed by Dunnett's *t*-test was used for statistical analysis (SPSS 13.0 software; SPSS, Chicago, USA), and the level of significance was set at * or $^{\#}P < 0.05$.

Results

CUR protects osteoblasts against H_2O_2-induced cell injury

The concentration-dependent effects of CUR and H_2O_2 on cell viability were first investigated. Treatment with CUR

at a concentration range from 0.0001 to 10 μM under normal condition for 48 h, did not significantly affect cell viability of osteoblasts apart from the dose of 0.01 and 0.1 μM [**Fig. 2(A)**]. However, treatment with H_2O_2 (400–600 μM) for 46 h significantly decreased cell viability in concentration-dependent manners and treatment with 400 μM of H_2O_2 decreased cell viability to 54.19% of the control [**Fig. 2(B)**]. Interestingly, pretreatment with CUR (0.1 to 10 μM) for 2 h significantly protected osteoblasts against H_2O_2 (400 μM)-induced cell injury and showed dose-dependent manners [**Fig. 2(C)**].

This is confirmed by the morphological study. As shown in **Fig. 2(D)**, treatment with H_2O_2 (400 μM) induced obvious morphological changes in osteoblasts. H_2O_2 induced pronounced cell damage as displayed by cell shrinkage and gradual detachment from culture dishes. Pretreatment of cells with CUR (0.1–10 μM) for 2 h dramatically alleviated the morphological changes. These findings suggest that CUR produces significant protection on H_2O_2-induced cell injury.

CUR attenuates H_2O_2-suppressed ALP activity

ALP is the earliest marker of osteoblast differentiation. As shown in **Fig. 3(A)**, there was a slight increase in ALP activity incubated with CUR in normal osteoblasts. However, incubation of with H_2O_2 (400 μM) for 46 h markedly decreased the ALP activity. Interestingly, pretreatment of cells with CUR for 2 h significantly attenuated H_2O_2 downregulated the level of ALP activity [**Fig. 3(B)**].

CUR suppressed H_2O_2-induced decrease of nodule formation

It is known that mineralized nodules formation is a definitive hallmark of osteoblastic differentiation. To determine the effect of CUR on osteoblast function, cells were stained with AR-S at the 14th day post-confluence for the identification of mineralized nodules. Under normal condition, Alizarin red S staining [**Fig. 4(A)**] and Alizarin red S staining density [**Fig. 4(C)**] were increased significantly at the concentration of 1 μM of CUR and number of mineralized nodules [**Fig. 4(E)**] was increased both in doses of 0.1 and 1 μM. Under oxidative stress condition, there was an apparent decrease in mineralized nodule formation in the presence of H_2O_2. H_2O_2-induced decrease in Alizarin red S staining [**Fig. 4(B)**], Alizarin red S staining density [**Fig. 4(D)**], and mineralized nodules [**Fig. 4(F)**] were significantly reversed by CUR at all doses, and 10 μM of

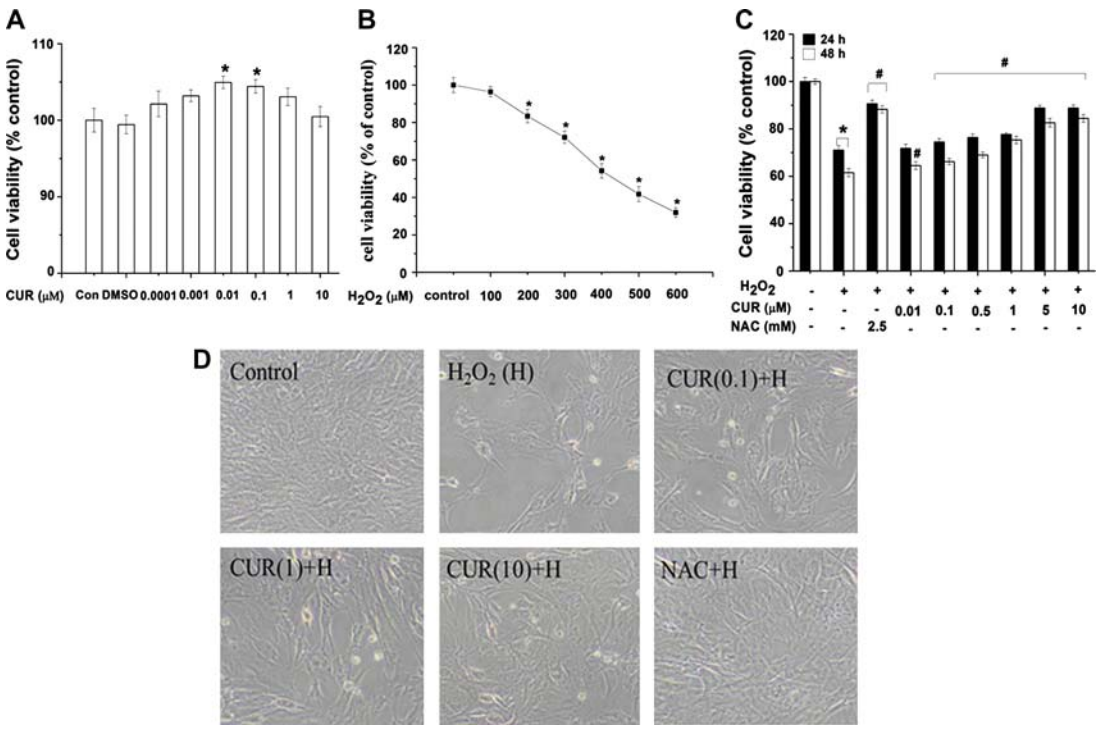

Figure 2 Protective effects of CUR on H_2O_2-induced cell injury in osteoblasts (A) Concentration-dependent effect of CUR on cell viability. Cells were seeded at density of 4×10^3 cells/well in 96-well plates. After 24 h, the cells were cultured in α-MEM supplemented with various concentrations of CUR for 48 h. (B) Concentration-dependent effects of H_2O_2 on cell viability. H_2O_2 at 400 μM significantly reduced cell viability after 46 h of incubation. (C) Pretreatment with CUR (0.01–10 μM, 2 h) alleviated H_2O_2-induced cell injury. Cells were pretreated with various concentrations of CUR for 2 h and then incubated with an additional 400 μM H_2O_2 for 46 h. The data were presented as the mean ± SD ($n = 6$); $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. the H_2O_2 group. (D) Morphological changes of osteoblasts incubated with CUR and/or H_2O_2 were observed and photographed using a light microscope. Magnification, ×100.

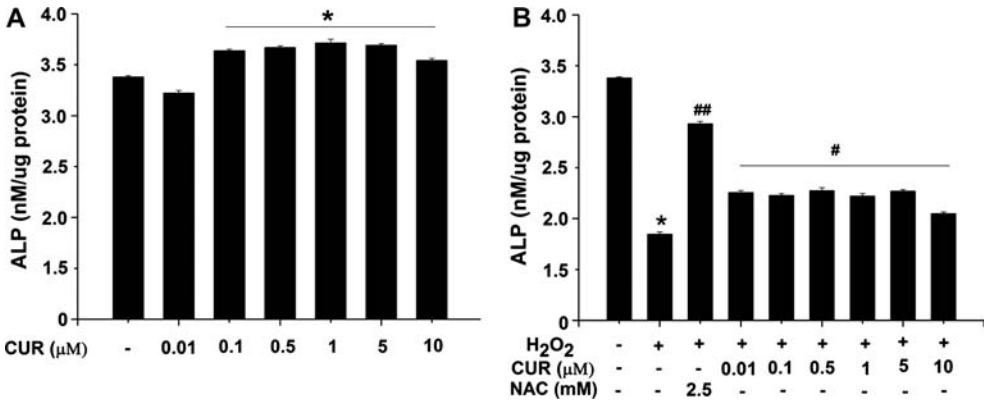

Figure 3 The activity of ALP are altered by CUR under normal condition (A) and oxidative stress condition (B) Cells were seeded in 96-well plates at the density of 4×10^3 cells/well and cultured with α-MEM containing 10% FBS. After 4 days, (A) the cells were treated with medium containing 0, 0.01, 0.1, 0.5, 1, 5, or 10 μM of CUR for 2 days and the activity of ALP was increased; (B) the cells were pre-treated with 0, 0.01, 0.1, 0.5, 1, 5, or 10 μM of CUR for 2 h, and then incubated with an additional 400 μM of H_2O_2 for 46 h. The data were presented as the mean ± SD ($n = 6$); $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. the H_2O_2 group; $^{\#\#}P < 0.01$ vs. the H_2O_2 group.

CUR produced the greatest effect, indicating that CUR increased the number and size of calcium deposition.

Antioxidant effects of CUR on osteoblasts

To investigate the antioxidant effects of CUR, we examined the effect of CUR on H_2O_2-induced ROS release and found that treatment with H_2O_2 (400 μM) for 46 h significantly increased ROS production. Exposure to CUR (0.1, 1, and 10 μM) for 2 h before addition of H_2O_2 significantly attenuated the effect of H_2O_2 in all groups [**Fig. 5(A)**]. We continued to examine the effect of CUR on the levels of MDA, an indicator of lipid peroxidation. As shown in **Fig. 5(B)**, CUR abolished H_2O_2-upregulated content of MDA. We further determined the effect of CUR on SOD and GSH-Px activity of osteoblasts. As shown in **Fig. 5(C,D)**, treatment with H_2O_2 (400 μM) significantly suppressed SOD and GSH-Px activities. These effects were also attenuated by CUR (0.1, 1, and 10 μM).

Effects of CUR on the expression of type I collagen, nuclear p-Runx2 and total Runx2 in H_2O_2-injury osteoblasts

To elucidate the effect of CUR on osteoblastic differentiation-related signaling under oxidative stress condition, we investigated the expression of Runx2, type I collagen and activation of Runx2. As shown in **Fig. 6**, treatment with H_2O_2 (400 μM) inhibited the expression of type I collagen and total Runx2, and activation of Runx2. Pretreatment with CUR (0.1−10 μM) reversed H_2O_2-induced inhibition on nuclear Runx2 phosphorylation completely, and increased the expression of type I collagen (1 μM CUR), but reduced the expression of total Runx2 of osteoblasts compared with H_2O_2 treatment group. These data suggest that CUR may protect osteoblasts against injury and promote their proliferation and differentiation.

Effects of CUR on the expression of RANKL and OPG in H_2O_2-treated osteoblasts

We also examined the effects of CUR on the expression of OPG and RANKL (receptor activator of nuclear factor κ-B ligand) under oxidative stress condition. As shown in **Fig. 7**, treatment with H_2O_2 (400 μM) stimulated the expression of RANKL and inhibited the expression of OPG. However, CUR significantly downregulated H_2O_2-stimulated protein expression of RANKL, and had a gentle effect on the protein expression of OPG in osteoblasts, suggesting that CUR may function in osteoclastogenesis.

The protective effects of CUR are mediated by NF-κB, p38 mitogen-activated protein kinase (p38 MAPK) and ERK.

To study the signaling mechanisms, we examined the involvement of MAPKs and NF-κB in the effects of CUR. As shown in **Fig. 8**, stimulation with H_2O_2 (400 μM) inhibited the phosphorylation of p38 MAPK, slightly altered the activation of ERK, and markedly increased the phosphorylation of IκBα in osteoblasts, the addition of CUR impaired H_2O_2-stimulated ERK signaling as well as NF-κB signaling and repaired H_2O_2-inhibited p38 MAPK signaling. These findings indicated that CUR enhanced the differentiation of osteoblasts under oxidative stress status through modulating phosphorylation of p38 MAPK, ERK, and NF-κB pathway.

Discussion

It is widely accepted that ROS greatly contributes to the pathogenesis of various diseases including osteoporosis [18]. H_2O_2, one of the main ROS, may diffuse across biological membranes and produce a wide range of injury. It has been reported that H_2O_2 induces apoptosis or necrosis of various types of cells [19,20]. Reduced bone formation is commonly associated with increased oxidative stress

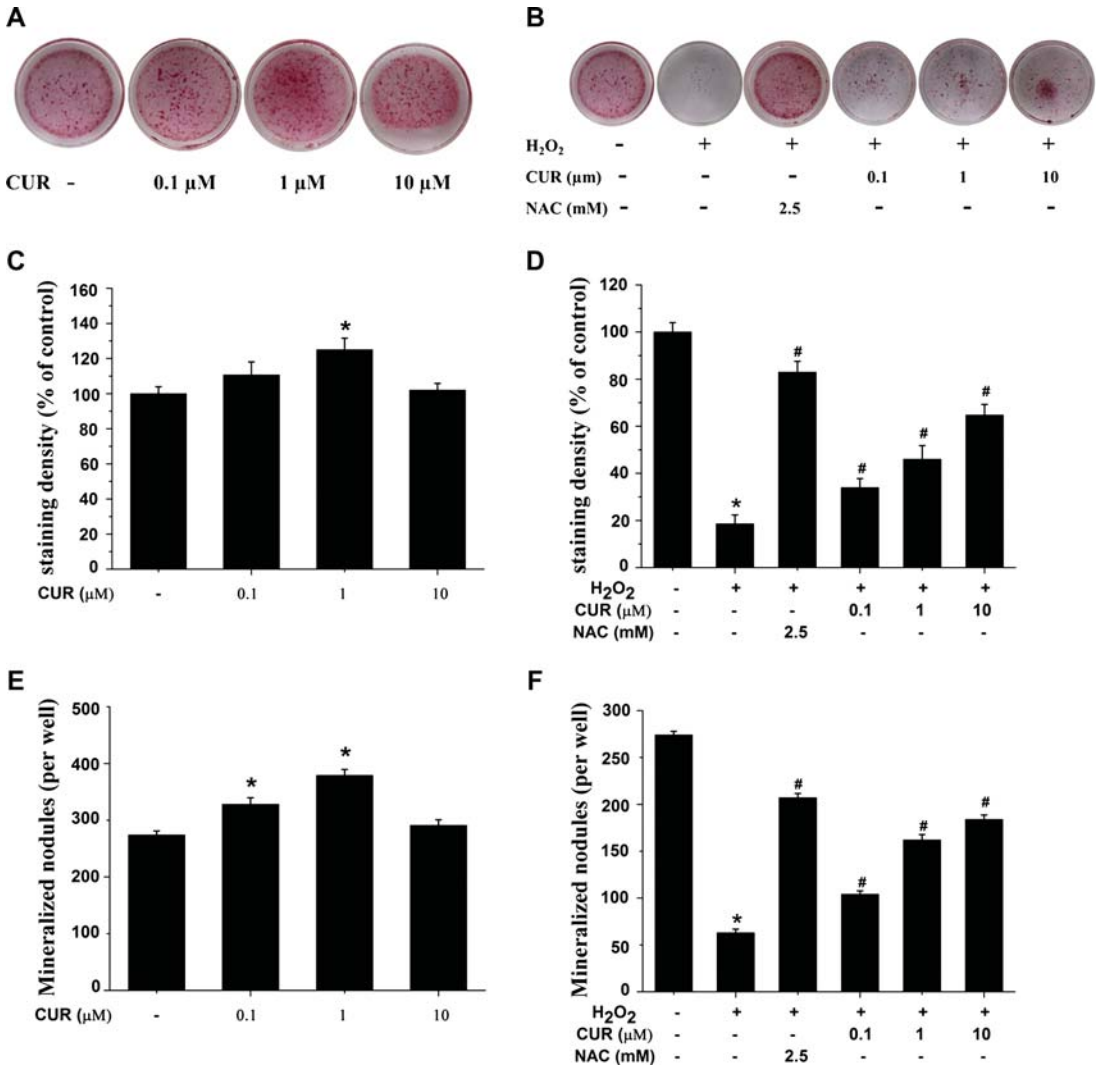

Figure 4 Staining density and number of mineralized nodules are altered by CUR under normal condition and oxidative stress condition (A,C,E) Under normal condition, cells were plated at 1×10^5 cells/well in 24-well plates and incubated with osteoblast differentiation medium for 10 days. Then, cells were continuously cultured with medium containing 0.3% BSA and CUR for another 4 days. Alizarin red S staining (A) and alizarin red S staining density (C) were increased at the concentration of 1 μM of CUR, and the number of mineralized nodules (E) was increased both in the doses of 0.1 and 1 μM. (B,D,F) Under oxidative stress conditions, cells were plated at 1×10^5 cells/well in 24-well plates and incubated with osteoblast differentiation medium for 10 days, Then, cells were continuously cultured with differentiation medium and various concentrations of CUR or NAC for 2 h prior to an additional 400 μM of H_2O_2 treatment for another 46 h. Alizarin red S staining (B), staining density (D), and nodule formation (F) were increased by all doses of CUR. The data were presented as the mean ± SD ($n = 6$). *$P < 0.05$ vs. control without any treatment group; #$P < 0.05$ vs. the H_2O_2 group.

in aged men and women [3]. A marked decrease in plasma antioxidants is found in aged osteoporotic women [4]. In this study, we first investigated the effect of various concentrations of CUR and H_2O_2 on cell viability. Treatment with CUR under normal conditions for 48 h did not significantly affect the cell viability of osteoblasts apart from the dose of 0.01 and 0.1 μM, showing that CUR (0.01 and 0.1 μM) may enhance the viability of osteoblasts and/or reduce the cell detachment from the substrate compared with control. Treatment cells with H_2O_2 can result in a variety of cell fates, such as decrease in cell viability, cell apoptosis, or cell necrosis. The necrosis cells detached from the substrate, leading to the decrease in number of osteoblasts. H_2O_2 (400 μM) treated for 46 h significantly decreased the cell viability in a concentration-dependent manner. In preliminary experiments, we found that when osteoblasts were treated simultaneously with CUR and H_2O_2, the viability of osteoblasts did not have significant improvement compared with H_2O_2-treated alone. However, interestingly, pretreatment with CUR (0.1–10 μM) for 2 h significantly protected osteoblasts against H_2O_2-induced cell injury with dose-dependent manners. This result may be related with the relative rate of CUR and H_2O_2 incorporation into the cells. H_2O_2 is one of the major ROS. It

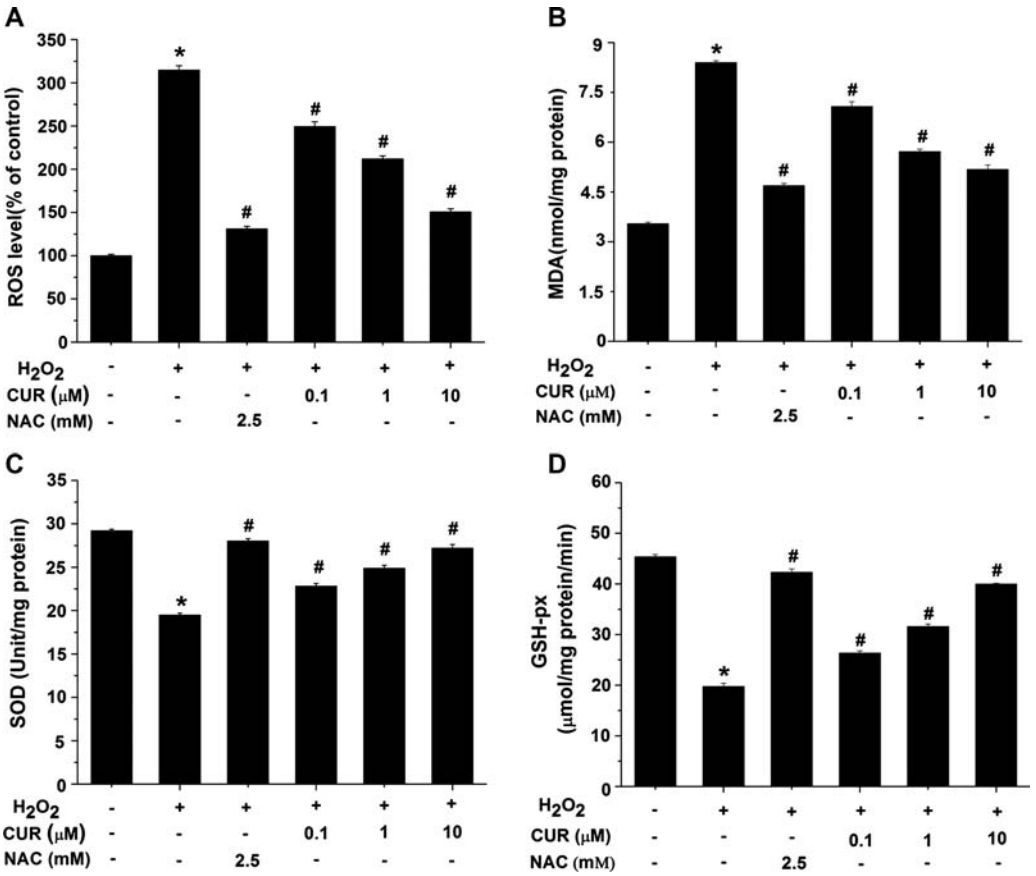

Figure 5 Antioxidant property of CUR in H_2O_2-treated osteoblasts (A) The effects of CUR on the production of intracellular ROS in H_2O_2-treated osteoblasts. Osteoblasts (3×10^5 cells/well in 6-well plates) were pre-incubated for 2 h with CUR (0.1–10 μM), and then exposed to 400 μM of H_2O_2 for 46 h. Pretreatment with CUR 2 h significantly suppressed ROS release induced by H_2O_2. The data were presented as the mean ± SD, $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. H_2O_2 only group. The effects of CUR on lipid peroxidation, SOD, and GSH-Px activities in H_2O_2-injured osteoblasts were displayed in (B–D), respectively. Osteoblasts (2×10^6 cells/100 mm dish) were seeded for 24 h, incubated with various concentrations of CUR for 2 h, and then treated with 400 μM of H_2O_2 for another 46 h. The data were presented as the mean ± SD ($n = 6$); $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. H_2O_2 only group.

facilely penetrates into cells and proceeds to react with intracellular ions such as iron and copper, to generate the highly reactive hydroxyl radicals that successively attack cellular components including lipids, proteins, and DNA to cause various oxidative damages. On the basis of these data, 400 μM of H_2O_2 was chosen to induce oxidative stress and CUR range (0.1–10 μM) was selected to investigate the effects of CUR on osteoblasts under oxidative stress condition in the subsequent assay.

Osteoblastic bone formation is thought to be mediated by two different processes: one is the formation of new osteoblasts, and the other is the activity of osteoblasts to produce bone matrix. Osteoblasts produce collagen, ALP, osteocalcin, and other matrix proteins. ALP is an important enzyme in the process of bone remodeling. It promotes mineralization of the matrix by decomposing phosphoric ester into inorganic phosphate, thus increasing the phosphorous concentration. Previous studies showed that H_2O_2-induced oxidative stress suppressed the differentiation process of osteoblasts in rabbit primary bone marrow stromal cells (BMSCs) and MC3-T3 cells [21,22]. In this study, we found that H_2O_2 significantly suppressed both activity of ALP and formation of calcium deposition, which were both reversed by CUR. These results demonstrate that CUR may promote osteoblast differentiation.

To confirm this, we further measured the expression of Runx2, type I collagen, and activation of Runx2. The differentiation of osteoblasts is modulated by Runt-related protein 2 (Runx2). Runx2 is a transcription factor that regulates transcription of its target genes such as osteocalcin, type I collagen, and osteopontin, and is thought to be essential for skeletal development [23]. Type I collagen is the major organic component of mineralized bone matrix, and is commonly used as an important biological marker of osteoblasts. We therefore also examined the expression of Runx2, type I collagen, and activation of Runx2. We found that CUR significantly reversed H_2O_2-suppressed nuclear Runx2 phosphorylation, and increased the expression of type I collagen (1 μM CUR) compared with H_2O_2 group, suggesting that CUR

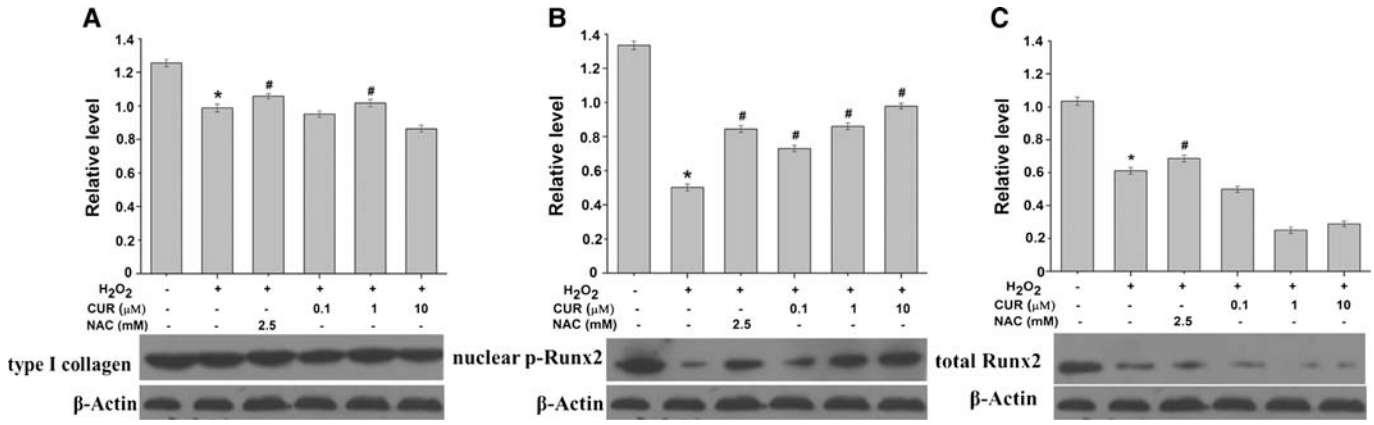

Figure 6 The effects of CUR on the expression of osteoblastic differentiation-associated molecular markers (type I collagen, nuclear p-Runx2, and total Runx2) Osteoblasts (2×10^6 cells/100 mm dish) were seeded for 24 h followed by incubation with various concentrations of CUR for 2 h, then treated with 400 μM of H_2O_2 for another 46 h. Lysates from the cells were subjected to western blot analysis. Blots were stripped and re-probed with anti-bodies against β-actin to correct for differences in protein loading. (A) Mean data (top) and representative gel (bottom) showing that CUR (1 μM) and NAC (2.5 mM) reversed H_2O_2-upregulated expression of type I collagen. (B) All dose of CUR and NAC (2.5 mM) reversed H_2O_2-upregulated expression of nuclear p-Runx2. (C) Mean data (top) and representative gel (bottom) showing that only NAC (2.5 mM) slightly reversed H_2O_2-downregulated expression of total Runx2, whereas CUR reduced the expression of total Runx2 compared with the H_2O_2 group. The data were presented as the mean ± SD ($n = 3$); $^*P < 0.05$ vs. H_2O_2 only group in type I collagen expression; $^{\#}P < 0.05$ vs. H_2O_2 only group in nuclear p-Runx2.

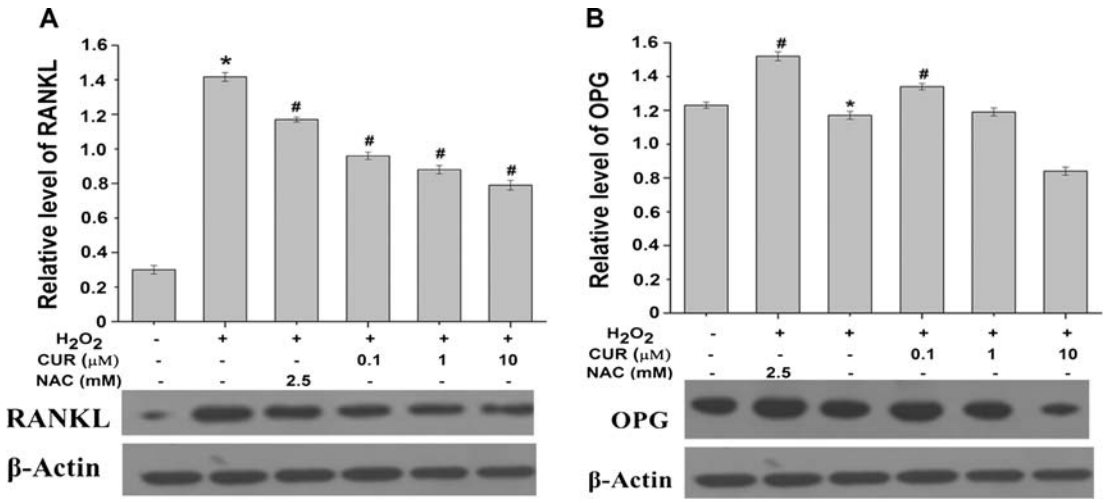

Figure 7 The effects of CUR on the expression of RANKL and OPG osteoblasts Cells were seeded for 24 h followed by incubation with various concentrations of CUR for 2 h, then treated with 400 μM of H_2O_2 for 46 h. Lysates from the cells were subjected to western blot analysis. Blots were stripped and re-probed with anti-bodies against β-actin to correct for differences in protein loading. (A) Mean data (top) and representative gel (bottom) showing that CUR (at the dose of 0.1, 1 and 10 μM) reversed H_2O_2-upregulated expression of RANKL. (B) Mean data (top) and representative gel (bottom) showing that CUR (at the dose of 0.1 and 1 μM) reversed H_2O_2-downregulated expression of OPG. The data were presented as the mean ± SD ($n = 3$); $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. H_2O_2 only group.

modulated osteoblast differentiation through increasing the expression of type I collagen and phosphorylation of nuclear Runx2. In addition, CUR reduced the expression of total Runx2 of osteoblasts compared with H_2O_2 treatment group. This is different from the report of the previous study that oxidative stress did not change the protein levels of total Runx2 during osteoblastic differentiation of BMSCs in rabbits [21]. These data suggest that CUR may act on nuclear Runx2 phosphorylation and type I collagen but not total Runx2.

ROS are chemically reactive molecules obtained from oxygen, and are capable of causing oxidative damage to biomacromolecules, leading to lipid peroxidation, oxidation of amino acid residues, formation of protein–protein cross-link and DNA oxidative damage. We examined the intracellular generation of ROS and MDA, an indicator

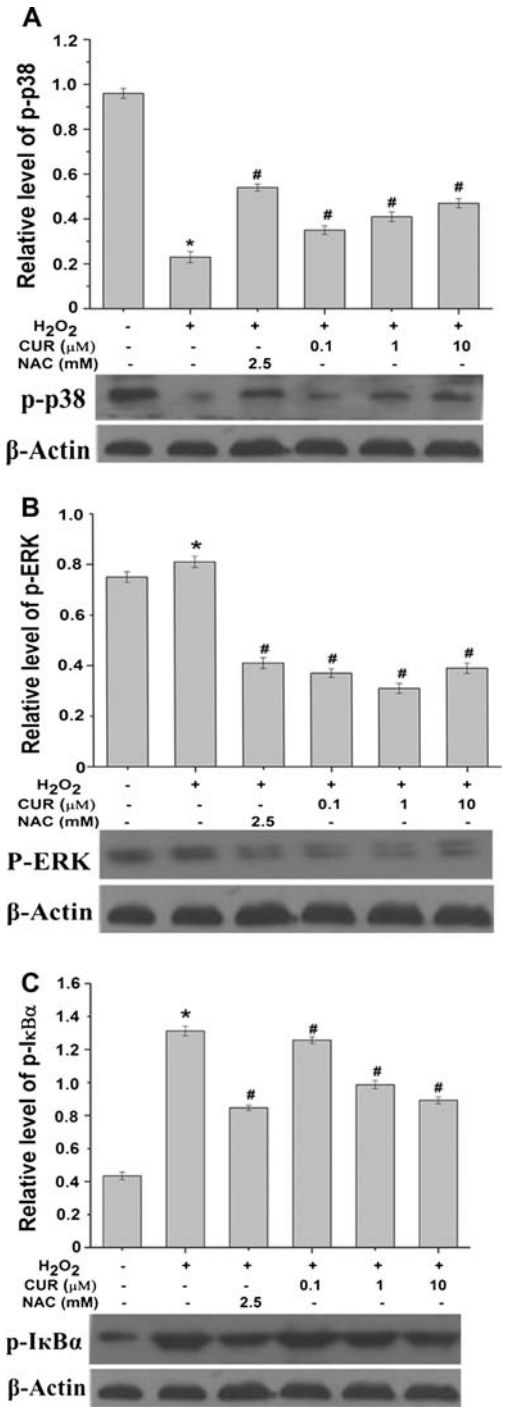

Figure 8 Possible signaling pathways of CUR on H_2O_2-injured osteoblasts Osteoblasts (2×10^6 cells/100 mm dish) were seeded for 24 h, incubated with various concentrations of CUR for 2 h, and then treated with 400 μM of H_2O_2 for 46 h. The expression of p38 MAPK (A), ERK1/2 (B), and NF-κB (C) was detected by western blot analysis with anti-phosphorylated p38 MAPK, ERK1/2, and IκBα anti-bodies, respectively. Mean data (top) and representative gel (bottom) showing that CUR decreased H_2O_2-activated IκBα and ERK phosphorylation, and increased H_2O_2-inhibited p38 MAPK phosphorylation in osteoblasts. The data were presented as the mean ± SD ($n = 3$); $^*P < 0.05$ vs. control without any treatment group; $^{\#}P < 0.05$ vs. H_2O_2 only group.

of lipid peroxidation, and found that CUR reduced H_2O_2-stimulated ROS production and MDA content. We continued to examine whether this effect is mediated by stimulation of the activity of antioxidant enzymes such as SOD and GSH-Px activities. GSH-Px is a major antioxidant enzyme that detoxifies hydrogen peroxide. In postmenopausal women with osteoporosis, GSH-Px activities was found to be lower, and estrogen deficiency lowered thiol antioxidant defenses in rodent bone, leading to accelerated bone loss with aging [24]. In addition, plasma level and activity of SOD, an enzyme that convert superoxide anion to hydrogen peroxide, were negatively associated with lumbar BMD in humans. CUR significantly attenuated H_2O_2-impaired SOD and GSH-Px activity. The enhanced SOD and GSH-Px activity by CUR may scavenge excessive superoxide derived from oxidative damage, ameliorating H_2O_2-impaired cell survival and proliferation.

Osteoblasts are not only involved in bone formation but also produce the RANKL and OPG to modulate the formation and differentiation of osteoclasts. RANKL is known to be the major factor responsible for osteoclast differentiation by providing a signal-to-osteoclast progenitors through the membrane-anchored receptor activator of NF-κB (RANK) to activate osteoclast differentiation and function. OPG blocks the interaction between RANKL and the RANK receptor. In other words, OPG inhibits osteoclastogenesis while RANKL supports bone resorption of osteoclasts. Therefore, the molecular triad of OPG, RANK, and RANKL plays a crucial role in bone remodeling and functions as a pivotal molecular link for osteoblast and osteoclast coupling [25,26]. In the primary mouse BMSCs and human osteoblast-like MG63 cell line, the elevation of ROS level stimulated RANKL mRNA and protein expression [21]. Chen *et al*. [27] found that ethanol-induced RANKL expression relied on increased intracellular levels of ROS in osteoclast precursors from BMSCs and osteoblast/pre-osteoclast co-culture. The potential sources of ROS promote osteoclastogenesis through RANKL expressed by osteoblasts, which support osteoclast differentiation and subsequent bone resorption [28]. We examined the effects of CUR on the protein expression of RANKL and OPG, and found that CUR prevented the increase of RANKL and attenuated the decrease of OPG in osteoblasts caused by H_2O_2, suggesting that CUR may act on osteoblasts to alter RANKL and inhibit osteoclastogenesis.

Previous studies demonstrated that H_2O_2-induced apoptosis was mediated by activation of ERK [29]. MAPKs play important roles in cell proliferation, differentiation, and apoptosis. NF-κB plays a critical role in the regulation of the cell cycle, cell adhesion, cytokine production, apoptosis, and other important cellular processes in

macrophages. The p38 MAPK has been reported to be necessary for the differentiation of osteoblastic MC3T3-E1 and mouse primary calvarial osteoblasts [30]. The activation of the ERK pathway contributed to apoptosis induced by H_2O_2 in oligodendrocytes [31], renal epithelial cells [32], and mesangial cells [33]. NF-κB plays a negative regulation role in osteoblast differentiation in MC3T3 cells [34] and human osteosarcoma cell line Saos-2 [35]. H_2O_2 stimulates ERK-dependent NF-κB activation, resulting in impaired osteoblastic differentiation [21], and exogenous metallothionein impaired H_2O_2-stimulated NF-κB signaling and prevented the hydrogen peroxide-induced inhibition of osteoblastic differentiation of primary mouse BMSCs [36]. Our results indicated that CUR enhanced the differentiation of osteoblasts under oxidative stress status through modulating phosphorylation of p38 MAPK, ERK, and NF-κB pathways.

Several risk factors (including smoking [37], hypertension [38], and diabetes mellitus [39]) for osteoporosis are associated with increased oxidative stress. Oxidative stress may play a role in the pathogenesis of osteoporosis with an association between increased levels of oxidative stress and reduced BMD. ROS generation has been shown to be important in the process of bone resorption. An *in vivo* experimental study in rats demonstrated that estrogen loss led to a reduced ability of osteoblasts to neutralize oxidants using antioxidant enzymes [40,41]. In normal or pathological aging, bone cells potentially become more vulnerable to basal or enhanced levels of oxidative stress as they mature, which could explain why older people are more susceptible to suffering bone fractures. Antioxidant therapies, especially when combined with existing treatments, could therefore represent a valuable approach in the management of diseased bone conditions associated with increased oxidative stress. CUR reduced H_2O_2-stimulated ROS production and MDA level, and significantly attenuated H_2O_2-impaired SOD and GPX activities of osteoblasts. These findings imply that CUR has powerful antioxidant activities, and can be considered as a potential natural resource for developing medicines or dietary supplements for the prevention and treatment of osteoporosis.

In summary, CUR reversed the dysfunction induced by oxidative stress in osteoblasts and its effect was associated with its antioxidant property. In addition, our study suggests that these effects mediated by CUR via p38 MAPK, ERK1/2, and NF-κB pathways may play key roles in the protection of osteoblasts. These results provide new insights into the osteoblast-protective mechanisms of CUR through reducing the production of ROS, suggesting that CUR may be developed as a bio-safe agent for the prevention and treatment of osteoporosis and other bone-related human diseases.

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