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Mitophagy is a protective response against oxidative damage in bone marrow mesenchymal stem cells

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ABSTRACT

Aims: Bone marrow mesenchymal stem cells (BMSCs) show great potential in clinical applications such as in intervertebral disc degeneration. Nevertheless, environmental stress during the BMSC transplant or in the injured tissues is a catastrophic factor that causes cell toxicity and poor survival of BMSCs. Mitophagy plays a vital role in maintaining cellular homeostasis and defending against oxidative stress because this process could control mitochondrial quality and quantity by eliminating dysfunctional or damaged mitochondria that can cause cell death. However, the accurate mechanisms of mitophagy in protecting BMSCs against the harshness of oxidative stress remain largely unknown.

Main methods: BMSCs were treated with H_2O_2 for various time periods. Mitophagy response was evaluated through the expression levels of LC3-II, p62 and mitophagosomal formation by using Western blot and fluorescence analysis. Cell apoptosis was examined by flow cytometry and TUNEL assay. The interactions of mitophagy and apoptosis and the possible signalling pathways were investigated through the co-treatment of mitophagy inhibitor or mitophagy activator with H_2O_2.

Key findings: Oxidative stress rapidly facilitated mitophagy through JNK at an early stage but decreased mitophagy and increased apoptosis at a late stage. Furthermore, mitophagy inhibition significantly enhanced the apoptosis in the cells treated by H_2O_2.

Significance: Induced mitophagy may play pivotal roles in protecting cells against oxidative stress in BMSCs.

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) can differentiate into mesodermal lineages cells, such as osteocytes, chondrocytes, adipocytes and other non-mesodermal lineage cells [1]. BMSCs show great potential in clinical applications, such as intervertebral disc degeneration (IVDD) [2]. Nevertheless, stress conditions, such as serum deprivation, hypoxia and oxidative stress during and after transplantation, cause the poor survival capacity of BMSCs. Moreover, the abnormal microenvironment of the IVDD with high oxidation level also enhances the apoptosis of the transplanted BMSCs [3,4]. Therefore, approaches to promote the survival capacity of transplanted BMSCs to strive against the severe stresses deserve further investigations [5].

Mitochondria, known as 'powerhouse of the cell', are essential to cell metabolic homeostasis and physiology. Considering that defective mitochondria are associated with a broad spectrum of pathologies, their quality and number control has evolved to restore and preserve energy metabolism [6,7]. Although cell metabolic homeostasis mediates responses to mitochondrial damage, persistent injuries trigger the elimination of the entire defective mitochondria through mitophagy, thus fine-tuning the mitochondrial number and preserving energy metabolism. Moderate mitophagy, a selective form of autophagy, is required to

Abbreviations: BMSCs, bone marrow mesenchymal stem cells; IVDD, intervertebral disc degeneration; ROS, Reactive oxygen species; caspase, cysteine aspartic acid protease; AMA, Antimycin A; CsA, Cyclosporin A; MAPK, mitogen-actived protein kinases; Baf A1, bafilomycin A1; JNK, Jun N-terminal kinase; mt△ψ, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; GFP, Green fluorescent protein; DMEM, Dulbecco's Modified Eagle Eedium; F12, Ham's F 12 nutrient medium; PI, Propidium Iodide; BCA, Bicinchonini Acid; TBST, Tris Buffered saline Tween; TUNEL, TdT-mediated dUTP nick end labeling

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Table 1
Primary antibodies for Western blot.

Target	Antibody (Company, catalog number, usage)
LC3	Abcam, ab48394, 1:1000
p62	Abcam, ab109012, 1:10,000
caspase3	CST, #9662, 1:1000
caspase7	CST, #9492, 1:1000
caspase9	CST, #9508, 1:1000
Bax	Abcam, ab32503, 1:100
Bcl-2	CST, #3498, 1:1000
phospho-JNK	CST, #4668, 1:1000
Total-JNK	CST, #9252, 1:1000
β-Actin	Proteintech, 20536-1-AP, 1:5000

withstand cellular stress inhibit apoptosis and is conducive to cell survival [7,8].

Mitophagy also plays a vital role in stem cell maintenance and differentiation [37]. Mitophagy impairment causes disorders in mitochondrial function and causes the accumulation of defective organelles, leading to cell death and more reactive oxygen species (ROS), which strongly indicates that mitophagy is more pivotal in stem cells. Additionally, ROS is a strong signal for the Jun N-terminal kinase (JNK) activation, which may mediate antioxidative responses, including the induction of mitophagy and cell death. However, the underlying mechanism of how mitophagy is induced in BMSCs under stressed conditions remains unclear. Furthermore, the precise role of mitophagy in BMSCs under oxidative situations is poorly understood.

In this study, we showed for the first time that oxidative stress rapidly facilitated mitophagy at an early stage, but prolonged oxidative exposure decreased mitophagy and increased apoptosis. We investigated the relationship between mitophagy and apoptosis and the possible signalling pathways involved in their interactions. We also suggested that the induction of mitophagy may play pivotal roles in protecting cells against the harshness of oxidative stress in BMSCs.

2. Materies and methods

2.1. Cell isolation and culture

According to the studies [9], we isolated BMSCs from the bone marrow of the femurs and tibias of Sprague-Dawley rats (4–6 weeks old and 150–200 g weight) after euthanization and expanded the cells for use between passages 3–6. Briefly, epiphyses were cut and bony shafts were flushed repeatedly with the use of a 5 ml syringe containing complete culture media (Dulbecco's Modified Eagle Medium/Ham's F

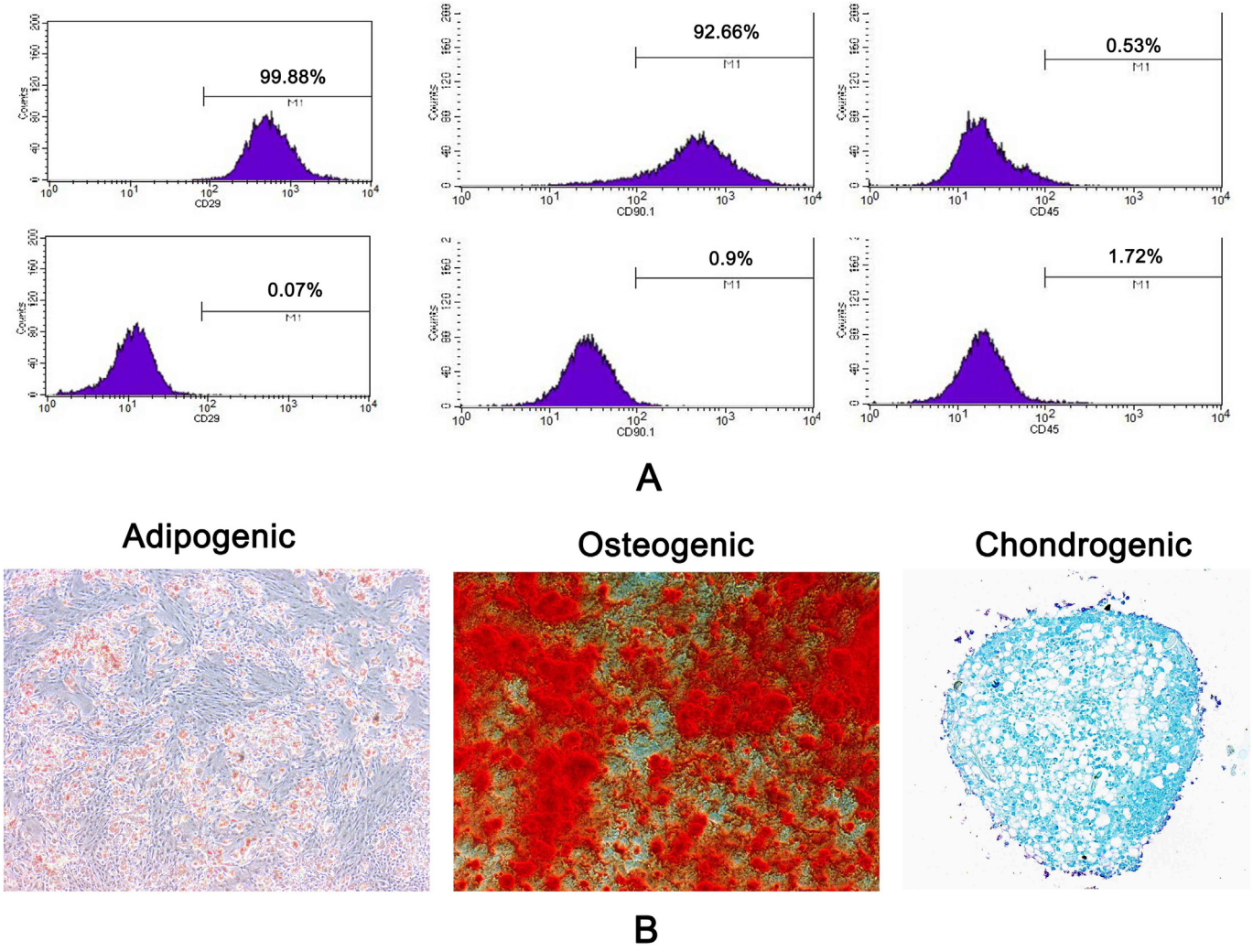

Fig. 1. Cell surface markers and differentiation capacity of BMSCs.
(A) Cells were positively stained with monoclonal antibodies against CD29 and CD90 and negative for CD45. The respective isotype controls were shown on the below groups. (B) Cells differentiated into adipocytes stained with oil red (left), mineralizing cells stained with alizarin red (middle) and chondrocytic lineage cells stained with Alcian blue (right).

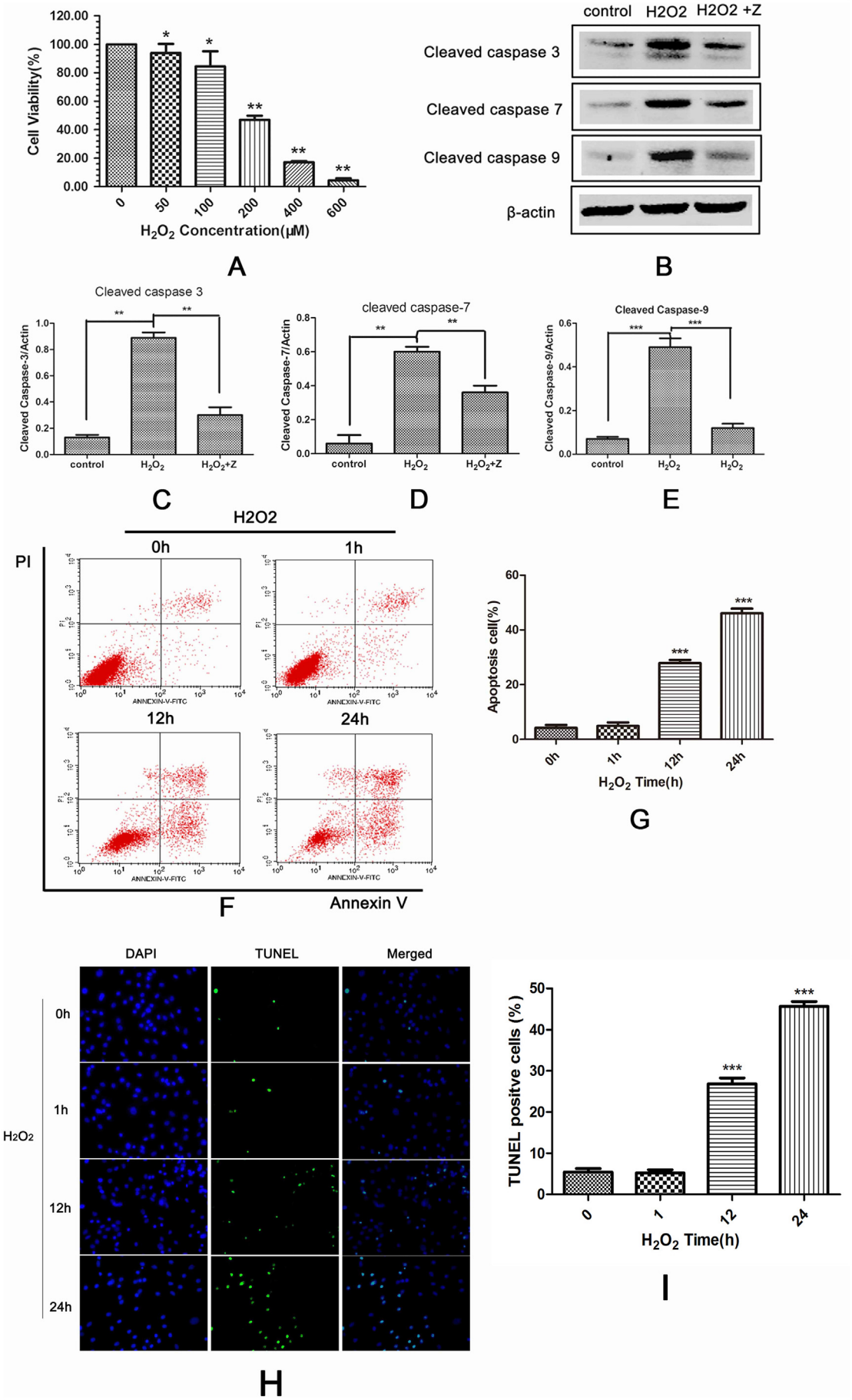

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Fig. 2. Prolonged treatment of H_2O_2 activates caspases and induces apoptosis in BMSCs.
(A) CCK-8 assay for the cell viability. BMSCs were treated with different concentrations of H_2O_2 for 24 h. (B–E) Western blotting analysis of the protein levels of cleaved caspase-3, -7 and -9. BMSCs were incubated in 200 μM H_2O_2 for 24 h. β-Actin was used as an internal control. The cells without H_2O_2 treatment were served as control. (F–G) BMSCs apoptosis was evaluated by flow cytometry using Annexin V-FITC/PI staining upon treatment with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). (H) TUNEL staining assay indicating apoptotic cells. (I) Cell apoptotic index was calculated as the number of TUBEL nuclei divided by the total number of DAPI nuclei. The results are presented as mean ± SD ($n = 3$). *p < 0.05; **p < 0.01; ***p < 0.001.

12 nutrient medium (DMEM/F12) and 10% fetal bovine serum (FBS) containing 1% penicillin/streptomycin and 1% glutamine). Cell suspension was filtered through a 70-μm stainless steel mesh to remove any bone spicules or muscle and cell clumps. Culture BMSCs in culture dishes at a density of 25×10^6 cells ml^{-1} and incubating the dishes in a humidified chamber (5% CO_2, 37 °C) without disturbing them. After 3 h, remove the nonadherent cells by replacing with fresh complete culture media. Thereafter, repeat this step every 24 h for up to 3–4 days. At 80–90% confluence, BMSCs were trypsinized and further expanded at a ratio of 1:2.

BMSCs during three to six expansion passages were exposed to H_2O_2 in the presence or absence of 10 μg/ml of Antimycin A (AMA, mitophagy inhibitor) [10], 10 μM of Cyclosporin A (CsA, mitophagy activator) [11] or 75 nM of bafilomycin A1 (Baf A1, autolysosomal maturation inhibitor) [12] for the duration of the experiment. Vehicle-treated or untreated cells were used as control groups.

2.2. Cell viability assay

The viability of BMSCs was determined by cell counting Kit-8 (CCK-8) assays. Cells were seeded in 96-well plates (2×10^4 cells/well) and incubated in 100 μl of complete culture medium overnight. When 80% confluence was achieved, different concentrations of H_2O_2 were added to the medium and incubated for 24 h. Each treatment was repeated in 8 parallel wells. Following H_2O_2 treatments, 10 μl solution from CCK-8 was added to each well and continuously incubated for 2 h in a humidified CO_2 incubator at 37 °C. Thereafter, the absorbance of the sample taken from each well was measured using a microplate reader at 450 nm.

2.3. Apoptosis incidence detection by flow cytometry and TUNEL assay

Apoptosis incidence was evaluated by using the Annexin V-FITC apoptosis detection kit (KeyGen) according to the manufacturer's instructions. Cells were seeded in 60-mm culture dishes until 90% confluence. Then they were treated with different concentration of H_2O_2 for the indicated time. After treatments, cells were harvested and washed twice with phosphate-buffered saline (PBS). Then, the cells were collected and re-suspended in buffer containing Annexin V-FITC and Propidium Iodide (PI). Thereafter, the samples were incubated for 15 min at room temperature in the dark, and quantified by flow cytometry (BD Accuri C6). The apoptotic rate was calculated by the percentage of early apoptotic (Annexin V + /PI-) cells plus the percentage of late apoptotic (Annexin V + /PI-) cells.

A TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using a TUNEL detection kit (Beyotime) according to the manufacturer's instructions. TUNEL staining was performed with fluorescein-dUTP to stain apoptotic cell nuclei, and DAPI was used to stain all cell nuclei for 3 min at room temperature. The cells whose nucleus was stained with fluorescein-dUTP were defined as TUNEL positive. Then, the slides were imaged under a confocal microscope.

2.4. Mitochondrial membrane potential measurement

The value of mitochondrial membrane potential (mt$\triangle\psi$) was measured by the dual-emission potential-sensitive probe, JC-1 staining, following the manufacturers' specifications (KGA602). Then, after treatment with different concentration of H_2O_2 for the indicated time, the BMSCs were collected and re-suspended in a mixture of 500 μl culture medium and 500 μl JC-1 staining fluid, and then incubated in the dark at 37 °C for 15 min. After washing twice with incubation buffer, cells were re-suspended in 500 μl incubation buffer and analyzed by flow cytometry (BD FACS Calibur, USA). Normal mitochondria having a high mt$\triangle\psi$, JC-1 formed red fluorescent aggregates, Nevertheless damaged or depolarized mitochondria, the sensor dye appeared as green fluorescent monomers.

2.5. GFP-LC3 transfection and Mito-Tracker Red staining

BMSCs were incubated at a density of 2×10^4 on 6-well plates and cultured up to 50% confluence. These cells were transfected with rLV-Green fluorescent protein (GFP)-LC3 (GeneChem, Shanghai, China) at a multiplicity of infection (MOI) of 100. The culture medium was changed after 24 h of transfection, when > 95% of the cells were alive. After 3 days, all transfected cells were treated with H_2O_2 for the indicated time.

Mito-Tracker Red CMXRos (KeyGEN) was used to stain mitochondria in live cells. BMSCs were incubated with Mtio-Tracker probes at the concentration of 50 nM for 30 min at 37 °C. Then, these cells were fixed with 4% paraformaldehyde, and washed by cold PBS for three times. Finally, nuclei were stained with 0.1 g/ml 4′,6-diamidino-2-phenylindole (DAPI) and imaged with a confocal microscope (Carl Zeiss LSM510, Tokyo, Japan). Mitophagy was evaluated by analyzing the formation of fluorescent puncta of autophagosomes in GFP-LC3 transfected cells.

2.6. Protein extraction and western blot analysis

Total protein of cell samples was extracted by whole-cell lysis assay (KeyGen), and protein concentrations were calculated by Bicinchonini Acid (BCA) protein assay kit (Beyotime, China). The extracted proteins were resolved in 10% sodium doecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% skimmed milk melted in TBS for 1 h and incubated overnight at 4 °C with primary antibodies (Table 1). After being washed with Tris Buffered saline Tween (TBST) for three times, the membranes were incubated with secondary antibodies (1:5000, ab6721; Abcam) for 1 h at room temperature. Then, the bands were detected with Two-color infrared laser imaging system (Li-COR Odyssey, USA) and assessed by image analysis software. Protein expression level was normalized by β-actin.

2.7. Statistical analysis

Data were presented as means ± SD (standard deviation). Statistical analyses were carried out using GraphPad Prism5. Differences among groups were evaluated using unpaired student's *t*-test. Differences were considered statistically significant when *p*-values < 0.05.

3. Results

3.1. Characteristics of BMSCs

These cells were analyzed for cell surface antigens. They were positively stained with monoclonal antibodies against CD29 and CD90

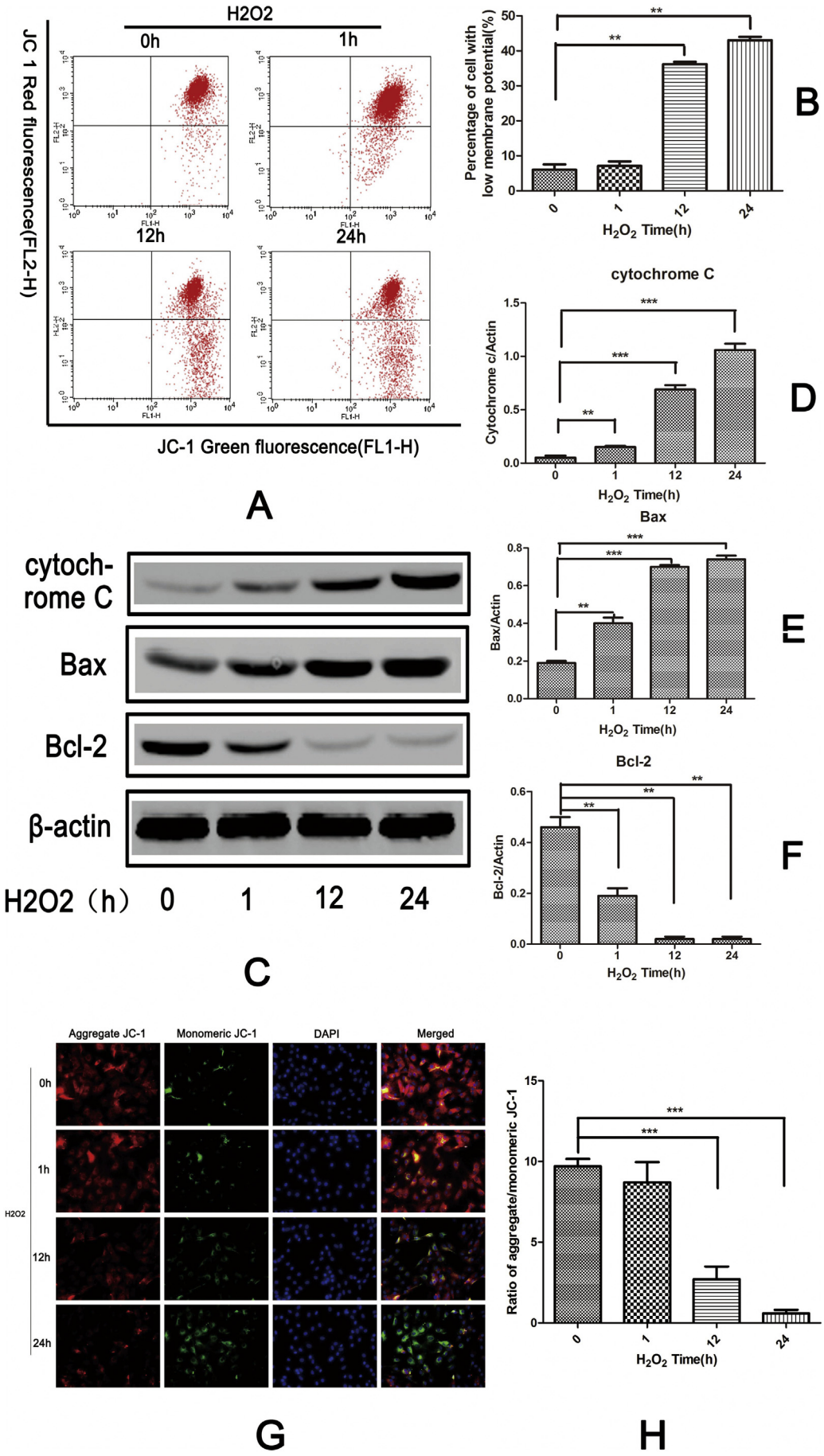

Fig. 3. Mitochondrial pathway has involved in the H2O2-induced apoptosis of BMSCs.

(A–B) Mtiochondrial membrane potential (mt△ψ) analyzed by flow cytometry through JC-1 staining. BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). The cells with green fluorescence indicated low mt△ψ. (C–F) Western blotting analysis for the protein expressions of Cytochrome c, Bax and Bcl-2. β-Actin was used as an internal control. BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). (G) Fluorescence microscopy and (H) quantitative measurements of the fluorescence intensity uncovered alteration of intracellular mt△ψ. mt△ψ was assessed by aggregates JC-1/monomers JC-1 ratios. The results were expressed as mean ± SD ($n = 3$). **p < 0.01; ***p < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

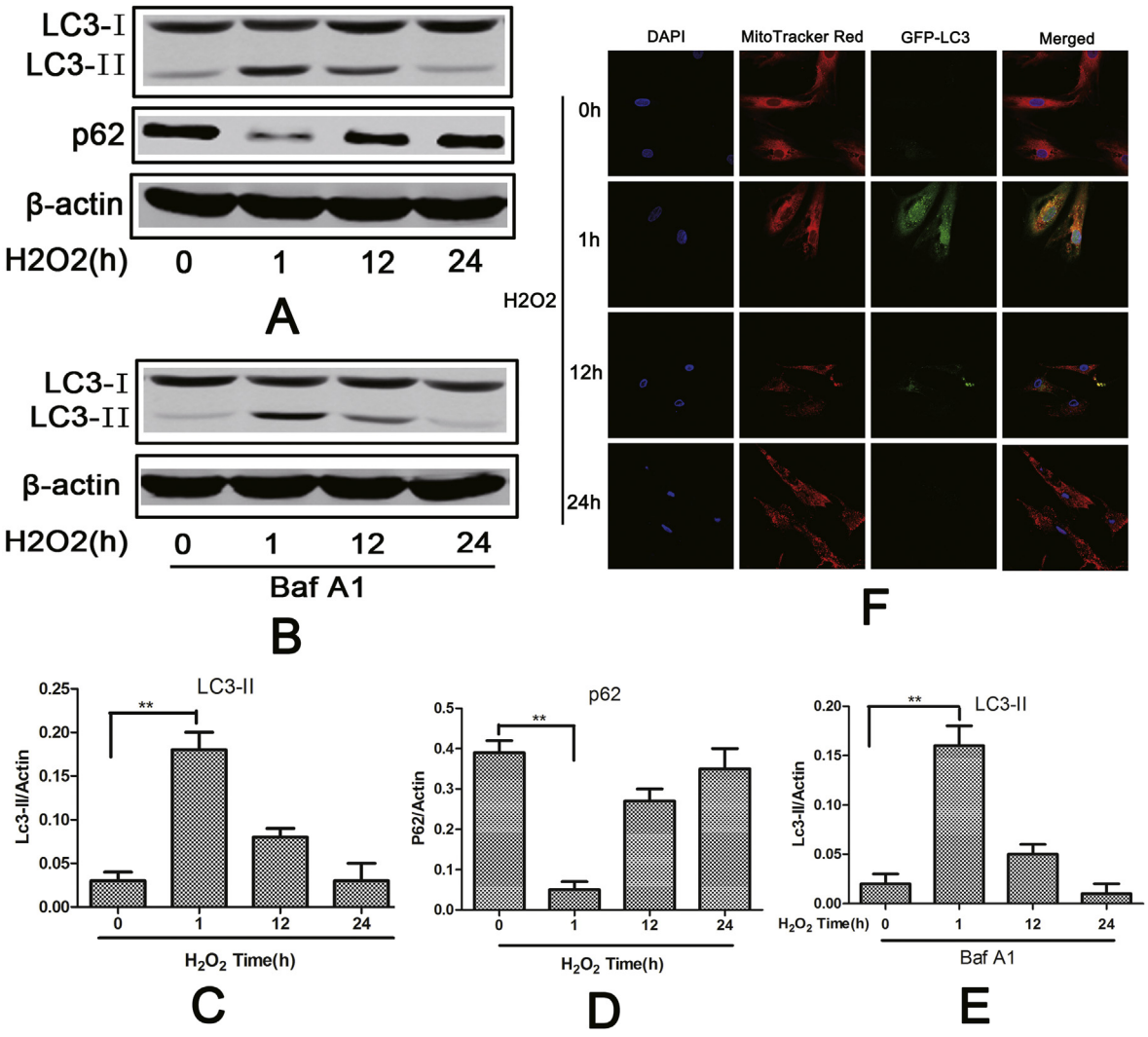

Fig. 4. Short-term oxidative stress induces mitophagy in BMSCs.
(A, C and D) Western blotting analysis for the protein expressions of LC3-II and p62. β-Actin was used as an internal control. BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). (B and E) Autophagic flux determination. BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h) in the presence of Bafilomycin A1 (Baf A1, 75 nM). (F) Colocalization analysis of confocal laser scanning microscopy images of DAPI, Mito Tracker Red and GFP-LC3. GFP-LC3 expressing BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). The results were expressed as mean ± SD (n = 3). **p < 0.01.

and negative for CD45 (Fig. 1A). In addition, cells were incubated for 28 days in the presence of agents triggering specific differentiation into adipocytes, osteoblasts and chondrocytes (Fig. 1B).

These results showed that these expanded cells were BMSCs, which maintained their phenotype to differentiate into multiple cell types.

3.2. Prolonged treatment of H_2O_2 activates caspases and induces apoptosis in BMSCs

H_2O_2 treatment to cultured cells is a commonly used model to evaluate oxidative stress susceptibility in various cell types [13,14]. In this study we used H_2O_2 to test the cytotoxicity of oxidative stress to BMSCs. CCK-8 assay showed a marked reduction of cell viability in BMSCs exposed in different concentrations of H_2O_2 (50, 100, 200, 400 and 600 μM) for 24 h. Considering that the LD_{50} values characterising cell viability corresponded to 200 μM H_2O_2 for BMSCs, we used this concentration in subsequent experiments (Fig. 2A).

Then, we assessed the apoptotic response of the BMSCs to oxidative stress. Western blot assay revealed that oxidative stress triggered the expression levels of cleaved caspase-3, -7 and -9. Meanwhile, the pan-caspase inhibitor, Benzyloxycarbonil-Val-Ala-Asp fluoromethylketone (Z-VAD-FMK), significantly inhibited the boosted activities of caspase-3, -7 and -9 in BMSCs (Fig. 2B–E). In addition, flow cytometric analysis by using Annexin V-FITC/PI staining and Tunel assay revealed an obvious increase of apoptosis in BMSCs with prolonged H_2O_2 treatment. Collectively, these results indicated that H_2O_2 can induce apoptosis in BMSCs.

3.3. Mitochondrial pathway is involved in the H_2O_2-induced apoptosis of BMSCs

Considering that H_2O_2 markedly increased the activities of caspase-3, -7 and -9, we speculated that the mitochondrial pathway should be involved in the H_2O_2-induced apoptosis of BMSCs. After treatment with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h), the mitochondrial membrane potential (mt△ψ) of BMSCs was detected with the specific mitochondrial dye JC-1. Flow cytometry and fluorescence microscopy for JC-1 staining revealed that the mt△ψ significantly decreased as indicated by the reduced JC-1 red fluorescence intensity and enhanced JC-1 green fluorescence intensity, in a manner

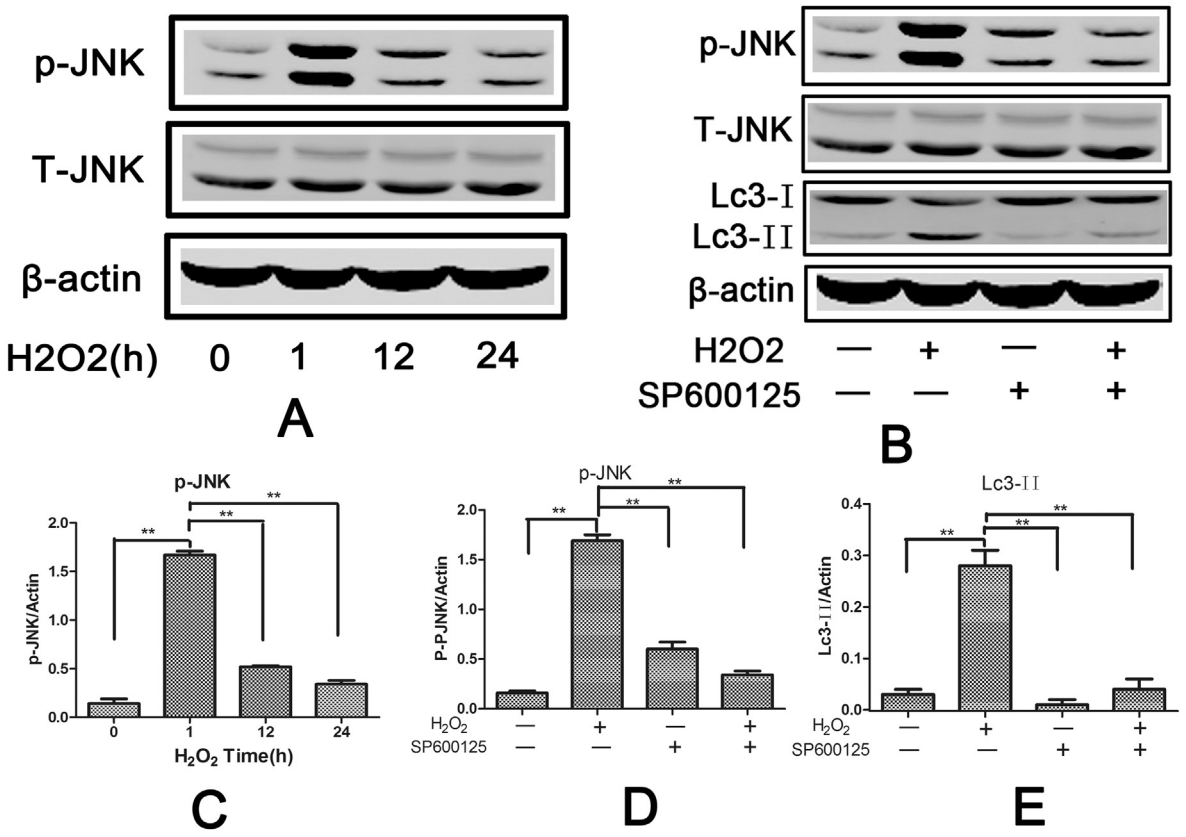

Fig. 5. H_2O_2 induces mitophagy in BMSCs through the JNK.
(A and C) Western blotting analysis for the protein expressions of p-JNK. β-Actin was used as an internal control. BMSCs were treated with 200 μM H_2O_2 for different time periods (0, 1, 12 and 24 h). (B, D and F) Western blotting analysis for the protein levels of p-JNK and LC3-II. β-Actin was used as an internal control. BMSCs were treated with 200 μM H_2O_2 for 1 h in the absence or presence of JNK inhibitor (SP600125). The results were presented as mean ± SD (n = 3). **p < 0.01.

corresponding to that of apoptosis incidence (Fig. 3A, B, G and H).

Furthermore, Western blot analysis indicated that H_2O_2 triggered the expression level of cytoplasm cytochrome *c*, which plays an important role in initiating apoptosis in the mitochondria. The expression level of proapoptotic protein Bax increased in BMSCs with the acting time of H_2O_2, whereas the antiapoptotic protein BCL-2 significantly decreased (Fig. 3C to F). All these results verified that the mitochondrial pathway was involved in the apoptosis of BMSCs under oxidative stress.

3.4. Short-term oxidative stress induces mitophagy in BMSCs

Mitophagy starts with the emergence of a double-membrane crescent that matures to a sealed double-membrane vesicle, which subsequently fuses with the lysosome, leading to the degradation of damaged mitochondria [7]. To investigate whether mitophagy was triggered by H_2O_2 in BMSCs, we measured the protein expression levels of LC3 and p62 in BMSCs.

LC3 is the mammalian autophagosomal homolog of yeast Atg8, which has two subtypes, LC3-I and LC3-II. The conversion of cytosolic-associated protein LC3-I into the membrane-bound LC3-II form is a crucial indicator of autophagosome activation. Accordingly, the detection of LC3-II can be applied to evaluate the formation of autophagosomes [15]. Western blot analysis showed that the expression level of LC3-II dramatically increased at the early stage of treatment with H_2O_2 and then gradually decreased (Fig. 4A and C). However, autophagosomes accumulation and increased LC3-II levels could result from increased autophagic flux or its defective fusion with lysosomes. LC3-II was analyzed during H_2O_2 treatment in the presence of bafilomycin A1 (Baf A1), a well-known inhibitor of autophagosomal lysosome degradation, to further investigate the role of H_2O_2 in the induction of mitophagy. Baf A1 can be used to rule out the possibility that the accumulation of LC3-II is caused by lysosomal disruption. Fig. 3B shows that Baf A1 further accumulated the H2O2-induced LC3-II in BMSCs even when the lysosome was disturbed, indicating that the H_2O_2-mediated conversion of LC3-II was not caused by the blockage of lysosomal degradation but due to an increase of autophagic flux (Fig. 4B and E).

In addition to LC3-II, p62, a substrate of mitophagy, which can be degraded by autolysosomes, is another common marker to study autophagic flux [15]. Mitophagy deficiency can cause the accumulation of p62 [13]. The protein expression of p62 was the least at 1 h, and then began to accumulate under prolonged oxidative stress (Fig. 4A and D).

As formation of autophagosome puncta containing LC3 has been a hallmark of mitophagy activation, confocal microscopy was used in BMSCs stably expressing GFP-LC3 to measure mitophagy. Colocalization of MitoTracker Red-stained mitochondria and GFP-LC3 dots was performed to examine mitophagy formation. In control groups, GFP-LC3 green fluorescence was predominantly dispersed throughout the cytoplasm. However, charactristic punctate fluorescent dots considerably increased with H_2O_2 treatment at the early stage, and then gradually declined (Fig. 4F).

All these evidence supported that H_2O_2 facilitated early mitophagy response in BMSCs.

3.5. H_2O_2 induces mitophagy in BMSCs through JNK

JNK is one of the three mitogen-activated protein kinases (MAPK) members [16] that transduces signals from the cell membranes to the nucleus in response to various stimuli, including oxidative stress [17]. Hence, we investigated whether JNK was involved in the mitophagy

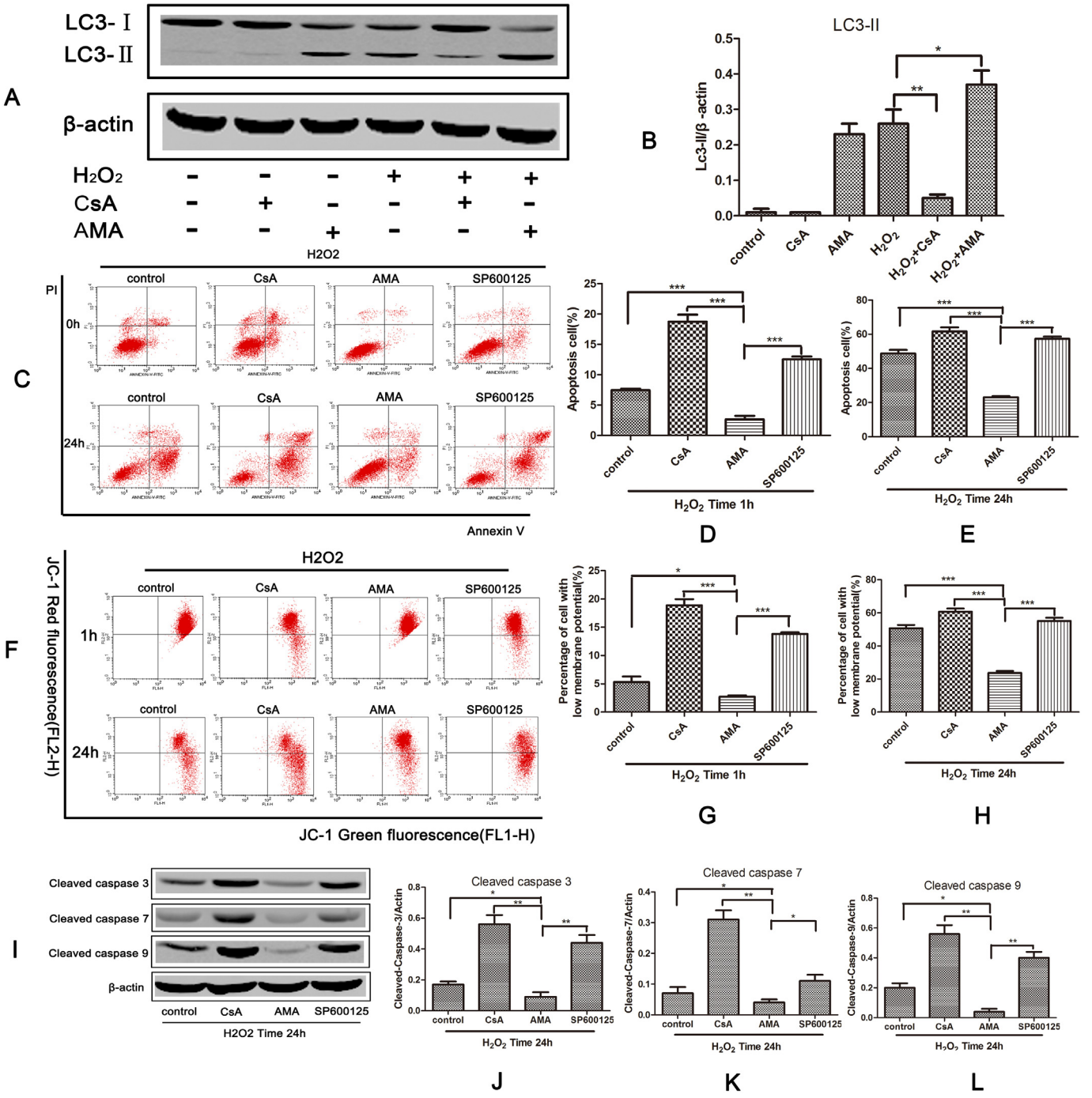

Fig. 6. Mitophagy inhibition enhanced H_2O_2-induced apoptosis in BMSCs.
BMSCs were treated with 200 μM H_2O_2 for 1 and 24 h in the presence of Antimycin A (AMA, 10 μg/ml) or Cyclosporin A (CsA, 10 μM) or SP600125 (10 μM) respectively. (A–D) Western blotting analysis for the protein levels of cleaved caspase-3, -7 and -9. β-Actin was used as an internal control. (E–G) Apoptosis incidence quantified by flow cytometry using Annexin V-FITC/PI. (H–J) mt△ψ analyzed by flow cytometry through JC-1 staining. (K and L) Western blotting analysis for the protein expression of LC3-II. β-Actin was used as an internal control. Data are expressed as mean ± SD (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001.

induced by H_2O_2 in BMSCs. Western blot analysis revealed that the phosphorylation of JNK, the activated form of JNK, immediately increased after H_2O_2 exposure at 1 h and then dropped gradually (Fig. 5A and C). H_2O_2 activated JNK in BMSCs in a manner corresponding to that of mitophagy.

To further determine the role of JNK in mitophagy induced by H_2O_2, JNK inhibitor SP600125 was used to inhibit JNK. SP600125 efficiently blocked the activation of JNK and significantly abrogated the effects of H_2O_2 on the accumulation of LC3-II protein (Fig. 5B, D and E). Hence, H_2O_2-induced mitophagy may through JNK.

3.6. Mitophagy inhibition enhanced H_2O_2-induced apoptosis in BMSCs

The relationship between mitophagy and apoptosis is complicated and varies across different cell types and different stimuli [18–21]. Mitophagy in BMSCs was activated immediately after H_2O_2 treatment and earlier than apoptosis. Cells were pretreated with Antimycin A (AMA, mitophagy activator), Cyclosporin A (CsA, mitophagy inhibitor), or SP600125 for 1 h before H_2O_2 exposure to investigate the interplay between mitophagy and apoptosis in BMSCs under oxidative stress. Western blot analysis showed that LC3-II in H_2O_2 treated cells was

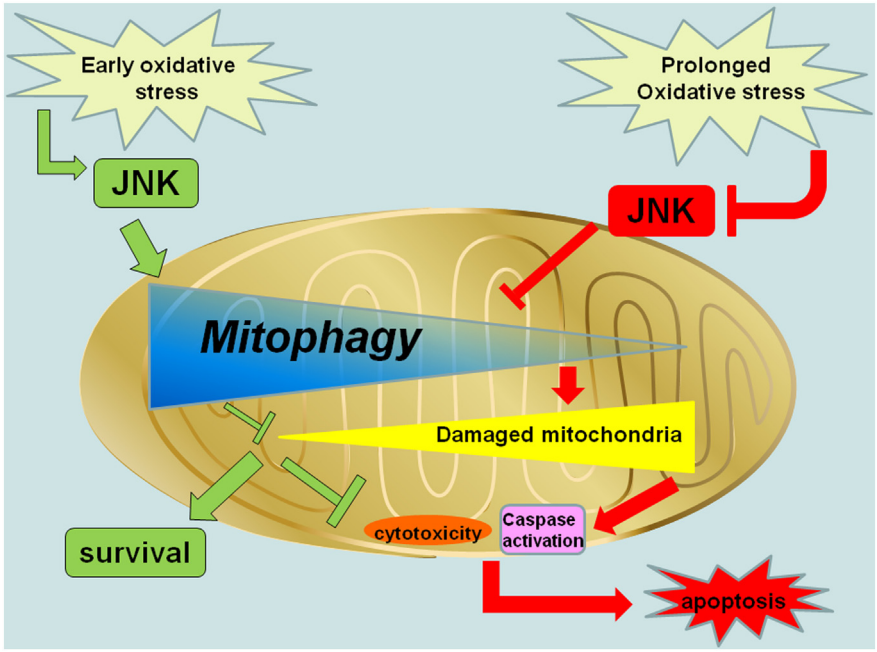

Fig. 7. Schematic diagram of mechanisms indicates the protective role of the mitophagy under oxidative stress in BMSCs.
Early exposure of BMSCs to H_2O_2 oxidative stress rapidly induces mitophagy through JNK and then eliminates damaged mitochondria. In addition, the increase of mitophagy protects BMSCs from oxidative stress-inducing cytotoxicity, caspase activation. Nevertheless, prolonged oxidative exposure shuts down the mitophagy via inhibition of JNK, following with the decrease of the elimination of damaged mitochondria and promotion of apoptotic cell death pathway.

significantly attenuated in the presence of CsA and was increased by AMA (Fig. 6A and B). Meanwhile, both the relative early and late stage apoptosis incidence escalated significantly when early mitophagy was inhibited in H_2O_2-treated BMSCs along with synchronous enhancement in the mt$\triangle\psi$ and the activation of caspase-3, -6 and -9. By contrast, AMA could attenuate cells apoptosis and increase mt$\triangle\psi$ and the cleavage of caspase-3, -6 and -9 (Fig. 6C–L).

These results revealed that mitophagy may be a preceding event for apoptosis in BMSCs treated with H_2O_2, and the suppression of early mitophagy could escalate the mitochondrial-mediated apoptosis in BMSCs under oxidative stress.

4. Discussion

Oxidative stress mediated by ROS has been increasingly recognised as a vital cellular stress with significant pathological implications in the progression of many human diseases [22,23]. Oxidative stress during BMSC transplant or in the injured tissue is a catastrophic factor that causes cell toxicity and poor survival of BMSCs [12]. ROS accumulation caused by the toxic effects of oxidative stress triggers mitophagy, and mitochondria are the major source and target of ROS [7]. Escalated ROS level could impair the healthy mitochondria by disturbing oxidative phosphorylation or mitochondrial permeability transition. Thereafter, the injured mitochondria can render cells to apoptosis through cytochrome *c*, Bax and activating caspase-9 that further processes caspase-3 and -7 to initiate a caspase cascade [7,24]. Mitophagy could control mitochondrial quality and quantity by eliminating dysfunctional or damaged mitochondria, which can generate ROS and even cause cell death [25]. However, the exact molecular machinery that facilitates mitophagy and the cross-talk between apoptosis and mitophagy in BMSCs remain poorly understood.

In our study, we showed that short-term treatment of H_2O_2 promoted mitophagy. Whereas, prolonged treatment of H_2O_2 blocked mitophagy and facilitated apoptosis in BMSCs, and the inhibition of mitophagy pharmacologically aggravated the reduction of mt$\triangle\psi$ and escalated H_2O_2-induced cell apoptosis in BMSCs. By contrast, mitophagy activator AMA ameliorated H_2O_2-induced mt$\triangle\psi$ decrease and alleviated cell apoptosis. Hence, mitophagy enhancement is an early event promoted by oxidative stress. However, sustained oxidative exposure reduced mitophagy, which caused mitochondrial irreversible damage and then enhanced cell apoptosis. Moreover, mitophagy played a pro-survival role during this process (Fig. 7). Our study systematically clarified the relationship between mitophagy and oxidative stress in BMSCs and elucidated the interplay of mitophagy with apoptosis in BMSCs under oxidative stress and suggested that mitophagy might be the cellular self-defensive mechanism for oxidative stress damage.

Despite the complex connections of mitophagy and apoptosis, many shared pathways and molecular mechanisms between them have been increasingly identified [26,27]. Mitophagy, a selective form of autophagy, is a highly conserved mechanism for eliminating dysfunctional or damaged mitochondria [6]. Currently, with increasing number of reports, mitophagy has been well known to have a protective role in various environmental stresses, including starvation, oxidative stress and hypoxia [28,29]. Mitophagy was rapidly increased in response to early oxidative stress. However, when H_2O_2 treatment was prolonged, mitophagy was reduced, dysfunctional mitochondria irreversibly accumulated and apoptosis was synchronously triggered. Moreover, the activation of cysteine aspartic acid protease (caspase) family played a crucial role in the execution and completion of apoptosis. Caspase-9 is an important member of the caspase family, and its cleaved form further promotes other caspase members, including caspase-3 and -7, to trigger cascade, leading to apoptosis. Executioner caspases (caspase-3, -7 and -9) were activated by prolonged treatment with H_2O_2. Furthermore, the promotion of mitophagy by AMA ameliorated the H_2O_2-induced caspase-3, -7 and -9 activations, whereas the inhibition of mitophagy by CsA escalated the activation and cytotoxicity. Hence, mitophagy plays an essential survival role in retarding the apoptosis of BMSCs under oxidative stress environment.

ROS production may also trigger JNK activation during cellular apoptosis progress [30,31]. As one of the three MAPK members, JNK is implicated in many cellular metabolisms such as cell proliferation, differentiation and apoptosis and can be activated via various extracellular stimuli, including mitogens, cytokines and ROS [32–34]. JNK is also involved in the regulation of mitophagy [35,36]. In our study, we found that short-term H_2O_2 treatment stimulated JNK expression level, whereas prolonged treatment of H_2O_2 inhibited it, corresponding to the variation of mitophagy. JNK inhibitor SP600125 efficiently blocked the activation of JNK and significantly abrogated the effects of H_2O_2 on the accumulation of LC3-II protein. Therefore, H_2O_2-induced mitophagy in BMSCs may through JNK.

In conclusion, we presented a novel finding that early oxidative stress enhanced mitophagy to protect cells, but mitophagy was reduced and apoptosis was increased once the cells had irreversible damage after long-term oxidative exposure (Fig. 7). Moreover, mitophagy enhancer AMA had a defensive effect against H_2O_2-induced caspase activation. However, the mitophagy inhibitor CsA treatment rendered cells more susceptible to H_2O_2-induced cell toxicity and cell death, which indicated the protective role of mitophagy in BMSCs under oxidative exposure. Furthermore, H_2O_2-induced mitophagy via JNK in BMSCs and the inhibition of JNK decreased mitophagy and promoted H_2O_2-induced apoptosis. These results help in understanding the mechanisms of mitophagy and apoptosis in BMSCs under oxidative stress exposure.

5. Conclusion

Induced mitophagy may play pivotal roles in protecting cells against oxidative stress in BMSCs. Targeting the mitophagy pathway in BMSCs may improve their survival capacity against oxidative stress during and after transplantation.

Declaration of Competing Interest

There were no financial conflicts of interest in all authors.

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