

Autophagy attenuates the oxidative stress-induced apoptosis of Mc3T3-E1 osteoblasts

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Abstract. – OBJECTIVE: The oxidative stress-induced osteoblast apoptosis plays an important role in the pathological process of osteoporosis, but the roles of autophagy in oxidative stress and apoptosis of osteoblasts remain unclear. This study aimed to observe the role of autophagy in oxidative stress injury of osteoblasts and the relationship between autophagy and apoptosis.

MATERIALS AND METHODS: Mc3T3-E1 cells were stimulated with different concentrations (0.1, 0.5, and 1 mM) of hydrogen peroxide. The cell viability was detected via cell counting kit 8 (CCK8) at different time points (0, 2, 6, 8, and 12 h), the apoptosis was detected via Western blotting and flow cytometry, and the autophagy was detected via macrophage-derived chemokine (MDC) and transmission electron microscope. The changes in expression of autophagy-associated protein, Beclin1, and LC3II/I ratio, were detected via Western blotting. Moreover, the intracellular reactive oxygen species (ROS) level and extracellular superoxide dismutase (SOD) level were observed using the autophagy regulators, rapamycin (Rap) and 3-methyladenine (3-MA), so as to clarify the interaction between autophagy and cellular oxidation.

RESULTS: Hydrogen peroxide-induced apoptosis and autophagy of osteoblasts were in dose- and time-dependent manners; the hydrogen peroxide inhibitors could inhibit the autophagy level, and autophagy inhibitor (3-MA) could significantly enhance the hydrogen peroxide-induced ROS level and apoptosis rate in cells. Besides, Western blotting confirmed that the cleaved caspase-3 and cleaved poly adenosine diphosphate ribose polymerase (PARP) proteins were increased. The autophagy inducer (Rap) partially inhibited the hydrogen peroxide-induced oxidative stress and apoptosis.

CONCLUSIONS: Autophagy inhibits the oxidative stress-mediated apoptosis of osteoblasts, which is a potential target for the osteoporosis treatment.

Key Words:

Osteoporosis, Oxidative stress, Autophagy, Apoptosis.

Introduction

Osteoporosis is a kind of systemic metabolic bone disease characterized by low bone mass, bone microstructure damage, increased bone fragility and proneness to fracture, which seriously affects the life quality of patients¹. Previous studies² mostly focused on the role of osteoclast hyperactivation in the pathogenesis of osteoporosis, but the role of osteoblast dysfunction in osteoporosis has gradually attracted attention in recent years. The viability of osteoblasts in postmenopausal osteoporosis patients and animal models with osteoporosis after spinal cord injury is decreased³, and the responses of osteoblasts to estrogen⁴ and leptin⁵ are significantly reduced. This proves that osteoblast dysfunction, besides the cytokines, may play a more important role in the pathogenesis of osteoporosis.

Oxidative stress refers to the increased reactive oxygen species (ROS) level in tissues or cells due to the excessive production of ROS or obstacle clearing in the body, which is closely related to the aging, cancer and diabetes mellitus. Oxidative stress can produce a series of changes in cells, activate apoptotic signaling pathways and lead to cell dysfunction⁶. Currently, studies have shown that oxidative stress is the common pathological basis of various types of osteoporosis. It is reported in the literature that the ROS level in bone tissues is increased in different degrees in the postmenopausal osteoporosis, senile osteoporosis and glucocorticoid-induced oste-

oporosis, suggesting that oxidative stress plays a vital role in pathological processes of various types of osteoporosis⁷.

Autophagy is closely related to the cellular damage repair, cell replication, proliferation, etc., which plays important roles in cell stress and environmental adaptation. At present, it has been confirmed that glucocorticoid⁸, high glucose⁹ and other stress factors can induce the autophagy of osteoblasts or osteocytes, and the inhibition of cell autophagy leads to increased apoptosis, suggesting that autophagy has a protective effect in these stress environment. The study on the role of osteoblast autophagy in oxidative stress injury is still in its infancy. Recently, investigations have found that autophagy inhibits the oxidative stress injury, and plays a protective effect on cells¹⁰. However, the excessive autophagy can activate apoptosis or convert into the autophagic death⁶. Therefore, it is necessary to further investigate the relationship among osteoblast autophagy, oxidative stress, and apoptosis.

Materials and Methods

Experimental Materials

Mc3T3-E1 cells were purchased from ATCC (Manassas, VA, USA); 3-methyladenine (3-MA) and rapamycin (Rap) were purchased from Sigma-Aldrich (St. Louis, MO, USA); the cell culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); the antibodies used in the experiments were bought from Cell Signaling Technologies (Danvers, MA, USA), and fluorescent antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

Cell Counting Kit 8 (CCK8) Assay

5×10^3 osteoblasts were inoculated into a 96-well plate (Corning, Corning, NY, USA), and when the cell density reached 80-90%, 0.1, 0.5 and 1 mM hydrogen peroxide were added, respectively; the treatment group added with double distilled water was set as the blank control. At 0, 2, 4, 6, 8 and 12 h after treatment, 120 μ L supernatant was taken from each well, respectively, and added into another new 96-well plate, followed by sample determination.

Electron Microscope Scanning of Autophagosomes

10^7 - 10^8 cells were washed with phosphate-buffered saline (PBS) for 2-3 times and fixed with

2.5% neutral glutaraldehyde (preparation of phosphate buffer, pH 7.2 and 7.4) at 4°C for 2-4 h. They were washed with 0.1 M PBS for two times (30 min/time), and fixed with 1% osmic acid at 4°C for 2 h. Next, cells were treated with gradient dehydration using acetone (50-70%), placed in the 70% acetone-prepared saturated uranyl acetate overnight, and dehydrated on the next day using acetone (100% \times 5) (10 min/time) at room temperature. They were soaked in the mixture of acetone and epoxy resin (1:1) for 1 h, and embedded into the embedding medium without 2,3-dimercaptopropanol-30 (DMP-30) for 2 h in the oven at 40°C. Next, they were embedded into the epoxy resin, followed by semi-thin section localization, ultrathin section lead staining, and observation via scanning electron microscope.

Detection of Autophagy Rate Via Flow Cytometry

Cells were inoculated onto a 6-well plate at a density of 2×10^5 per well. After cells covered 90% of the plate, the medium was replaced with the Dulbecco's modified Eagle medium (DMEM) containing 1% fetal bovine serum (FBS) for incubation for 12 h to synchronize cells. After different stimulation conditions, the cells were washed with PBS for 3 times at room temperature (5 min/time). Then, the cells were incubated with 0.05 mM macrophage-derived chemokine (MDC) at 37°C for 10 min, and washed with PBS for 4 times (5 min/time) to remove the excess MDC. After that, the cells were digested with trypsin, centrifuged and resuspended using PBS. The autophagy rate was detected via flow cytometry within 30 min at the excitation wavelength of 488 nm.

SOD Detection

After grouping and treatment, the culture supernatant was collected. After the reagents were added according to the instructions of superoxide dismutase (SOD) kit, the mixture was shaken evenly, followed by water bath at constant temperature (37°C) for 35 min; 2 mL developer was added, mixed and placed for 10 min, followed by zero setting using distilled water; the optical density (OD) at 550 nm was measured. SOD activity (U/mL) = $(OD_{\text{control tube}} - OD_{\text{measured tube}}) \div 50\% \times \text{dilution ratio of reaction system} \times \text{dilution ratio before sample test}$.

ROS Detection

After grouping and treatment, the original medium was discarded and cells were washed with

pre-cooled D-Hanks solution at 4°C for 1-2 times. DCFH-DA (2',7'-Dichlorofluorescein diacetate) fluorescent probe was added into the cells using serum-free 1640 medium at a ratio of 1:1000. The positive control group was added with Rosup and placed in an incubator for 30 min, followed by washing of cells for 1-2 times and collection of cells. The cells were resuspended using the 200 μ L serum-free medium, and fluorescence intensity of each group of cells was detected using flow cytometer. The average fluorescence intensity was analyzed using CELL Quest™ software.

Detection of Apoptosis Rate Via Flow Cytometry

Cells were treated with different concentrations of hydrogen peroxide, digested using trypsin at different time points, centrifuged and resuspended using BD binding buffer (Franklin Lakes, NJ, USA); the cell concentration was adjusted into 10^6 /mL, and the samples with 10^5 cells were taken and incubated in Annexin V-PE solution and AAD (7-amino-actinomycin D) solution in a dark room at room temperature for 15 min; then, BD binding buffer was added. The apoptotic rate was measured using flow cytometer after 30 min.

Western Blotting

The protein was extracted using the protein extraction kit from cells collected from each group, and the protein concentration was determined using bicinchoninic acid (BCA) method. After protein quantification, 5 μ L 5 \times loading buffer solution was added, and the mixture was boiled at 99°C for 5 min, followed by separation via sodium dodecyl sulfonate polyacrylamide gel electrophoresis. The concentration of spacer gel is 5%, while that of lower separation gel is 10%; the separation was performed under constant voltage, 90 V for spacer gel for 90 min and 150 V for separation gel for 50 min, until the bromophenol blue reached the bottom of the gel. The gel, filter paper and polyvinylidene difluoride (PVDF) membrane were placed in order, followed by membrane transfer in the electrophoresis tank. After that, the membrane was removed and labeled, and then sealed in 5% skimmed milk powder for 1 h and incubated at 37°C in a shaker. The membrane was washed with phosphate-buffered saline (PBS) for 3 times to wash off the sealing solution, and the primary antibody (diluted at 1:850) was added dropwise onto the membrane in a dark place at 4°C overnight. On the next day, the primary antibody was recovered, and the

membrane was washed with PBST for 3 times (5 min/time). Then, the secondary antibody horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG), diluted at 1:5000, was added for incubation in a shaker at 37°C for 2 h. After that, the secondary antibody was discarded, and the membrane was washed with PBST (PBS and Tween 20) for 3 times (15 min/time). Finally, the image was developed, and the gray image scale was scanned using ImageJ.

Statistical Analysis

Statistical Product and Service Solutions 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical processing and analysis. Measurement data were presented as mean \pm standard deviation ($\bar{x} \pm s$). One-way analysis of variance was used for the comparison among groups, while *t*-test was used for pairwise comparison. $p < 0.05$ suggested that the difference was statistically significant.

Results

Effect of Hydrogen Peroxide on Survival of Osteoblasts

The effects of hydrogen peroxide on the proliferation, apoptosis and death of osteoblasts were observed at 2, 4, 6, 8, and 12 h after treatment with hydrogen peroxide. The optical density was determined using lactate dehydrogenase (LDH) colorimetric method, and the inhibition rate of cell growth was calculated using the formula. With the increase of hydrogen peroxide concentration and the prolongation of time, the growth of osteoblasts was significantly inhibited (Figure 1A). At the same time, the results of flow cytometry showed that the apoptotic rate of osteoblasts was increased after the treatment with hydrogen peroxide for 12 h, mainly the early and intermediate apoptosis (Figure 1B-C). Western blotting of apoptosis-associated proteins showed that the ratios of cleaved caspase-3/caspase-3 and cleaved poly adenosine diphosphate ribose polymerase (PARP)/PARP were significantly increased with the prolongation of action time of hydrogen peroxide (Figure 1D-E).

Effect of Hydrogen Peroxide on Autophagy of Osteoblasts

Mc3T3-E1 cells were treated with different concentrations of hydrogen peroxide, and the changes in autophagy at different time points

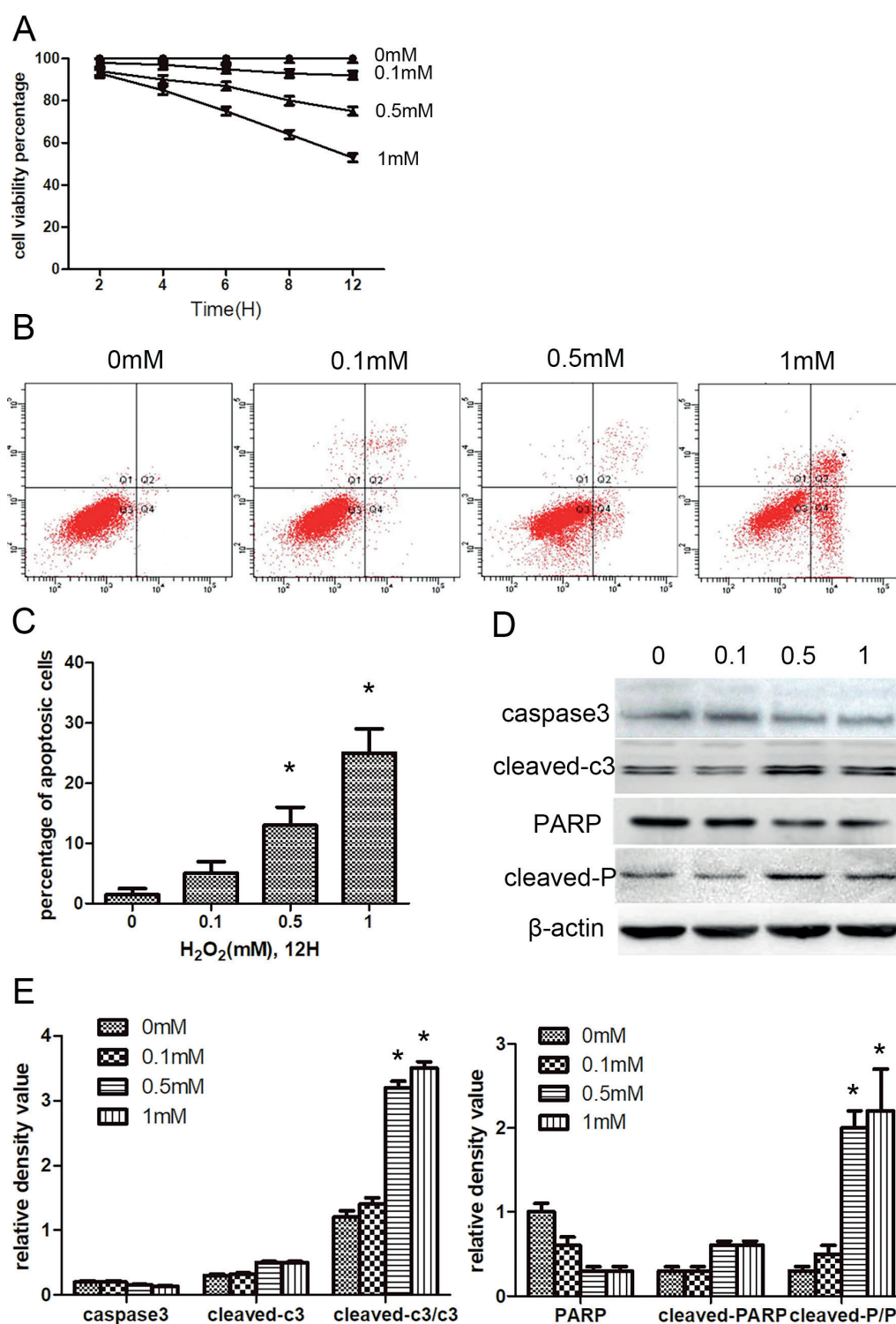


Figure 1. Effect of hydrogen peroxide on osteoblast apoptosis. **A**, Detection of the cell viability after action with different concentrations of hydrogen peroxide (0, 0.1, 0.5 and 1 mM) for different time (0, 2, 4, 6, 8 and 12 h) via CCK8 assay. **B**, Detection of the apoptotic levels after action with different concentrations of hydrogen peroxide (0, 0.1, 0.5 and 1 mM) for 8 h via flow cytometry. **C**, Data statistics of early apoptosis ratio via flow cytometry. **D**, Detection of expressions of apoptosis-related proteins, caspase-3, cleaved caspase-3, PARP and cleaved PARP, via Western blotting. **E**, Gray value analysis of caspase-3, cleaved caspase-3, PARP and cleaved PARP; ratios of cleaved caspase-3/caspase-3 and cleaved PARP/PARP. *Compared with blank group, $p < 0.05$.

were detected via Western blotting and MDC staining. The results revealed that the typical autophagosomes were found in cells under transmission electron microscope after treatment with 0.1, 0.5, and 1 mM hydrogen peroxide for 8 h (Figure 2A). After treatment with hydrogen peroxide, MDC staining (Figure 2B) and flow cytometry were used to detect the fluorescence intensity (Figure 2C). The results showed that the number of autophagosomes was increased significantly. Moreover, the results of Western blotting revealed that the ratio of LC3II/LC3I and expression of Beclin1 were increased in a hydrogen peroxide concentration-independent manner (Figure 2D-E).

Effect of Oxidative Stress on Autophagy

The oxidative stress of osteoblasts was inhibited using catalase (CAT). It was found that after the pretreatment with CAT (20 µg/mL) for 1 h, MDC staining showed that the number of autophagosomes was decreased significantly (Figure 3A), the percentage of autophagic fluorescent cells was decreased (Figure 3B), and the ratio of LC3II/LC3I and expression of Beclin1 were significantly decreased compared with those in control group (Figure 3C-D). These studies suggested that hydrogen peroxide causes the oxidative stress and induces the activation of osteoblast autophagy, while CAT can inhibit these processes.

Effect of Autophagy on Oxidative Stress

The effects of autophagy on apoptosis and oxidative stress levels were observed using autophagy blocker (3-MA) and autophagy activator (Rap). After pre-treatment with 100 nM Rap and 5 mM 3-MA for 1 h, the apoptosis level was detected via flow cytometry. The results showed that the apoptosis in 3-MA group was significantly increased compared with that in hydrogen peroxide group, but it decreased after application of Rap (Figure 4A-B). The intracellular ROS level was decreased after application of Rap, while 3-MA blocked the autophagy and led to the increased intracellular ROS level (Figure 4C). The extracellular SOD level had similar changes in both groups (Figure 4D). Western blotting was used to detect the apoptosis-associated protein expressions. The activated caspase-3 and PARP expressions were increased after treatment with hydrogen peroxide for 8 h. The activated caspase-3 and PARP expressions were further raised after the autophagy was blocked via 3-MA, while the activated caspase-3 and PARP

were partially down-regulated after autophagy was up-regulated via Rap. These results suggested that autophagy may inhibit the oxidative stress-induced apoptosis.

Discussion

Under normal physiological conditions, the body's oxidation system and antioxidant system are balanced. Physiological concentrations of ROS play important roles in cell growth, development and differentiation regulation. However, under pathological conditions, such as aging and estrogen withdrawal, the excessive ROS will be produced. The excessive ROS oxidizes the intracellular proteins, lipids and DNA, leading to apoptosis. Currently, studies have confirmed that oxidative stress is related to the diabetes mellitus, cancer, and aging¹¹. In addition, oxidative stress is also one of the important causes of osteoporosis¹². It has been reported that ROS levels in osteoporosis patients and osteoporotic animal model are closely related to bone mineral density⁷. Another work has found that the increased oxidative stress level and insufficient bone formation are direct causes of age-related osteoporosis¹³.

Hydrogen peroxide, as an active oxygen preparation, can easily get through the cell membrane, and bind to intracellular iron ions to form high-activity oxygen free radicals, which is a classic model to induce the cell oxidation stress. Low-concentration hydrogen peroxide promotes the osteoblast proliferation and differentiation¹⁴, whereas high-concentration hydrogen peroxide inhibits the cell proliferation, and even leads to apoptosis and death¹⁵. In this study, it was also found that hydrogen peroxide inhibited the cell proliferation, and the osteoblast apoptosis was in time- and concentration-dependent manners under the action of certain hydrogen peroxide.

Autophagy is an important mechanism of cell self-protection, which helps cells maintain the synthesis and degradation cycles and promotes cell survival through the lysosomal degradation mechanism¹⁶. First, the changes in autophagy under the action of hydrogen peroxide were detected to clarify the relationship between oxidative stress and autophagy. In *in vitro* experiments, it was found that the expressions of autophagy-related genes in osteoblasts were significantly increased after treatment with hydrogen peroxide for 2-6 h, and then

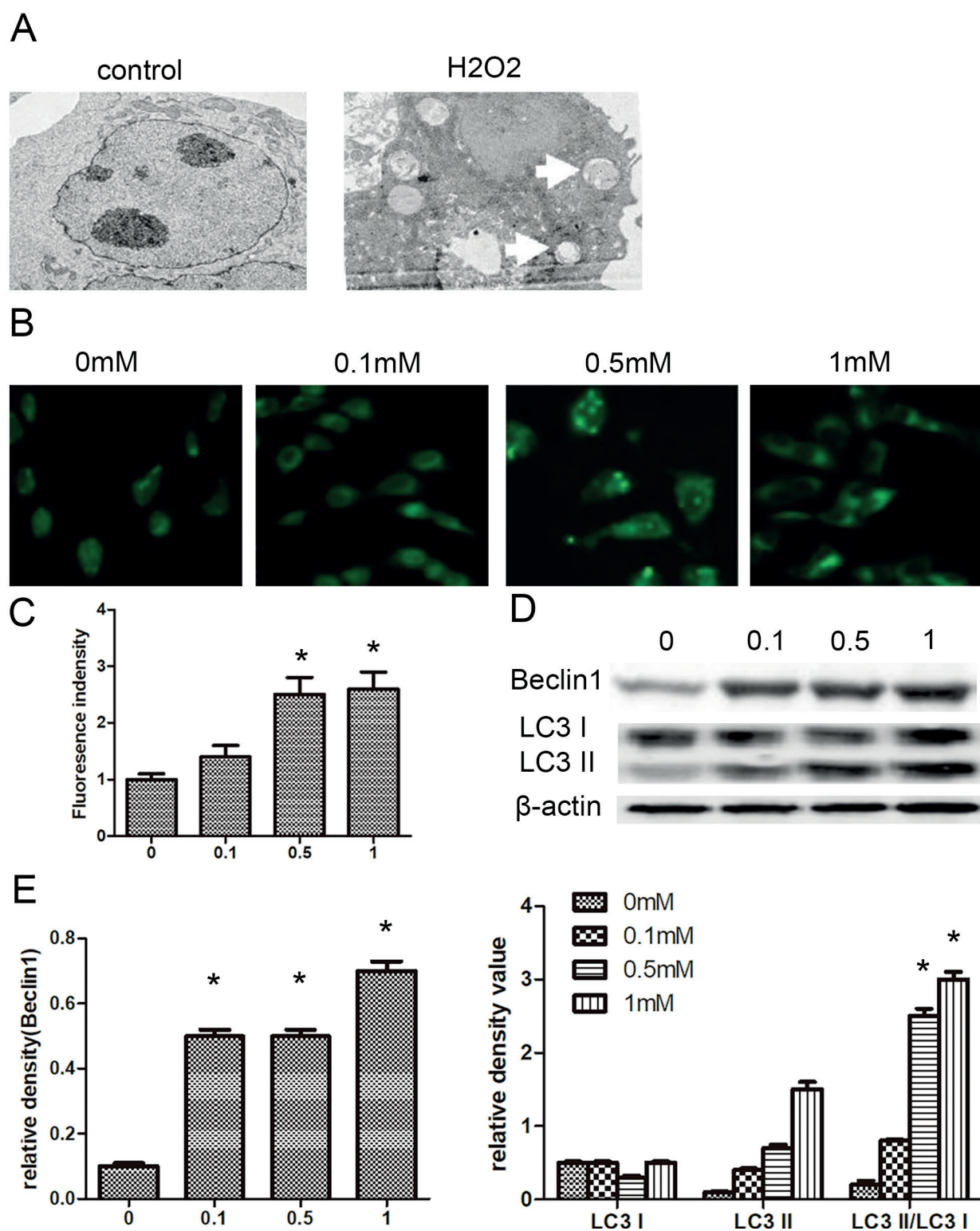


Figure 2. Effect of hydrogen peroxide on osteoblast autophagy. **A**, Detection of intracellular autophagosome formation via transmission electron microscope after treatment with hydrogen peroxide for 8 h ($\times 500$). **B**, Detection of autophagy fluorescence via fluorescence microscope after treatment with different concentrations of hydrogen peroxide (0, 0.1, 0.5 and 1 mM) for 8 h ($\times 400$). **C**, Data analysis of autophagy fluorescence intensity. **D**, Detection of expressions of autophagy-related proteins, Beclin1, LC3I and LC3II, via Western blotting. **E** Gray value analysis of Beclin1 expression and LC3II/LC3I ratio. *Compared with blank group, $p < 0.05$.

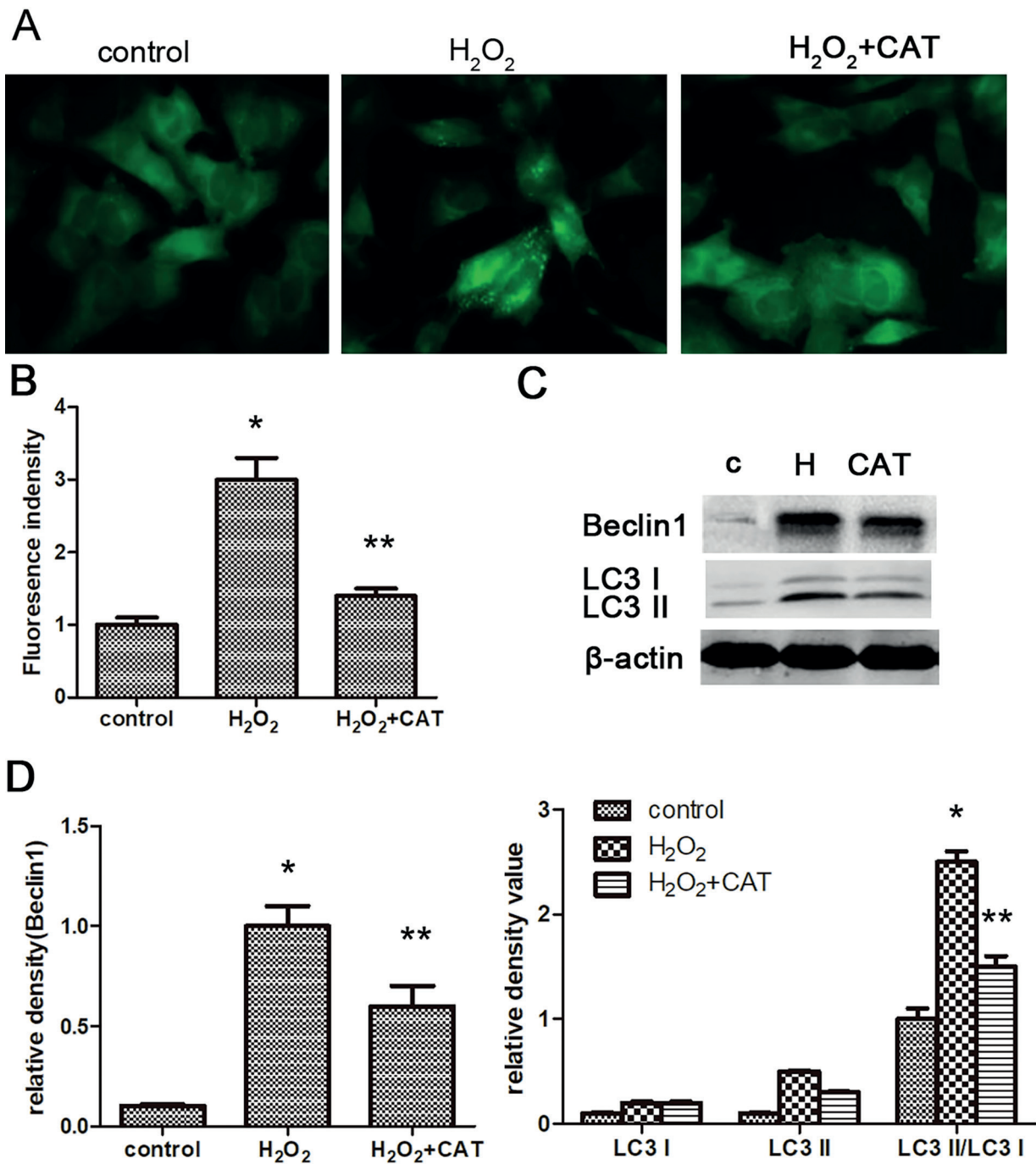


Figure 3. Effect of inhibiting hydrogen peroxide on autophagy. **A**, Observation of autophagy fluorescence in control group, hydrogen peroxide (0.5 mM) group and hydrogen peroxide + CAT group via fluorescence microscope ($\times 400$). **B**, Data analysis of autophagy fluorescence intensity. **C**, Detection of expressions of autophagy-related proteins, Beclin1, LC3I and LC3II, via Western blotting. **D**, Gray value analysis of Beclin1 expression and LC3II/LC3I ratio. *Compared with blank group, $p < 0.05$; **compared with hydrogen peroxide group, $p < 0.05$.

decreased gradually. This process can be reversed by catalase. The results preliminarily indicate that hydrogen peroxide can induce autophagy. In order to clarify the role of autophagy in the oxidative

stress of osteoblasts, different levels of autophagy were blocked. The results showed that the level of ROS in osteoblasts was further increased and the apoptosis was also increased after autophagy was

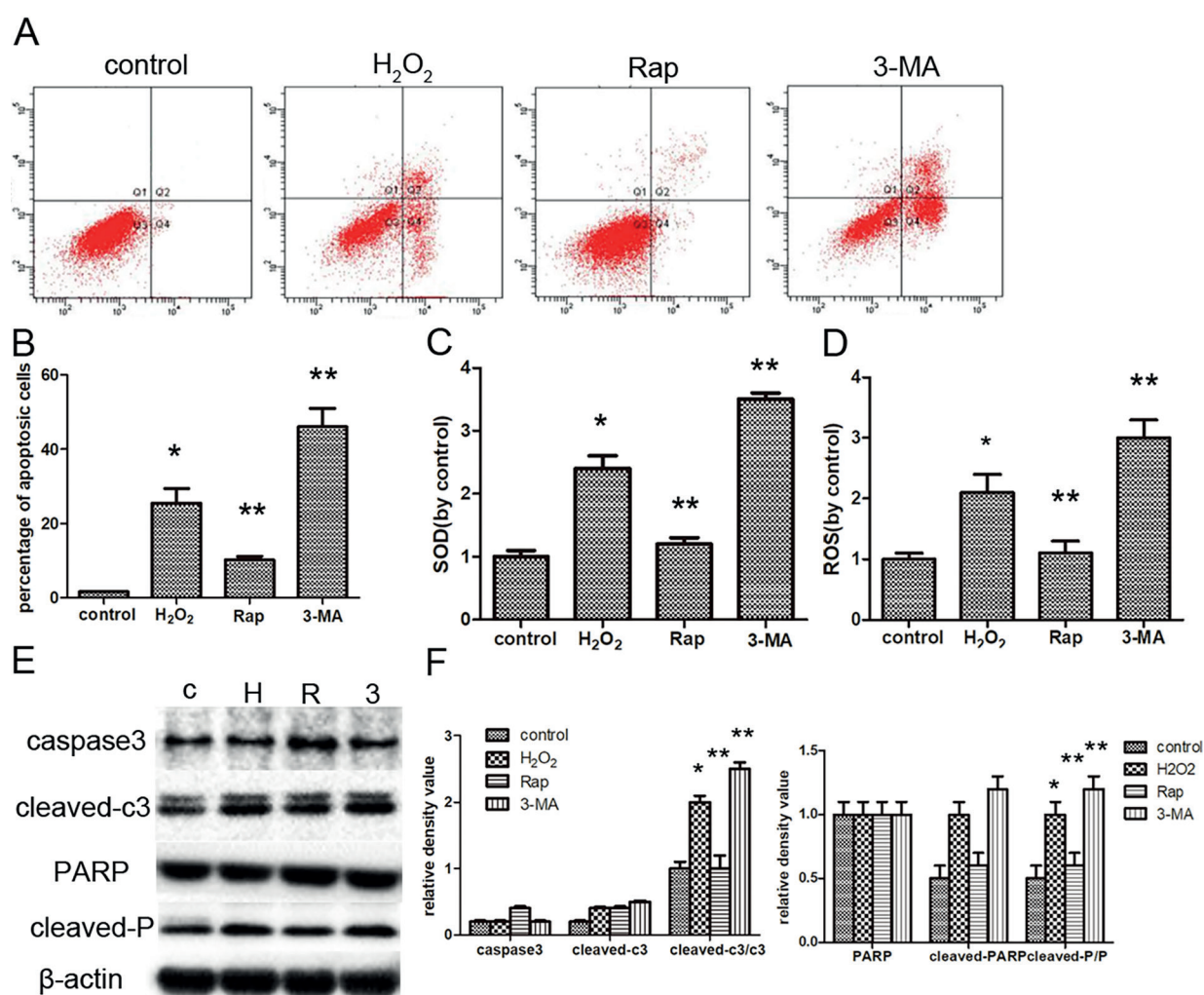


Figure 4. Effects of regulating autophagy on apoptosis and oxidative stress. **A**, Detection of apoptosis levels in control group, hydrogen peroxide group, Rap or 3-MA pretreatment group via flow cytometry. **B**, Data statistics of early apoptotic cell proportion. **C**, Data analysis of extracellular SOD concentration. **D**, Data analysis intracellular ROS fluorescence. **E**, Detection of expressions of apoptosis-related proteins, caspase-3, cleaved caspase-3, PARP and cleaved PARP, via Western blotting. **F**, Gray value analysis of caspase-3, cleaved caspase-3, PARP and cleaved PARP; ratios of cleaved caspase-3/caspase-3 and cleaved PARP/PARP. *Compared with blank group, $p < 0.05$; **compared with hydrogen peroxide group, $p < 0.05$.

blocked via autophagy inhibitor (3-MA) under the stimulation of hydrogen peroxide. The autophagy inducer (Rap) decreased the ROS level in osteoblasts and reduced apoptosis. Besides, the overexpression of autophagy-associated protein, Beclin1, could partially alleviate the oxidative stress injury of osteoblasts, and inhibit the osteoblast apoptosis. However, excessive autophagy can also lead to apoptosis¹⁰. In this study, the level of autophagy was induced moderately, which reduced the apoptosis through the lysosomal degradation mechanism. If the level of autophagy is further increased, it is likely to cause irreversible damage to cells and increase apoptosis.

Conclusions

These results suggest that the moderate autophagy inhibits oxidative stress injury and apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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