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Mangiferin protects osteoblast against oxidative damage by modulation of ERK5/Nrf2 signaling

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ABSTRACT

Oxidative stress has currently been proposed as a risk factor associated with the development and proression of osteoporosis. In this study, we identify the effect of mangiferin (MAN) on apoptosis and differentiation of osteoblast-like MC3T3-E1 cells insulted by H_2O_2. We firstly found that MAN can promote cell proliferation of MC3T3-E1 cells in a time- and dose-dependent manner and stimulate the phosphorylation of ERK5. Cells were divided as five groups: control, H_2O_2 (100 μM, control), H_2O_2 + MAN (5 μM), H_2O_2 + MAN (10 μM), and H_2O_2 + MAN (20 μM). MAN can significantly decrease H_2O_2-induced apoptosis and elevated ROS level of MC3T3-E1 cells. The expressions of caspase-3, caspase-9 and Bax/Bcl-2 were increased with H_2O_2 treatment, and MAN can reverse these changes. In addition, Nrf2 and its downstream target effectors (HO1, NQO1) were dramatically attenuated in MC3T3-E cells treatment with H_2O_2, while MAN can significantly increase the expression of Nrf2, HO1 and NQO1. The expression of ERK5 was down regulated by RNA interference in MC3T3-E1 cells, and we found that MAN (20 μM) pretreatment didn't make remarkable decrease in cell apoptosis or expressions of apoptosis-related proteins in H_2O_2-insulted siRNA-ERK5 cells. This study indicated that MAN can protect osteoblast against oxidative damage by modulation of ERK5/Nrf2 signaling, which can be new agent for osteoporosis.

1. Introduction

Oxidative stress has currently been proposed as a risk factor associated with the development and proression of osteoporosis, and the underlying mechanism involved in regulation of Nrf2/HO-1 and NF-κB signaling pathway in osteoporosis mediated by oxidative stress has been studied extensively [1,2]. There is increasing evidence that increase of reactive oxygen species (ROS) accumulation leads to oxidative stress under conditions of aging, or some illnesses, or use of medicines and subsequently suppress induction of Nfr/HO-1 pathway activation and activation of NF-κB signaling, which contributes to development and progression of osteoporosis [3–5]. As a result, antioxidants may be adopted as a novel therapeutic approach in the prevention and treatment of osteoporosis.

Mangiferin (MAN) a naturally occurring polyphenol commonly found in both mango and papaya is a natural immunomodulator [6]. MAN exhibits a viariaty of pharmacological activities including anti-radiation [7], anti-tumor [8], anti-diabetes [9], anti-inflammatory [10] and immunoregulation [11]. Previous study reported that MAN attenuated osteoclast formation and bone resorption by attenuating RANKL-induced signaling, inhibited RANKL-induced activation of NF-κB, and abrogates RANKL-induced ERK phosphorylation [12]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor regulating antioxidant genes by binding to antioxidant response elements and it plays an important role in response to oxidative stress [13]. The activation of Nrf2 will promote expression of several phase II and anti-oxidative enzymes including heme oxygenase 1 (HO-1), which was as a feedback to reduce the oxidative injury [14]. ERK5 MAP kinase play important role in osteoclast apoptosis [15], and the phorphorylation of ERK5 induces the phorphorylation of Bad. The p-Bad is binding with 14-3-3 protein in the cytoplasm, which prevents it from translocating to mitochondria where it can induce activation of caspase-3 [16]. In addition, it also acts as the upstream gene regulating Nrf2 activation [17]. We previously found that MAN promoted cell

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proliferation and the phosphorylation of ERK5, and we aim at exploring the protective effect of MAN on H_2O_2-insulted MC3T3-E1 cells and clarifying the mechanism.

2. Materials and methods

2.1. Mangiferin

Mangiferin (MAN, CAS#: 4773-96-0) with a purity of 98% was obtained from Shanghai Yuanye Biotech Co., Ltd (Shanghai, China). It was dissolved in an appropriate amount of dimethylsulfoxide (DMSO) and diluted to the desired concentrations before utilization, with the final concentration of DMSO kept below 0.5%.

2.2. Cell culture

Osteoblast-like MC3T3-E1 cells were purchased from National Infrastructure of Cell Line Resource (Beijing, China). MC3T3-E1 cell line was cultured in α-minimum essential medium supplemented with 10% fetal bovine serum 100 U/ml penicillin and 100 lg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were regularly split and subcultured up to 80–90% confluence before experimental procedures.

2.3. Cell viability assay

MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product] was used to estimate cell viability. Cells were seeded in a 96-well plate at 1×10^5/well and treated with α-MEM (Basal), H_2O_2 (100 μM, control), H_2O_2 + MAN (5 μM), H_2O_2 + MAN (10 μM), and H_2O_2 + MAN (20 μM). After treatment for 0, 6, 12, 24, 48 and 72 h, cells were gently washed twice with PBS. Cells were incubated with 200 μl fresh medium containing 0.5 mg/ml MTT in the last 4 h of the culture period tested at 37 °C in the dark. The media were decanted and then washed twice with PBS. The produced formazan salts were dissolved with dimethyl sulphoxide, and the absorbance at 490 nm was measured using an ELISA reader.

2.4. TUNEL assay

To identify apoptotic cells, a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed. After treatment, MC3T3-E1 cells cultured on coverslips fixed with paraformaldehyde (4% in PBS, pH = 7.4), and exposed to 0.1% Triton X-100 in 0.1% sodium citrate. Next, cells were incubated with 50 μl TUNEL reaction mix (In Situ Cell Death Detection kit, POD, Roche, IN, USA), followed by DAPI Staining Solution (Beyotime Institute of Biotechnology, China). Coverslips were mounted on glass slides with Antifade Mounting Medium (Beyotime Institute of Biotechnology, China) for fluorescence microscopy.

2.5. Flow cytometric analysis for apoptosis

Cells were seeded in a 6-well plate. After RNA interference treatment, MAN pretreatment for 4 h and H_2O_2 treatment for 24 h, cells were washed out with fresh medium. Apoptosis was examined by Annexin V-fluorescein isothiocyanate staining (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The fluorescein isothiocyanate (FITC) fluorescence intensity of 10,000 cells was measured using a Becton-Dickinson FACS Caliber flow cytometer (BD Biosciences). Apoptosis ratio was calculated as the ratio of apoptotic cell number to total cell number.

2.6. Detection of intracellular ROS

Cells were seeded in a 6-well plate and treated with α-MEM (Basal), H_2O_2 (100 μM, control), H_2O_2 + MAN (5 μM), H_2O_2 + MAN (10 μM), and H_2O_2 + MAN (20 μM). After MAN pretreatment for 4 h and H_2O_2 treatment for 24 h, cells were gently washed twice with PBS to exclude the interference of added H_2O_2. Intracellular ROS were quantified by employing ROS-sensitive dye, DCFH-DA. Fluorescent images were acquired from a laser confocal microscope (Zeiss LSM 510 META), and the intensity on regions of interest was measured.

2.7. RNA interference

ERK5-siRNA cell transfection was performed according to the guide lines of Santa. Briefly speaking, MC3T3-E1 cells were cultured in 6-well plate for 24 h (cell confluence: 70–80%) and then cultured in base medium. 100 μl transfection medium containing 6 μl of ERK5-siRNA (Santa Cruz Biotechnology, USA) was mixed with another 100 μl transfection medium containing 6 μl siRNA transfection (Santa) reagents and was incubated for 30 min. The siRNA mixture was added with 0.8 ml siRNA transfection medium. MC3T3-E1 cells were washed with siRNA transfection medium and the mixture, and then cultured in cell incubator for 6 h. Cell growth and transfection were observed by fluorescence microscope (Olympus, Japan). Cell culture medium was replaced by 20% FBS medium and then incubated for 24 h. Transfected cells were finally cultured in 10% FBS medium.

2.8. Western blot

Cells were obtained and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rcf for 10 min at 4 °C and the supernatant (20–30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies against caspase-3/-8 (Abcam), Bcl-2 (Abcam), Bax (Abcam), ERK5 (Abcam), phosphorylation-ERK5 (Abcam), Nrf2 (Abcam), HO-1 (Abcam), NQO1 (Abcam) and GAPDH (Fermentas). Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China).

2.9. Statistical analysis

All results are expressed as mean ± SD. Multiple comparisons were done by Student *t*-test. Differences yielding P values less than 0.05 were considered statistically significant. Statistical analysis was performed using SAS statistical software (SAS Inc., NC, USA).

3. Results

3.1. MAN exhibits no cytotoxicity on MC3T3-E1 cells and promotes phosphorylation of ERK5

We firstly identify the effect of MAN on the proliferation of MC3T3-E1 cells by MTT assay as previously described. As shown in Fig. 1A, after treatment of MAN (0, 1, 2, 5, 10, 20 and 50 μM) for 6, 12, 24 and 48 h, MAN over the dose of 5 μM could obviously increase the cell viability of MC3T3-E1 cells at 24 and 48 h ($P < 0.05$). in addition, MAN could promote the cell proliferation in a dose- and time-

dependent manner. 5, 10 and 20 μM of MAN were determined for the further investigations. We then found that 5, 10 and 20 μM of MAN could significantly promote the phosphorylation-ERK5 (Fig. 1B).

The effect of MAN on cell viability of MC3T3-E1 cells insulted by H_2O_2 treatment was also detected. As shown in Fig. 1C, H_2O_2 induced a significant decrease in cell viability of MC3T3-E1 cells at 24, 48 and 72 h compared with the control group. However, 5, 10 and 20 μM of MAN treatment can reduce the oxidative damage, and the cell viabilities of MAN treatment groups were increased notably compared as the H_2O_2 treatment group at 48 and 72 h ($P < 0.05$).

3.2. Effects of MAN on ROS level and apoptosis of H_2O_2-insulted MC3T3-E1 cells

The generation of ROS in cells easily leads to mitochondrial apoptosis. As Fig. 2B exhibited, MDA levels were significantly soared by the H_2O_2 treatment after 24 h compared the control group. MAN effectively revised the MDA level in comparison with the H_2O_2 group. We also identify the ROS level of MC3T3-E1 cells as previously described. The ROS level was significantly increased by H_2O_2 treatment, while MAN notably decreased the ROS level in a dose-dependent manner (Fig. 2A).

H_2O_2 shows cytotoxicity in MC3T3-E1 cells, and we explored the protective effect of MAN on H_2O_2-insulted MC3T3-E1 cells. Cell apoptosis was identified by flow cytometry analysis (Fig. 2B) and TUNEL (Fig. 3). Figs. 2B and 3 showed that cell apoptosis rate of control, H_2O_2 and MAN treated groups. H_2O_2 resulted in an apoptosis rate of 36.42± 8.96% compared with the control group of 6.77± 0.94%. However, MAN decreased the apoptosis rate as 32.15± 4.32% (5 μM), 20.17± 2.68% (10 μM) and 16.24± 3.17% (20 μM). MAN displayed protective effect of H_2O_2-insulted MC3T3-E1 cells.

3.3. Effects of MAN on the expressions of apoptosis-related proteins and Nrf2 signaling

Protein expression of caspase-3, caspase-9 and Bax/Bcl-2 was identified by western blot analysis. We can see in Fig. 4A, steep increase in the activities of caspase-3 and caspase-9 was observed in MC3T3-E1 cells treated with H_2O_2. The increasing activation of caspase-9 indicated H_2O_2 could induce the apoptosis initiation mediated by mitochondria and/or death receptor. However, treatment of MAN (5, 10 and 20 μM) dramatically suppressed the activation of caspase-3 and caspase-9 induced by H_2O_2 in a dose-dependent manner. In addition, H_2O_2 treatment also notably resulted in increased expression of Bax/Bcl-2 in comparison with the control cells (Fig. 4A). As expected, it was suppressed after addition of MAN (10 and 20 μM). In conclusion, the results demonstrate that MAN is an effective inhibitor of H_2O_2-induced apoptosis in osteoblastic cells. Expressions of Nrf2 and its downstream targets (HO-1 and NQO-1) was decreased in MC3T3-E1 cells with H_2O_2 treatment, MAN (5, 10 and 20 μM) dramatically increased the expressions of Nrf2, HO-1 and NQO-1 (Fig. 4B).

3.4. MAN decreases the H_2O_2-induced apoptosis of MC3T3-E1 cells through ERK5 signaling

With the aim of determining the protective effect of MAN

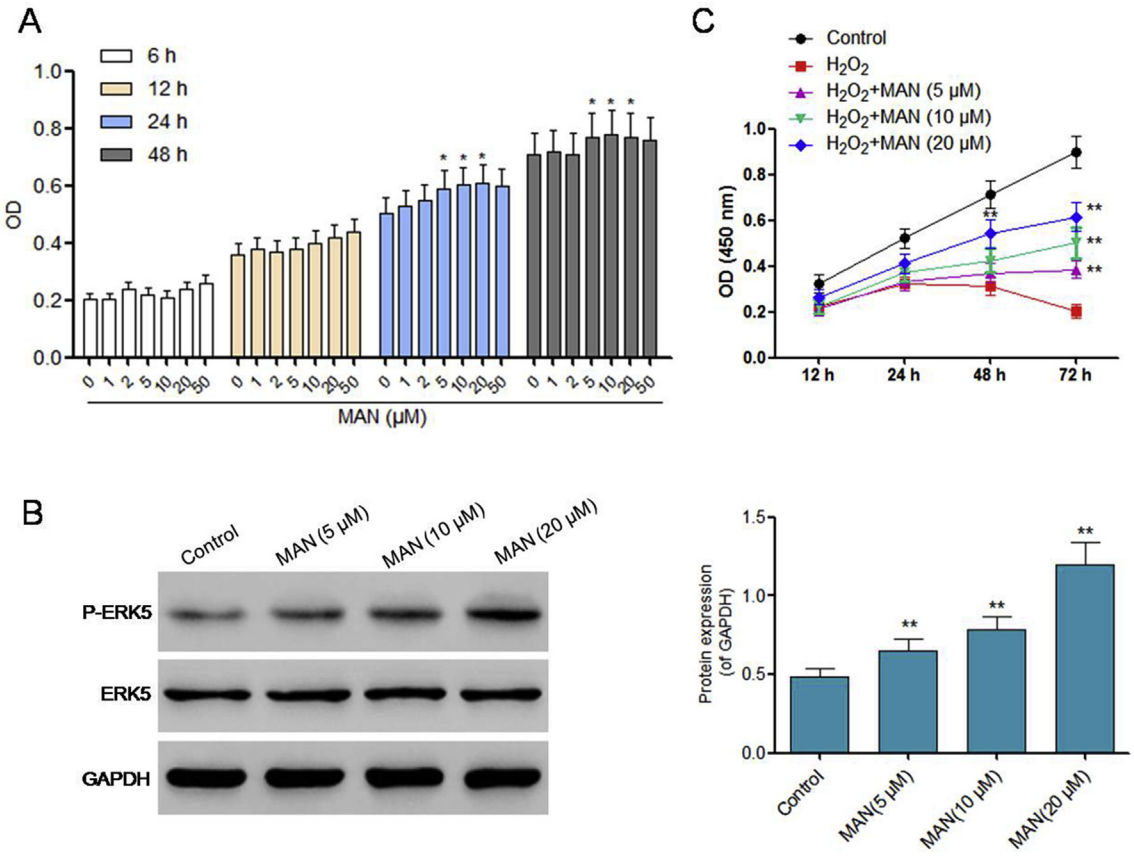

Fig. 1. Effect of MAN on cell viability and p-ERK5 of MC3T3-E1 cells. (A) After MC3T3-E1 cells were exposed to various concentrations of MAN (0, 1, 2, 5, 10, 20 and 50 μM) for 6, 12, 24 and 48 h, cell viability was determined by MTT assay. (B) P-ERK5 and ERK5 expressions were identified by western blot analysis. (C) MAN protected MC3T3-E1 cells against H_2O_2-induced injury. Data were presented as mean ± SD, n = 6, $^*P < 0.05$, $^{**}P < 0.01$ *versus* H_2O_2 treated MC3T3-E1 cells.

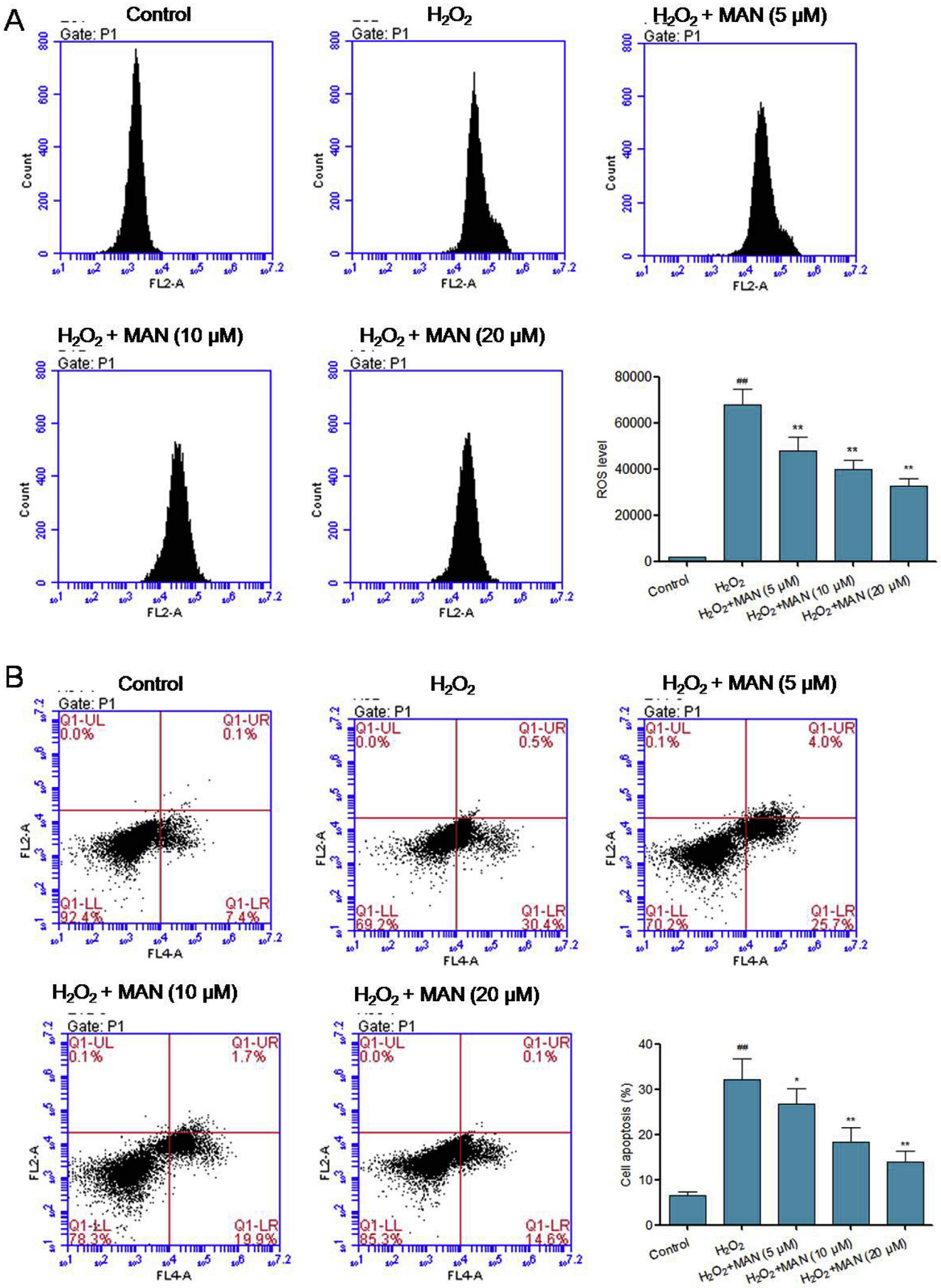

Fig. 2. Effect of MAN on ROS level and apoptosis in H_2O_2-insulted cells. Cells treated with different dose of MAN (5, 10 and 20 μM) for 2 h, then exposed to H_2O_2 for 24 h (A) ROS level and (B) cell apoptosis were identified by flow cytometry. Data were presented as mean ± SD, n = 6, $^{\#\#}P < 0.01$, *versus* control; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* H_2O_2 treated MC3T3-E1 cells.

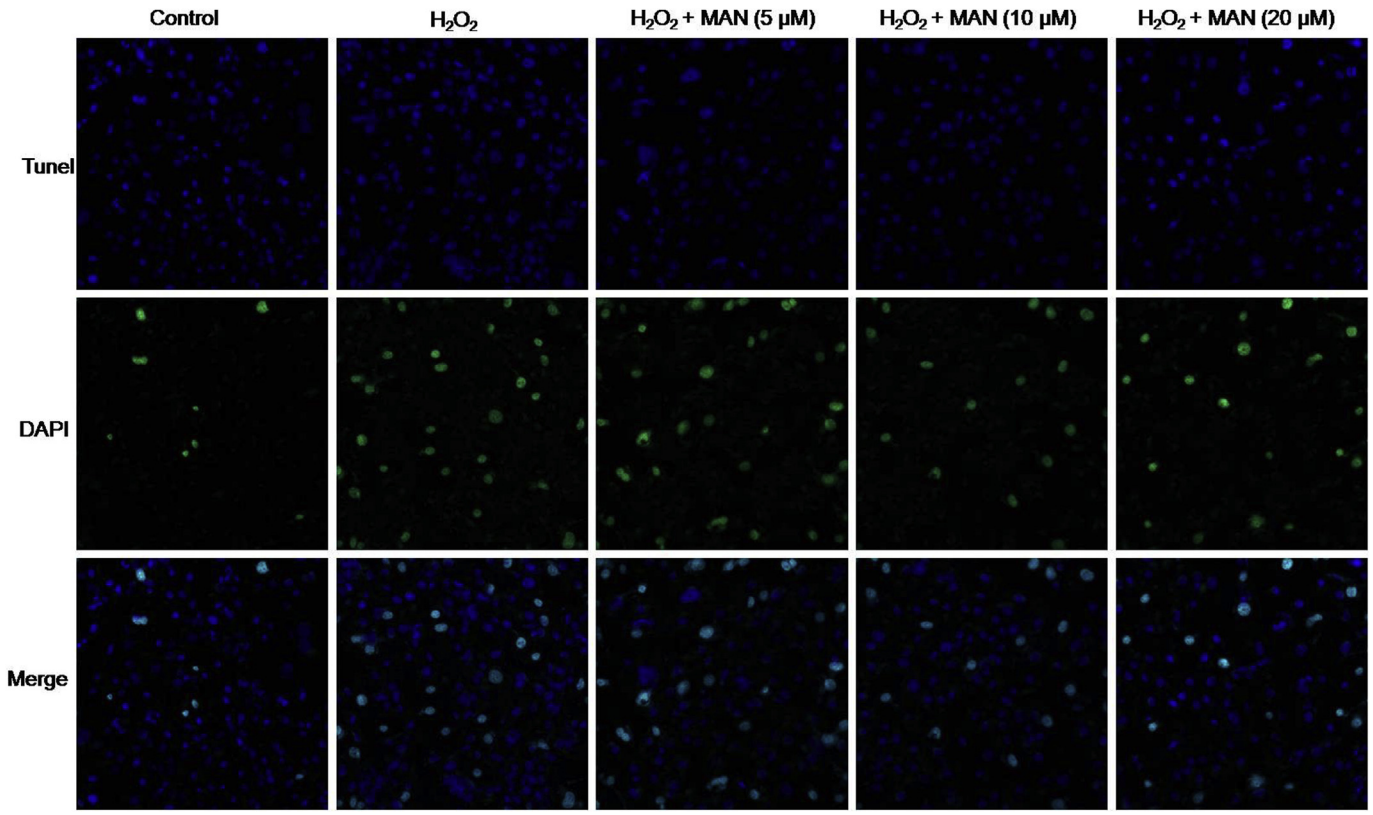

Fig. 3. Cell apoptosis detected by TUNEL.

against oxidative damage via ERK signaling, we then detected the protective effect of MAN on siRNA-ERK5 cells and normal cells. mRNA expression of ERK5 was significantly decreased in MC3T3-E1 cells after RNA interference treated for 48 h (data was not shown). Cells with siRNA-ERK5 treatment were exposed to H_2O_2 for 24 h, the apoptosis rate of H_2O_2 treated siRNA-ERK5 group was decreased compared with the control group. MAN (20 μM) effectively attenuated the apoptosis of H_2O_2-insulted normal cells. However, MAN (20 μM) pretreatment didn't make remarkable decrease in H_2O_2-insulted siRNA-ERK5 group (Fig. 4C).

Protein expressions of caspase-3/-9 and Bax/Bcl-2 were also assessed by western blot analysis. As shown in Fig. 4D, there is notable increase of caspase-3, caspase-9 and Bax/Bcl-2 in H_2O_2 treated siRNA-ERK5 MC3T3-E1 cells compared with the control cells ($P < 0.01$). MAN (20 μM) pretreatment can effectively reverse the increased expression of caspase-3, caspase-9 and Bax/Bcl-2 in H_2O_2-insulted normal cells, while MAN (20 μM) pretreatment didn't make remarkable increase in the expression of caspase-3, caspase-9 and Bax/Bcl-2 of H_2O_2-insulted siRNA-ERK5 cells.

4. Discussion

The results described in this report were summarized as follows: (1) MAN attenuated H_2O_2-induced cell apoptosis in osteoblastic cells and promoted the phosphorylation of ERK5; (2) H_2O_2 treatment in MC3T3-E1 cells significantly suppressed the expression of Nrf2 and inhibited the downstream targets, which can be revised by MAN; and (3) MAN protected MC3T3-E1 cells against oxidative damage via ERK/Nrf2 signaling.

Oxidative stress is a pivotal pathogenic factor for agerelated bone loss in mice and rats, leading to an increase in osteoblast and osteocyte apoptosis, and a decrease in osteoblast numbers and the rate of bone formation [18–20]. In addition, a series of study suggested an important association between oxidative stress and the pathogenesis of osteoporosis in humans [21–23]. It is also reported that the increased oxidative stress can reduce bone mineral density in men and women who are 55 years or older [24]. Along with the above evidence, we expect to find natural product which shows anti-osteoporosis and demonstrate the mechanism. We firstly find that MAN (5, 10 and 50 μM) can promote the proliferation and the phosphorylation of ERK5. Previous study reported that the phosphorylation of ERK5 contributes to decrease the apoptosis of osteoblast [25].

Mitochondria play a crucial role in the complex process of apoptosis [26,27]. High levels of ROS induce mitochondrial dysfunction and apoptosis. During this process, mitochondrial membrane pores are opened, resulting in the loss of mitochondrial membrane potential (MMP). The loss of MMP causes an increase in the permeability of the mitochondrial membrane, followed by the release of pro-apoptotic molecules such as cytochrome *c*. Cytochrome *c* releasing from mitochondrial interacts with ATP, Apaf-1 and caspase 9, and subsequently activates caspase-3, which consequently elicits caspase-dependent apoptotic cell death [28,29]. The western blot analysis results suggest that the protein levels of caspase-3 and caspase-9 increased after treatment with H_2O_2. Bcl-2 family members are crucial to regulating the mitochondrial death pathway [30,31] and include anti-apoptotic proteins Bcl-2 and Bcl-xL and pro-apoptotic proteins Bax, Bak and Bid. Apoptosis inhibitory protein Bcl-2 residing on the outer mitochondrial membrane suppresses cytochrome *c* release through inhibiting mitochondrial permeability transition and/or stabilizing the outer mitochondrial membrane barrier function. Bax/Bcl-2 expression was also significantly increased by treatment with H_2O_2 compared with the control cells. In our study, MAN can

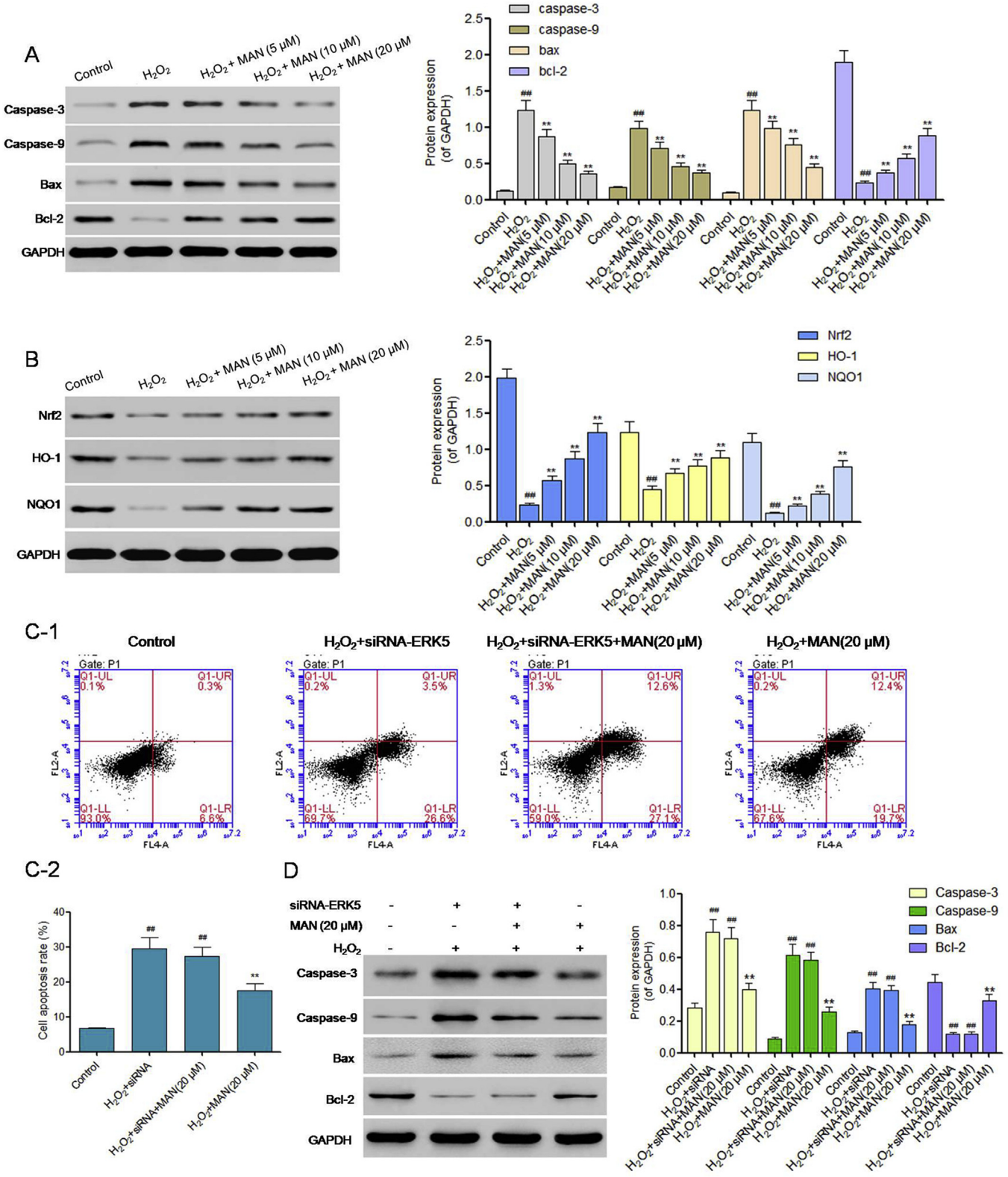

Fig. 4. MAN decreases the H_2O_2-induced apoptosis of MC3T3-E1 cells through ERK5 signaling. Cells treated with different dose of MAN (20, 50 and 100 μM) for 2 h, then exposed to H_2O_2 for 12 h. (A) Caspase-3/-9 and Bax/Bcl-2 expression expressions were determined by western blot. (B) Protein expression of Nrf2, NQO-1, HO1 and NF-κB was evaluated by western blot. (C) Cells with siRNA-ERK5 treatment were exposed to H_2O_2 for 24 h. Effect of MAN on H_2O_2-insulted siRNA-ERK5 cells and normal cells was assessed, cell apoptosis was detected by flow cytometry analysis. (D) Caspase-3/-9 and Bax/Bcl-2 expression expressions were determined by western blot.
Data were presented as mean ± SD, n = 6, ##P < 0.01, *versus* control, **P < 0.01 *versus* H2O2 treated MC3T3−E1 cells.

suppress the expression of caspase-3/-9 and Bax/Bcl-2 expression, which indicated that MAN can protect the MC3T3-E1 cells against caspase- and mitochondrial death pathway dependent apoptosis induced by oxidative stress.

Nrf2 signaling pathway is reported as one of the major regulators of cytoprotective responses to endogenous and exogenous stresses caused by ROS and electrophiles [32,33]. Thus, we determined the effects of H_2O_2 on the protein expression of Nrf2 and the downstream genes expression by western blot analysis. The results showed that the protein expression of Nrf2 and the downstream effectors (HO1, NQO1) were dramatically attenuated in MC3T3-E cells treatment with H_2O_2. It is reported that ERK participates in the process of apoptosis in osteoblasts [16] and it also the upstream gene regulating Nrf2 activation [17]. The phosphorylation of ERK5 can inhibit the activity of caspase-3 and the inhibition of ERK5-FasL actually gives rise to cell apoptosis of osteoblasts [16]. In this study, we found that MAN (20 μM) effectively attenuated the apoptosis of H_2O_2-insulted normal cells. However, MAN (20 μM) pretreatment didn't make remarkable decrease in H_2O_2-insulted siRNA-ERK5 group, which indicated that MAN decreases the H_2O_2-induced apoptosis of MC3T3-E1 cells through ERK5/Nrf2 signaling.

In summary, results from this cell-based study reveal that MAN show the capability of anti-oxidative through the regulation of ERK5/Nrf2-mediated responses in MC3T3-E1 cells. This study also provides protocols for screening natural products on oxidative and anti-oxidative responses in osteoblast.

Disclosure

The authors report no conflicts of interest in this work.

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