Effect of Oxidative Stress-Induced Autophagy on Proliferation and Apoptosis of hMSCs

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Research Article

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Abstract

Background: Objective: To observe the effect of H2O2 induced oxidative stress on autophagy and apoptosis of human bone marrow mesenchymal stem cells (hBMSCs).

Method: The hBMSCs were separated and cultured by density gradient centrifugation combined with adherence method. They were divided into blank group (with medium only), 3-MA (autophagy inhibitor) pretreatment group (with 2 ml of 5 mM 3-MA medium), H2O2 Intervention group (add 2ml medium containing 0.05mM H2O2), H2O2+3-MA treatment group (add 2ml medium containing 5mM 3-MA, then add 2ml medium containing 0.05mM H2O2). DCFH-DA staining was used to detect cellular reactive oxygen species (ROS) levels, and CCK-8 analysis was used to detect the effects of different concentrations (0, 50, 100, 200, 400 μmol/L) of H2O2 on the proliferation of hBMSCs; Monodansylcadaverine (MDC) Fluorescent amine probe staining, Lysosome Red Fluorescent Probe (Lyso-Tracker Red) staining to observe the level of autophagy; Immunofluorescence staining to detect the expression of LC3A/B; Flow cytometry (Annexin V/PI) to detect cell apoptosis Circumstances; Protein chip detection of autophagy-related proteins; Western blot detection of Beclin1, mTOR, p-mTOR, LC3A/B, and Cleaved caspase-3 protein expression.

Result: After treating hBMSCs with different concentrations of H2O2 (0, 50, 100, 200, 400 μmol) for 24h, 48h, and 72h, with the increase of H2O2 concentration, the cell proliferation ability decreased; while with the extension of time, the cell proliferation ability increased not significantly; 50μmol cell proliferation ability is the strongest. Compared with the blank group and 3-MA group, the H2O2 intervention group increased the level of intracellular ROS, increased autophagosomes, and significantly decreased the apoptosis rate; up-regulated Beclin1, mTOR, LC3A/B and Cleaved caspase-3 protein expression, and down-regulated p-mTOR Protein expression level. Compared with the autophagy inhibitor 3-MA group, the H2O2+3-MA group increased the level of intracellular ROS, increased autophagosomes, and did not significantly increase the apoptosis rate; up-regulated the protein expression of Beclin1, mTOR, LC3A/B and Cleaved caspase-3 Down-regulate the expression of p-mTOR protein.

Conclusion: H2O2 can induce hMSCs to produce oxidative stress response. Under oxidative stress conditions, hMSCs can promote protective autophagy and reduce cell apoptosis or the level of apoptosis caused by excessive autophagy.

Introduction

Bone mesenchymal stem cells (bone mesenchymal stem cells, BMSCs) are pluripotent stem cells with multidirectional differentiation potential [1]. In recent years, due to its high proliferation ability and high differentiation ability, it has attracted widespread attention in stem cell research. At present, the use of bone marrow mesenchymal stem cell transplantation to treat injuries has proven to be very promising [2]. However, clinical practice and experiments have shown that ischemia, hypoxia, and inflammatory cell infiltration at the injured site will produce a large amount of ROS, forming an oxidative stress microenvironment. Oxidative stress is the key cause of the death of transplanted bone marrow...
mesenchymal stem cells [3]. The important mechanism is that the oxidative stress microenvironment of the osteonecrosis area increases the production of ROS and/or reduces the ability to remove ROS, which leads to an imbalance between ROS production and removal [4].

Autophagy is a lysosomal pathway that can degrade proteins and organelles, and recover intracellular organelles and proteins to maintain the energy steady-state time of cell stress [5]. In autophagy, organelles and degraded fragments fall into double-membrane autophagosomes. Autophagy is the process of maintaining cell homeostasis under oxidative stress conditions. There is a relationship between autophagy deficiency and many neurodegenerative diseases, cardiovascular diseases, aging and cancer. Some stress states, such as hypoxia and cell serum deprivation, can induce autophagy and prolong cell lifespan [6]. This experiment intends to activate the oxidative stress response by H2O2, regulate the autophagy level of human bone mesenchymal stem cells (hBMSCs), and observe the effect of autophagy on the proliferation and apoptosis of hMSCs under oxidative stress.

**Materials And Method**

**Reagents and antibodies**

Human bone tissue specimens were taken from the femoral bone marrow of patients undergoing hip and knee replacement surgery at the Third Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine. They were collected aseptically during the operation and stored in liquid nitrogen for later use. The study patients all gave informed consent to this experiment, signed an informed consent form, and the study was approved by the medical ethics committee of our hospital. This experimental study was registered by China Clinical Trial Research, registration number: CHiCTR2000037969.

DMEM high sugar medium(Gibco,#11965092), Penicillin-Streptomycin(Gibco,#15070063), fetal bovine serum (FBS,Gibco,#10099133),Tissue ROS detection kit(Beibo Biological,#BB-470532),cell reactive oxygen detection kit(Biyuntian,#S0033S),cell apoptosis detection kit (KGI Bio, #KGA108-1), CCK8 reagent (Dongren Chemical, #CK04), superoxide Substance detection kit (Biyuntian,S0060), autophagy staining detection kit (Solebao, #G0170-100T), Lyso-Tracker Red(Solebao,#L8010-50μl); Goat Anti-Rabbit IgG H&L (HRP)(Jackson,#111-035-003),Beclin-1 (D40C5) Rabbit mAb(CST,#3495T), mTOR (7C10) Rabbit mAb(CST, #2983T), Phospho-mTOR (Ser2448) (D9C2) Rabbit(CST, #5536T), LC3A/B (D3U4C) XP® Rabbit mAb(CST,#12741T), Cleaved Caspase-3 (Asp175) Antibody(CST, #9661T), 3-MA(MCE, #HY-19312), Endonuclease SgrAI(NEB, #R0603S), Endonuclease EcoRI(NEB, #R3101V).

**Isolation, culture and grouping of hBMSC**

Remove the bone marrow specimen from the liquid nitrogen, thaw it, pipette the bone marrow evenly to make a cell suspension, add 25 mL of IMDM culture medium containing 10% FBS, and inoculate it in a 175 cm2 culture flask at 37°C, 5% CO2 incubator. nourish. Collect logarithmic growth phase human bone marrow mesenchymal stem cells (hBMSCs) cells, count them, resuspend the cells in DMEM low-sugar complete medium, adjust the cell concentration to 1×105 cells/ml, inoculate a 6-well plate, add 2ml cell
suspension to each well. Incubate overnight at 37°C and 5% CO2. Discard the old culture medium and proceed as follows: blank group: add 2ml fresh DMEM low sugar complete medium; 3-MA pretreatment group: add 2ml DMEM low sugar complete medium containing 5mM 3-MA to pre-condition for 30 minutes, discard the culture. Add 2ml DMEM low sugar complete medium; H2O2 intervention group: add 2ml DMEM low sugar complete medium containing 0.05 mM H2O2; 3-MA pretreatment group + H2O2 group: add 2ml DMEM low sugar complete containing 5mM 3-MA. The medium was pretreated for 30 minutes, the medium was discarded, and 2ml of DMEM high-glycosylated complete medium containing 0.05mM H2O2 was added. Cells in each group were cultured at 37°C and 5% CO2 for 48 hours.

**CCK-8 analysis detects the effect of different concentrations of H2O2 on the proliferation of hBMSCs**

Collect hBMSCs cells, count them, resuspend the cells in DMEM high glucose complete medium, adjust the cell concentration to 1×10^5 cells/ml, inoculate in 96-well plates, add 0.1 ml cell suspension to each well, and incubate overnight at 37°C, 5% CO2. Discard the old medium, add 0.1ml of DMEM high-sugar complete medium containing 0, 0.05, 0.1, 0.2, 0.4 mM H2O2, and continue culturing at 37°C and 5% CO2. After 24h, 48h, 72h, discard the culture medium in the well, add 0.1ml DMEM high glucose complete medium containing 10% CCK8, incubate for 2-3h at 37°C, 5% CO2, measure OD450 on the microplate reader, and Draw cell growth curve.

**DCFH-DA detects cellular ROS level**

Dilute DCFH-DA (2,7-Dichlorodihydrofluorescein diacetate) with serum-free medium at 1:1000 to make the concentration 10μM. After the hBMSCs cells were treated with H2O2 for 48h, the cells were collected and suspended in DCFH-DA at a cell concentration of 10^6-2×10^7/ml, and incubated in a cell incubator at 37°C for 20 min. Mix by inversion every 3-5 min to make the probe and the cells fully contact. The cells were washed three times with serum-free cell culture medium to fully remove the DCFH-DA that did not enter the cells. Flow cytometry detection, 488nm excitation wavelength, 525nm emission wavelength, real-time detection of fluorescence intensity.

**Autophagy staining detection(MDC)**

After 48 hours of induction treatment of the above groups of cells, the culture medium was discarded, and the cells were washed with Wash Buffer. Add monodansylcadaverine(MDC) staining solution and incubate in the dark at 37 °C and 5% CO2. Add Wash Buffer to wash the cells 2-3 times. Observe under a fluorescence microscope (Ex/Em=355nm/512nm), count and take pictures.

**Autophagy Lysosome Red Fluorescent Probe (Lyso-Tracker Red)**

Take a small amount of Lyso-Tracker Red and add it to the DMEM high-glycemic medium at a ratio of 1:20000. After each group of cells is induced for 48 hours, the culture medium is discarded, and an appropriate amount of Lyso-Tracker Red working solution is added at 37 °C, 5% CO2. Incubate for 30-120 min under dark conditions. Discard the cell surface staining solution and add fresh DMEM high-sugar
complete medium. Observe under a fluorescence microscope. Lysosomes are stained with bright and strong fluorescence.

**Immunofluorescence staining to detect LC3A/B**

After the above groups were induced and cultured for 48 hours, the cell culture medium was discarded, and the cells were fixed with freshly prepared 4% paraformaldehyde for 10 minutes after washing with PBS three times. Wash with PBS three times, 5min each time. Permeabilize the cells with 0.2% triton X-100 (prepared in PBS) for 10 minutes. Wash with PBS three times, 5min each time. Blocked with 2% BSA for 30 min, washed twice with PBS. Add rabbit anti-LC3A/B monoclonal antibody (1:400) and incubate at room temperature for 1h. Wash with PBS three times, 5min each time. Add FITC-labeled secondary antibody (1:500) and incubate at room temperature for 30-45min. Wash with PBS four times, 5min each time. Add 0.5μg/mL DAPI (prepared in PBS) for staining for 10 minutes. Wash three times with PBS to remove excess DAPI. Observe with a fluorescence microscope and take pictures and record.

**Annexin V-FITC/PI cell apoptosis detection**

After H2O2 treatment of hBMSCs cells for 48 hours, the cells were trypsinized without EDTA, centrifuged at 2000 rpm for 5 min at room temperature, and 1-5×10⁵ cells were collected and washed twice with PBS. Add 5μl PI dye solution to 50μl Binding Buffer and mix well. Add PI staining solution to the cell pellet, mix well, and react for 5-15 min in the dark at room temperature. After the reaction, add 450μl of Binding Buffer and mix well. Add 1μl Annexin V-FITC and mix well, and react for 5-15 min in the dark at room temperature. Detect with flow cytometer, excitation wavelength Ex=488 nm; emission wavelength Em=530 nm, Annexin V-FITC fluorescence signal is green, use FL1 channel detection; excitation wavelength Ex=488nm, emission wavelength Em≥630 nm, PI red Fluorescence is detected by the FL3 channel.

**Gene expression profiling chip to detect autophagy-related proteins**

After the above groups of cells were cultured for 48 hours after intervention, the cells were collected, and gene expression profiling chips were performed to detect autophagy-related proteins.

**Western blot detection of Beclin1, mTOR, p-mTOR (Ser2448), LC3A/B, Cleaved caspase-3 protein expression**

After the cells of each group were treated with conditioned medium and hydrogen peroxide, the protein was extracted from the phosphorylalted protein lysate, electrophoresed, transferred and blocked after quantification, and Beclin1, mTOR, p-mTOR, LC3A/B, Cleaved caspase-3 were added. Incubate the antibody overnight at 4°C, wash with TBST, incubate the secondary antibody for 2 hours at room temperature, develop and expose the eECL, and use Image J software to calculate the band gray value. Use Beta-action as an internal reference to calculate the relative protein amount.

The above experiments were repeated three times.
Statistical Analysis

SPSS 20.0 software was used for statistical analysis. Mean±SD was used to represent measurement data. All data were tested for normality and homogeneity of variance. The comparison between groups was tested by t, and the non-parametric test was used when the analysis of variance was not satisfied. The detection level was α= 0.05, P<0.05, the difference is statistically significant.

Result

Effects of different concentrations of H2O2 on the proliferation ability of hBMSCs

After treating hBMSCs with different concentrations of H2O2 (0, 50, 100, 200, 400 μmol) for 24h, 48h, and 72h, the CCK-8 test results showed that the cell proliferation ability decreased with the increase of H2O2 concentration, and the cell proliferation ability decreased with time. The increase is not obvious (Figure 1). It shows that the oxidative stress response induced by H2O2 leads to a significant decrease in the survival rate and proliferation ability of hBMSCs. When the H2O2 concentration is 50μmol, the cell proliferation ability is the strongest, so the follow-up experiment chooses 50μmol H2O2 as the intervention group.

Cell reactive oxygen species (ROS) levels in each group

DCFH-DA staining method was used to detect the content of ROS in cells and observe the changes of oxidative stress levels in cells. DCFH-DA staining results showed that compared with the blank group and 3-MA group hBMSCs, the fluorescence intensity of hBMSCs in the H2O2 group was significantly increased in other groups (Figure 2). It shows that after H2O2 interferes with hBMSCs, it leads to an increase in the level of cellular oxidative stress and activates the cellular oxidative stress response.

Effect of H2O2 on the autophagy level of hBMSC

The effect of H2O2 on hBMSC was observed by electron microscope, and it was found that autophagosomes increased after H2O2 treatment. Compared with the autophagy inhibitor 3-MA group, the autophagosomes in hBMSC treated with H2O2 also increased (Figure 3). The MDC staining method was used to detect the effect of H2O2 on hBMSC autophagy. The results showed that there were fewer green fluorescent autophagosomes in the blank group and 3-MA group, while the cells in the H2O2 group showed different sizes, dense and dense autophagy-related autophagy. Green particles (Figure 4). Lyso-Tracker Red method was used to detect the effect of H2O2 on hBMSC autophagy lysosomes. The results showed that the blank group and 3-MA group had fewer red fluorescent autophagy lysosomes and weaker red light, while the H2O2 group showed large cells Different, dense and densely stained autophagosome particles with obvious red light (Figure 5). It shows that H2O2 can enhance the green and red fluorescence of hBMSC autophagosomes; compared with the 3-MA group, the autophagy lysosomes and fluorescence increase in the H2O2+3-MA group, indicating that H2O2 can reverse the green color caused by the autophagy inhibitor 3-MA, The effect of reducing red fluorescence.
The effect of H2O2 on the apoptosis rate of hBMSC

Use Annexin V-FITC/PI apoptosis kit and flow cytometer for detection. The results showed that compared with the blank group (17.98%), the apoptosis rate of the H2O2 group (6.53%) decreased ($P < 0.05$), and compared with the 3-MA group (4.85%), the apoptosis rate increased ($P < 0.05$); Compared with the 3-MA+H2O2 group (6.2%) in the H2O2 group (6.53%), the apoptosis rate was not significantly increased ($P > 0.05$) (Figure 6). It shows that after H2O2 interferes with hBMSCs, it activates the oxidative stress response and reduces the level of cell apoptosis; at the same time, it induces autophagy in cells, leading to an increase in the rate of cell apoptosis.

Immunofluorescence staining to observe the effect of H2O2 on hBMSC autophagy protein LC3A/B

The results showed that the autophagy protein LC3A/B in the blank group and the 3-MA group had less red and blue fluorescence, and weaker red and blue light, while the cells in the H2O2 group showed different sizes, dense and dense stains, red light, Autophagosome particles with obvious blue light (Figure 7). It shows that H2O2 can enhance the red and blue fluorescence of hBMSC autophagy protein LC3A/B; compared with the 3-MA group, the autophagy lysosomes and fluorescence increase in the H2O2+3-MA group, indicating that H2O2 can reverse the autophagy inhibitor 3- MA.

Protein chip detection of autophagy-related proteins

Compared with the control group, a total of 5 autophagy-related proteins were significantly up-regulated in human BMSC after 3-MA intervention; compared with the control group, a total of 15 autophagy-related proteins were significantly upregulated in human BMSC after H2O2 treatment Up-regulation; Compared with the control group, human BMSCs were pretreated with 3-MA and then treated with H2O2. A total of 8 autophagy-related proteins were significantly down-regulated (Figure 8). It shows that after H2O2 treatment, human BMSC can significantly up-regulate the expression of autophagy-related proteins, which indicates that H2O2 can induce autophagy.

Expression of H2O2 on autophagy and apoptosis-related proteins of hBMSC

Western blot showed that: compared with the blank group and 3-MA group, H2O2 treatment of hBMSCs can up-regulate Beclin1, mTOR, LC3A/B, Cleaved caspase-3 protein expression, and down-regulate p-mTOR protein expression; and autophagy inhibition Compared with the 3-MA group, the H2O2+3-MA group can up-regulate Beclin1, mTOR, LC3A/B, Cleaved caspase-3 protein expression, and down-regulate the p-mTOR protein expression (Figure 9). It shows that H2O2 can up-regulate the expression of hBMSC autophagy-related proteins and can reverse the changes in protein expression caused by the autophagy inhibitor 3-MA.

Discussion
Oxidative stress mediated by reactive oxygen species is increasingly recognized as a direct cause of the occurrence and development of many human diseases, including orthopedic diseases, cardiovascular diseases, neurodegenerative diseases and cancer[7]. Oxidative stress during BMSC transplantation or in injured tissues has been shown to be a catastrophic factor leading to the cytotoxicity and low survival rate of BMSCs. It has been reported that the accumulation of reactive oxygen species caused by the harmful effects of oxidative stress has been proposed to trigger autophagy in different organs or cell types. Oxidative exposure can also lead to increased apoptosis [8]. However, the molecular mechanism that promotes autophagy and the relationship and interaction between apoptosis and autophagy in BMSCs are still unclear.

In this study, we treated hBMSCs with different concentrations of H2O2 (0, 50, 100, 200, 400 μmol) for 24h, 48h, and 72h. The results showed that as the concentration of H2O2 increased, the cell proliferation ability decreased; and as time passed, the cells The increase in proliferation capacity is not obvious. It shows that the oxidative stress response induced by H2O2 leads to a significant decrease in the survival rate and proliferation ability of hBMSCs. When the concentration of H2O2 is 50μmol, the cell proliferation ability is the strongest, so the follow-up experiments choose 50μmol concentration of H2O2 to interfere with hBMSCs. Short-term low-concentration H2O2 promotes autophagy, which is a cellular self-protection immune mechanism. Long-term or high-concentration H2O2 can block autophagy and promote BMSC apoptosis, indicating that autophagy enhancement is the key to rapid upregulation of oxidative exposure. Phagekine expression activates the early response, and continuous oxidative stress causes irreversible damage, then reduces expression autophagy and enhances cell death through apoptosis.

Oxidative stress is a pathological state in which the body's pro-oxidation and anti-oxidation are out of balance, the production of free radicals increases, and the antioxidant capacity of tissues and organs decreases. It can cause wound damage and is not conducive to healing [9]. ROS is a general term for oxygen-containing free radicals and peroxides that form free radicals related to oxygen metabolism in organisms, and its oxidizing properties are very active. ROS can cause membrane lipid peroxidation, protein cross-linking and degradation, DNA cleavage, and mitochondrial dysfunction of BMSCs [10]. Under physiological conditions, the body continuously produces reactive oxygen species, and the body's antioxidant system continuously scavenges the reactive oxygen species, which is in a state of dynamic equilibrium and will not cause harm to the body. However, when harmful stimuli occur, a large amount of reactive oxygen species will be produced, and the antioxidant system's ability to remove these reactive oxygen species is limited, eventually leading to oxidative damage [11]. Excessive ROS will increase mitochondrial membrane permeability, mitochondrial swelling, mitochondrial permeability (mMPTP) opening and mitochondrial DNA (mtDNA) damage [4]. This experiment shows that low-concentration H2O2 pretreatment activates the oxidative stress response, which can significantly increase the resistance of hBMSCs to oxidative stress, reduce the production of intracellular ROS, and reduce cell oxidative stress damage.

Autophagy is a lysosome-dependent degradation pathway, an evolutionarily conserved mechanism and an essential cell homeostasis process. Autophagy is also a highly controlled catabolic mechanism used
to flip unwanted and dysfunctional proteins through lysosomal mechanisms. Autophagosomes then fuse with lysosomes to form autolysosomes, the contents of which are hydrolyzed and degraded by lysosomal enzymes [12]. More and more evidences indicate that autophagy is a key steady-state response to maintain general cellular processes under stress conditions. Like oxidative stress, different types of tissues and cells have different degrees of tolerance. In recent years, with the deepening of research, autophagy has become widely known as a protective process under various environmental stresses such as starvation, oxidative stress, and hypoxia. Some studies have shown that BMSCs have the ability to resist oxidation system [13-14]. Therefore, we believe that autophagy may be a key factor in enhancing cell tolerance to oxidative stress. In this study, it was found that after the intervention of H2O2 in hBMSCs, the cells exhibited dense and dense autophagosome particles of different sizes, which could reverse the tendency of autophagy inhibitor 3-MA to decrease intracellular autophagosomes. Excessive autophagy will induce cell apoptosis. The occurrence of cell apoptosis is a complex process involving many factors, such as ROS production, mitochondrial depolarization, chromatin agglutination and nuclear division [4]. After H2O2 interferes with hBMSCs, it activates the oxidative stress response and reduces the level of cell apoptosis, which proves that low-concentration H2O2 pretreatment can significantly increase the cell survival rate of hBMSCs after oxidative stress damage and inhibit cell apoptosis. But it also induces autophagy in cells, leading to an increase in the rate of apoptosis.

Beclin1 is a key positive regulator of autophagy. Studies have shown that Beclin 1 mediated autophagy/apoptosis mutual feedback signal pathway regulates the balance between autophagy and apoptosis, and Beclin1 may be the intersection of mutual feedback effects [15]. Mammalian target of rapamycin (mTOR) is a key negative regulator of autophagy, and its activity is regulated by multiple signaling pathways. Studies have confirmed that autophagy has a negative feedback regulation effect on mTOR [16-17]. Caspase-3, which exists in the cytoplasm in the form of zymogen, is activated to generate the active fragment cleaved caspase-3 and the cleavage of PARP1 is regarded as one of the signs of cell apoptosis [18]. LC3 is a substrate that initiates autophagy and forms autophagosomes. The conversion of LC3A to LC3B represents the process of autophagy, and a higher amount of LC3B indicates an increase in the formation of autphagic vacuoles in the cell [19]. This experiment found that H2O2 can up-regulate the expression of hBMSC-related proteins Beclin1, mTOR, LC3A/B, Cleaved caspase-3, down-regulate the expression of p-mTOR protein, and reverse the protein expression caused by the autophagy inhibitor 3-MA Variety. It shows that oxidative stress induces autophagy, increases the level of autophagy, and inhibits cell apoptosis to a certain extent. Autophagy has a controversial role in cell survival and cell death regulation: it can be a defense mechanism against environmental stimuli, and sometimes it can be a cell death pathway, depending on the environment [20]. The results of our experiments and others have shown that if oxidation activates the intrinsic protective autophagy process, cells will be more able to withstand oxidative stress. On the other hand, if oxidation cannot induce the autophagy-lysosomal degradation pathway or the induced autophagy is a pro-death process, the cells are more sensitive to oxidative exposure [21-22].

Conclusion
To sum up, our data showed that oxidative stress has a dual effect on the survival of hMSCs. The autophagy induced by it has a protective effect on cells, prompting hMSCs to produce protective autophagy, reducing the rate of apoptosis and the level of apoptosis caused by excessive autophagy. However, the specific mechanism of autophagy induced by oxidative stress in hMSCs remains to be explored in depth.

**Abbreviations**

hMSCs: human bone marrow mesenchymal stem cells; ROS: Reactive oxygen species; mTOR: Mammalian target of Rapamycin. DCFH-DA: 2,7-Dichlorodihydrofluorescein diacetate; MDC: Monodansylcadaverine. Lyso-Tracker Red: Autophagy Lysosome Red Fluorescent Probe.

**Declarations**

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Not applicable.

**Authors’ contributions**

Hewei Wei conceived and designed the experiments; Zhijun Liu, Shaojin Liu, and Weipeng Zhen performed the experiments; Zhijun Liu and Shaojin Liu performed data analysis; Zhihao Liao and Sheng Chen contributed to sample collection; Zhijun Liu and Shaojin Liu wrote the paper; Hewei Wei assisted with writing and proofreading. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Ethics approval and consent to participate**

This study was approved by the Internal Review and the Ethics Boards of Guangzhou University of Chinese Medicine and The Third affiliated hospital, Guangzhou University of Chinese Medicine. Informed written consent was obtained from all study subjects. This study was registered with the China Clinical Trial Registry, registration number ChiCTR-2000033948.

**Consent for publication**

Not applicable.
**Competing interests**

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

The effect of different concentrations of H2O2 on cell proliferation at 24h, 48h, 72h after treatment of hMSCs (*Compared with the blank group, P<0.05; #Compared with 24h, P<0.05).

**Figure 2**
DCFH-DA detects cell ROS content, and flow cytometry analyzes DCF fluorescence intensity.

|       | H2O2 | - | - | + | + |
|-------|------|---|---|---|---|
| 3-MA | -    | + | - | - | + |

**Figure 3**
Electron microscopic observation of autophagosomes after H2O2 treatment of hBMSCs.

|       | H2O2 | - | - | + | + |
|-------|------|---|---|---|---|
| 3-MA | -    | + | - | - | + |

**Figure 4**
MDC detects the effect of H2O2 on autophagy induced by hBMSC.

|       | H2O2 | - | - | + | + |
|-------|------|---|---|---|---|
| 3-MA | -    | + | - | - | + |

**Figure 5**
Lyso-Tracker Red detects the effect of H2O2 on autophagy induced by hBMSC.

|          | H2O2 | 3-MA |
|----------|------|------|
| Untreated| -    | -    |
| 3-MA     | -    | +    |
| H2O2     | +    | -    |
| H2O2     | +    | +    |

**Figure 6**

H2O2 induces hBMSC apoptosis.

|          | H2O2 | 3-MA |
|----------|------|------|
| Untreated| -    | -    |
| 3-MA     | -    | +    |
| H2O2     | +    | -    |
| H2O2     | +    | +    |

**Figure 7**

Immunofluorescence staining to observe the effect of H2O2 on hBMSC autophagy
Figure 8

The effect of H2O2 on the regulation of hBMSC autophagy protein.
Figure 9

H2O2 expression of hBMSC autophagy and apoptosis-related proteins.