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Original Article

Apoptosis of bone marrow mesenchymal stem cells caused by hypoxia/reoxygenation via multiple pathways

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Abstract: The irreversible loss of cardiomyocytes remains a key problem to resolve, which forms the cellular basis of cardiac dysfunction. MSCs transplantation brings out a promising potential for myocardial renovation with less limitations. However, this cell transplantation therapy is limited by its poor viability after transplantation. Apoptosis is thought to be the major factor that affects the efficiency of MSCs transplantation. Therefore, exploring the process of apoptosis and the underlying mechanisms of MSCs in the 'harmful' microenvironment is significant for the sake of improving the efficiency of MSCs transplantation therapy. A hypoxia/reoxygenation (H/R) model of MSCs had been established. TUNEL, Hoechst staining and MTT were used for the evaluation of morphological changes, cell viability and apoptosis. Mitochondrial transmembrane potential was detected by JC-1 using the fluorescence microscopy system. The protein expression of cytochrome c, p-ERK, p-AKT, Bcl-2, Bax, p-JNK, HIF-1α and VEGF was assessed for the analysis of protein changes using the Western blot. In our study, H/R insult lead to apoptosis and cell viability lost in a time-dependent manner in MSCs. Multiple pathways were involved in the apoptosis of MSCs, including cytochrome c released from mitochondria to cytosol, mitochondrial transmembrane potential lost. In addition, p-ERK and p-AKT were downregulated, while Bcl-2, p-JNK and VEGF were upregulated. H/R induced the apoptosis in MSCs is through multiple pathways. These multiple pathways will be helpful for understanding and explaining the process and mechanism of apoptosis in MSCs.

Keywords: MSCs, apoptosis, cytochrome c, transmembrane potential, ERK, Akt, JNK, Bcl-2, Bax, HIF-1α, VEGF

Introduction

Myocardial ischemia disease is known as a major killer in the world. Despite therapeutic progress of cardiovascular diseases, the irreversible loss of cardiomyocytes remains a key problem to resolve, which forms the cellular basis of cardiac dysfunction. Several kinds of stem cells have been demonstrated their potential effects to the cardiomycytes replacement, including induced pluripotent stem cells, adipose-derived stem cells, and so on [1-3]. However, there are some limitations in these cell transplantation therapies, such as ethical concerns, tumorigenesis, histocompatibility and inadequate tissue supplement. Bone marrow mesenchymal stem cells (MSCs) transplantation brings out a promising potential for cardiac repair with less limitations. Some groups have reported that MSCs can differentiate into vascular endothelial cells and cardiomyocytes such that they can improve the heart function [4]. In addition to promoting angiogenesis and limiting adverse structural remodeling in the infarcted heart, the transplantation of MSCs also alters the ion channel expression and mitigates the electrophysiological remodeling [5]. Meanwhile, MSCs could improve cardiac conduction by upregulation of connexin 43 through the paracrine signaling [6]. Although the MSCs transplantation therapy was regarded as a new frontier in regenerative medicine in cardiology [7], it was limited by the poor viability of MSCs after transplantation. As reported, only mild to moderate improvement of heart function was observed in the majority of studies, and even no improvement in some clinical trials [8]. In mice model, the majority of the transplanted MSCs were readily lost during the following 4 days after the transplantation [9]. This circum-

stance reflected that the harmful, proapoptotic microenvironment in the infarcted heart was involved in the dysfunction or loss of MSCs. Apoptosis is thought to be a major factor which affects the transplantation efficiency of MSCs [10]. The influencing factors for the apoptosis of MSCs in the peri-infarct region, in a large part, during to the endogenous and microenvironmental factors, such as poor blood supply, ischemia-reperfusion injury and inflammatory response [11].

Therefore, exploring the mechanism of MSCs apoptosis in peri-infarct myocardium is important for the interruption of the pathologic process and further improvement of the efficiency of cell therapy. Hypoxia and survival growth factor withdrawal caused apoptosis via the caspase-dependent manner in transplanted MSCs [12]. However, the molecular mechanisms of MSCs have not been fully elucidated at present, as there are plenty of apoptosis-induced factors in the microenviroment of myocardium. In General, two pathways of apoptosis have been delineated in other cell lines (i.e., sensory hair cell) [13]. The mitochondrial pathway involved the release of proteins, such as cytochrome c from the mitochondria to cytosol [14]. Cytochrome c release was usually in association with regulation of mitochondrial membrane proteins, such as the Bcl-2 family [15]. Cytochrome c bound Apaf-1 in the cytosol, leading to the oligomerization and activation of caspase family. Another is the extrinsic pathway, including FAS and its receptor. However, a study showed that Fas pathway seemed not to be involved in the apoptosis of MSCs, as agonistic Fas mAb treatment failed to induce apoptosis of MSCs [16].

Meanwhile, we presume that other pathways may play an important role in apoptosis of MSCs, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), Akt, Hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF), especially in a hypoxia condition. ERK is a chain of proteins in cell that communicates a signal from a receptor on the surface to the DNA in the nucleus of cell. The ERK signaling pathway is widely involved in various cellular functions, including growth, differentiation, inflammation and apoptosis. In addition, ERK can be activated by hypoxia and may be involved in the response to hypoxia [17]. HIF is activated when a cell is short of oxygen. HIF stimulates the release of VEGF [18]. Recently, much attention has been paid on the role of Akt as a kind of survival signal [19]. Akt, known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration [20].

To date, studies of MSCs have focused on the restoration of heart function and the potential of cell differentiation and migration. How the microenvironment of myocardial infarction induced apoptosis in MSCs and the processes of cell response after MSCs transplantation are not fully illustrated. As ischemia/reperfusion has been a widespread phenomenon in clinic with therapeutic progress, in this study, we used hypoxia/reoxygenation (H/R) and survival growth factor withdrawal to imitate the microenviroment of myocardial ischemic-reperfusion and detect the processes of MSCs response and its underlying mechanism.

Materials and methods

Isolation and proliferation of MSCs

Isolation and proliferation of MSCs were performed according to previously described methods [21]. In brief, we humanely killed male 80g Sprague-Dawley rats and harvested bone marrow by flushing their femoral and tibial cavities with phosphate-buffered saline (PBS). Bone marrow cells were prepared by gradient centrifugation at 900 g for 30 minutes on Percoll (GE Healthcare, Sweden) of a density of 1.073 g/ml and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. The adherent, spindle-shaped MSCs population expanded to $> 5 \times 10^7$ cells within 3 passages. Cells were determined by fluorescence activating cell sorting (FACS, Beckman Coulter, USA) analysis before the experiments, using directly conjugated antibodies against anti-rat CD44, anti-CD45 and anti-CD90 (PE, Caltag, USA). All cells in the study were cultured to 3 passages. All animals in this study received humane care and the study was approved by Zhejiang university ethics committee.

Hypoxia/reoxygenation protocol

To imitate ischemia/reperfusion injury of MSCs, we used hypoxia/reoxygenation (H/R) treatment on MSCs with 6 hours of hypoxia (< 0.5% O_2) in a hypoxic GENbox Jar (Billumps-Rothenberg, Del Mar, CA), followed by 12 hours of reoxygenation (21% O_2). The oxygen level in the chamber was monitored with an oxygen analyzer. Before H/R, cells were washed with PBS and placed in serum free DMEM. To investigate whether H/R has long-term effects on MSCs, we tested the apoptosis induced by 24 hours hypoxia and 24 hours reoxygenation (H/R 24 h) in the MSCs.

TUNEL procedure

Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL, Roche Diagnostic, USA) was performed with a detection kit according to the manufacturer's instructions. Briefly, after the cells were treated with H/R, the cells were fixed with 4% paraformaldehyde at room temperature for 60 minutes and subsequently permeabilized with 0.1% Triton X 100 for 30 minutes. After the cells were incubated with TUNEL reaction mixture for 60 minutes at 37°C in a humid chamber, the cells were incubated in Converter-peroxidase (POD) for 30 minutes at 37°C, and then added a diaminobenzidine (DAB) POD substrate .The percentage of TUNEL-positive cells in relation to the total number of cells was determined by counting at least 200 cells in 3 different fields.

Assessment of morphological changes

Chromosomal condensation was assessed using the chromatin dye Hoechst 33342 (Sigma-Aldrich, USA). Cells were fixed for 30 minutes in PBS containing 1% glutaraldehyde. After being fixed, the cells were washed and then exposed to 5 μg/ml Hoechst 33342 for 30 minutes at room temperature. All samples were observed using a fluorescence microscope. Apoptotic cells were characterized by morphological changes including nuclei thickening and cell shrinkage.

Cell viability assay

MSCs were seeded in 96-well plates at a density of 5×10^4 cells/well. After synchronization by serum-free medium treatment for 24 hours, the cells were treated with H/R. Then the cells were added with 500 μg/ml 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) each well and cultured for 3 hours in a CO_2 incubator. Cells having functional mitochondrial succinate dehydrogenase could convert MTT to formazan which generated a blue color when dissolved in dimethyl sulfoxide. After plates were shaken to dissolve the purple formazan producer, the intensity was measured with a microplate reader at absorption wavelength of 570 nm.

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential was assessed using the lipophilic cationic probe 5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-tetraethyl-imidacarbocyanine iodide (JC-1, BioVision, USA), a sensitive fluorescent dye. The red emission of the dye was attributable to a potential-dependent aggregation in the mitochondria, reflecting $\Delta\Psi m$. Green fluorescence reflected the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. Treated MSCs were loaded with 10 μM JC-1 for 15 minutes by incubation at 37°C and were monitored by the ZESIS fluorescence microscopy system.

Isolation of mitochondria and cytosol

The preparation of mitochondrial and cytosolic fractions was achieved using a mitochondria/cytosol fractionation kit (BioVision, USA) according to the manufacturer's protocol. Briefly, cells were collected and then resuspended in 1 ml of 1x cytosol extraction buffer, and homogenized in an ice-cold tissue grinder for 60 passes. The resultant supernatants were further centrifuged. Supernatants were used as the cytosolic fraction, while the pellets, which were then resuspended in 100 μl of the mitochondrial extraction buffer, used as the mitochondrial fraction.

Western blot analysis

For detection of the protein expression, disposed cells were washed and scraped into 50 μl lysis buffer. After being quantified by BCA reagent, equivalent proteins for each sample were resuspended in 10 μl of electrophoresis sample buffer and subjected to SDS-PAGE in

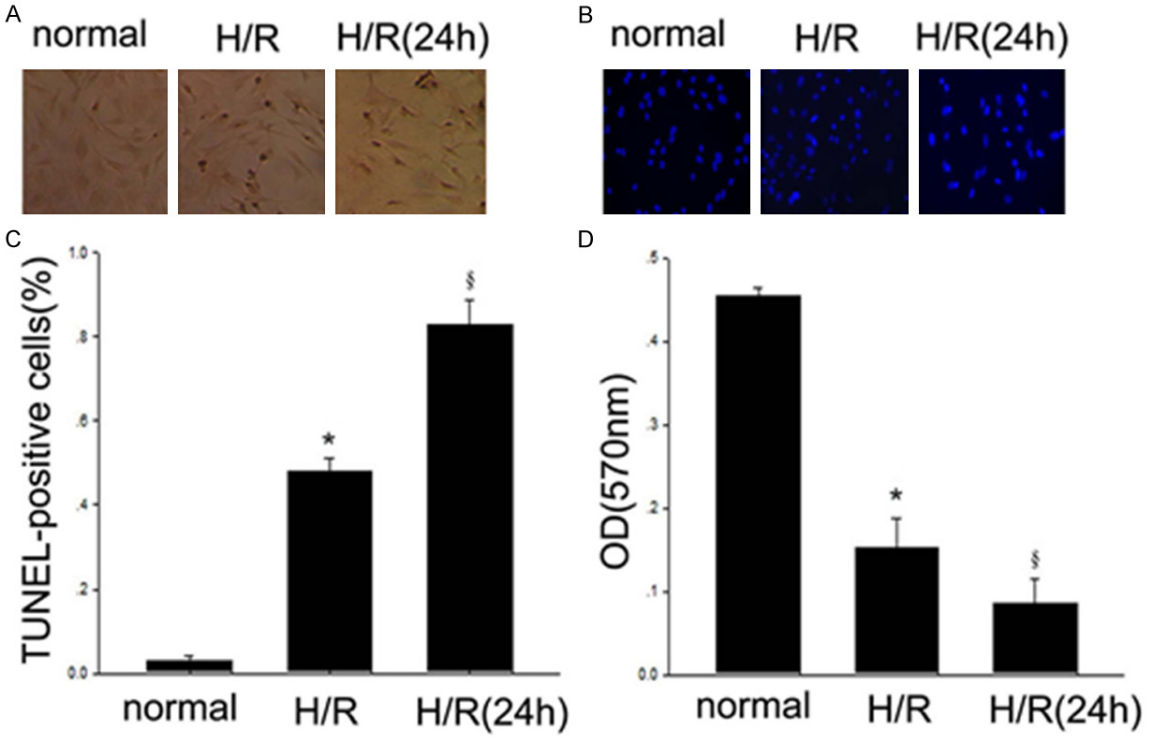

Figure 1. H/R induced apoptosis of MSCs. A. DNA strand breaks assessed by TUNEL staining of MSCs. Shown are photograph of MSCs of normal MSCs, H/R and 24 h H/R. Brown nucleus indicates TUNEL-positive (apoptotic) cell. B. Morphological changes assessed by Hoechst 33342.Shown are photograph of MSCs of normal MSCs, H/R and 24 h H/R. Chromatin condensation together with decrease in cell size indicates TUNEL-positive (apoptotic) cell. C. Quantification of apoptotic MSCs by TUNEL staining. D. Quantitative analysis of cell viability by MTT. *$P < 0.05$ *vs.* normal cell, §$P < 0.05$ *vs.* H/R group. Data shown are means ± SE representative of 5 independent experiments.

12-15% acrylamide minigels. Proteins were then transferred to PVDF membrane in a transfer buffer. After blocking with 5% skim milk, PVDF membrane was washed in TBS containing 0.1% Tween 20 and incubated with a protein-specific antibody at room temperature followed by a secondary antibody. Primary antibodies were used as follows: antibodies specific to Bcl-2, Bax, Akt, p-Akt (Cell Signaling Technology, USA), HIF-1α (R&D Systems, USA), VEGF, β-actin, ERK, p-ERK and p-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the secondary antibody incubation, the membranes were rinsed and the bound antibodies were detected using enhanced chemiluminescence (ECL, Santa Cruz Biotechnology, USA) followed by autoradiography. Image pro plus 5 software was used to semiquantify protein in every lane.

Statistical analysis

Statistical analysis was performed with one-way anova followed by Bonferroni multiple-comparison test. The results were considered to be significant at a value of $P < 0.05$.

Results

H/R induced apoptosis, morphological changes and decreases cells viability

After the MSCs transplantation, multiple 'harmful' factors could lead to apoptosis. Among these, the major factors were the deprivation of nutrients, oxygen fluctuation and inflammation. To study the effects of these stimuli, MSCs were exposed to culture conditions represented by H/R and the nutrients deprivation was analyzed by TUNEL and morphological changes-Hoechst 33342. Three generation of MSCs were stained by TUNEL method, about 3% of normal MSCs were positive (**Figure 1A**). When MSCs cultured in serum-free media and insulted by hypoxia for 6 hours and reoxygenation for 12 hours, the number of TUNEL-positive MSCs was significantly increased (≈ 48.2%, *$P < 0.05$ *vs.* normal cell) (**Figure 1A**). To further investi-

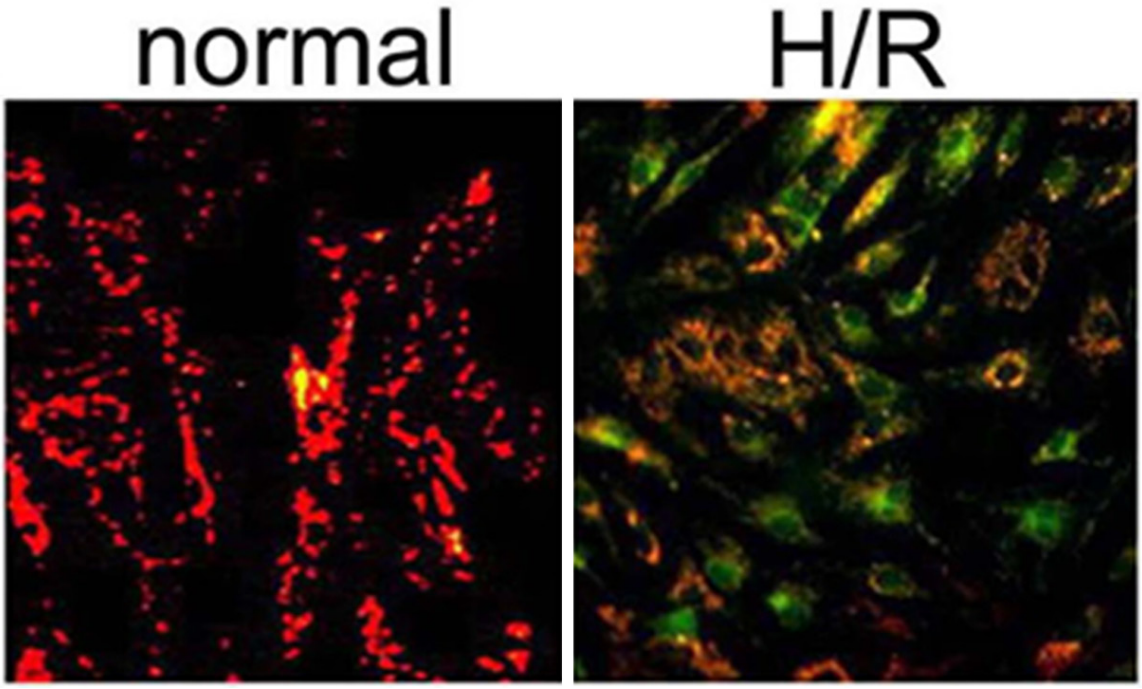

Figure 2. H/R induced mitochondrial membrane potential of MSCs, mitochondrial membrane potential (ΔΨm) of MSCs exposed to H/R was determined using the potential-sensitive fluorescent probe JC-1. Each panel shows an overlay of 2 images; orange-yellow color denotes colocalization of red (aggregate) and green (monomer) fluorescence signals. Normal MSCs exhibited punctate red staining indicative of coupled mitochondria with a normal ΔΨm. MSCs after H/R developed a diffuse green staining pattern, representative of reduced ΔΨm. Results are representative of 1 experiment from a total of 3 experiments performed.

gate long-term effects of H/R on MSCs, we tested the apoptosis induced by 24 h hypoxia and 24 h reoxygenation, the apoptotic index further increased (83% ± 5.6%, §$P < 0.05$ *vs.* H/R; **Figure 1A**). As shown in **Figure 1B**, most cells from the normal group had big and regular nuclei, with only a few showing apoptotic nuclei with condensed chromatin. In the cells exposed to H/R, there was clear evidence of chromatin condensation together with decrease in cell size, which was the characteristic of apoptosis. H/R also decreased the viability of the MSCs in a time-dependent manner ($^{*}P < 0.05$ *vs.* normal cells; **Figure 1D**).

Influence of H/R on mitochondrial membrane potential (ΔΨm)

To determine the influence of H/R on mitochondrial dysfunction in MSCs, we assessed ΔΨm by using the potential-sensitive fluorescent probe JC-1. Normal MSCs exhibited punctate red staining indicative of coupled mitochondria with a normal ΔΨm (**Figure 2**). After insulted by H/R, MSCs developed a diffuse green staining pattern, which was a representative of reduced ΔΨm (**Figure 2**).

Effects of H/R on translocation of cytochrome c

Cytochrome c was an important protein which was released from mitochondria to cytosol. Once cytochrome c was released, it bound with apoptotic protease activating factor-1 and ATP, which created a protein complex known as an apoptosome. In this study, cytochrome c was translocated from mitochondria to cytosol when MSCs were subjected to H/R (≈ 1.4-fold *vs* normal cells, $P < 0.05$ *vs.* normal cells) (**Figure 3**). Combined with ΔΨm changes, we presumed that apoptosis of MSCs induced by H/R through the mitochondrial pathway.

Effects of H/R on expression of Bcl-2 and Bax

To investigate the changes of apoptosis regulatory proteins about the mitochondria pathway, the expressions of Bcl-2 and Bax were studied. We firstly examined the expression of Bcl-2 pro-

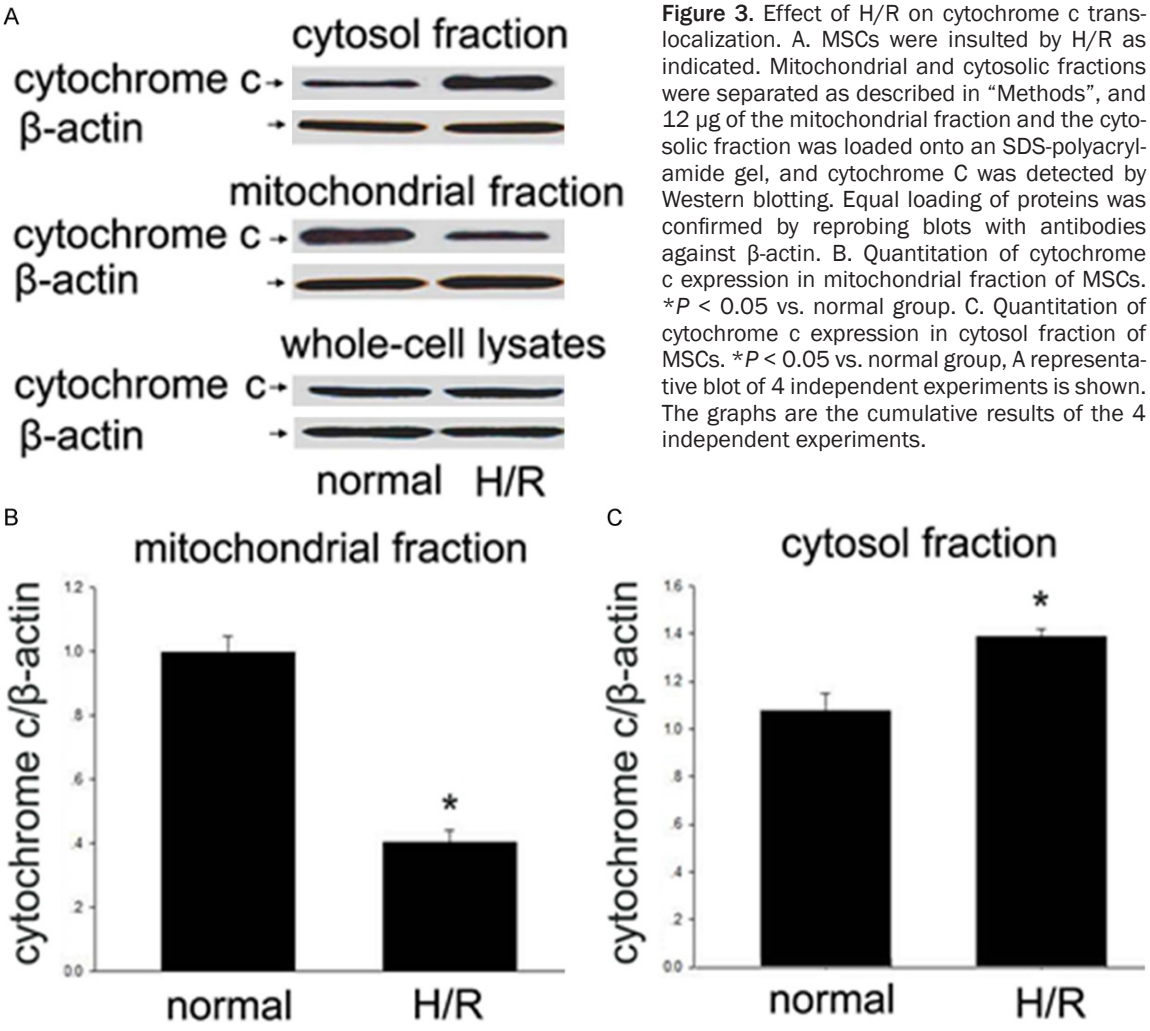

Figure 3. Effect of H/R on cytochrome c translocalization. A. MSCs were insulted by H/R as indicated. Mitochondrial and cytosolic fractions were separated as described in "Methods", and 12 μg of the mitochondrial fraction and the cytosolic fraction was loaded onto an SDS-polyacrylamide gel, and cytochrome C was detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. B. Quantitation of cytochrome c expression in mitochondrial fraction of MSCs. **P* < 0.05 vs. normal group. C. Quantitation of cytochrome c expression in cytosol fraction of MSCs. **P* < 0.05 vs. normal group, A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

teins in MSCs which were exposed to H/R. As shown in **Figure 4A**, Bcl-2 expression was upregulated at H/R by 4.5-fold vs. normal cells (*P* < 0.05 *vs.* normal cell). At the same time, Bax expression was upregulated at H/R by 1.9-fold *vs.* normal cells (*P* < 0.05 *vs.* normal cell) (**Figure 4**). This result reflected that Bcl-2 and Bax may participate in the regulation of mitochondrial pathway in MSCs.

Effects of H/R on p-ERK-1/2 and p-JNK in MSCs

To further determine the effects of H/R on MSCs, we tested ERK and JNK activity using an antibody specific to phosphorylated (activated) form of ERK and JNK. As shown in **Figure 5**, ERK phosphorylation decreased in MSCs after H/R by 0.8 fold compared with normal cells (*P* < 0.05 *vs.* normal cell) (**Figure 5**). JNK had been reported as an oxidant sensitive kinase. Thus, we assessed whether the H/R induced apoptosis in MSCs through JNK pathway. As shown in **Figure 6**, JNK phosphorylation increased after H/R in MSCs by 1.4 fold compared with normal cells (*P* < 0.05 *vs.* normal cell). This data suggested that ERK and JNK phosphorylation were involved in apoptosis in MSCs (**Figure 6**).

Effects of H/R on Akt expression in MSCs

Akt was a serine/threonine-specific protein kinase that played a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. To further determine the underlying mechanism of the effects of H/R on MSCs, we tested the Akt expression, a molecule involved in the transduction of anti-apoptotic signals. The results showed that the p-Akt content decreased in the MSCs after H/R treatment by 0.39 ± 0.14 fold versus normal cells (*P* < 0.05, H/R *vs* normal cells; **Figure 7**).

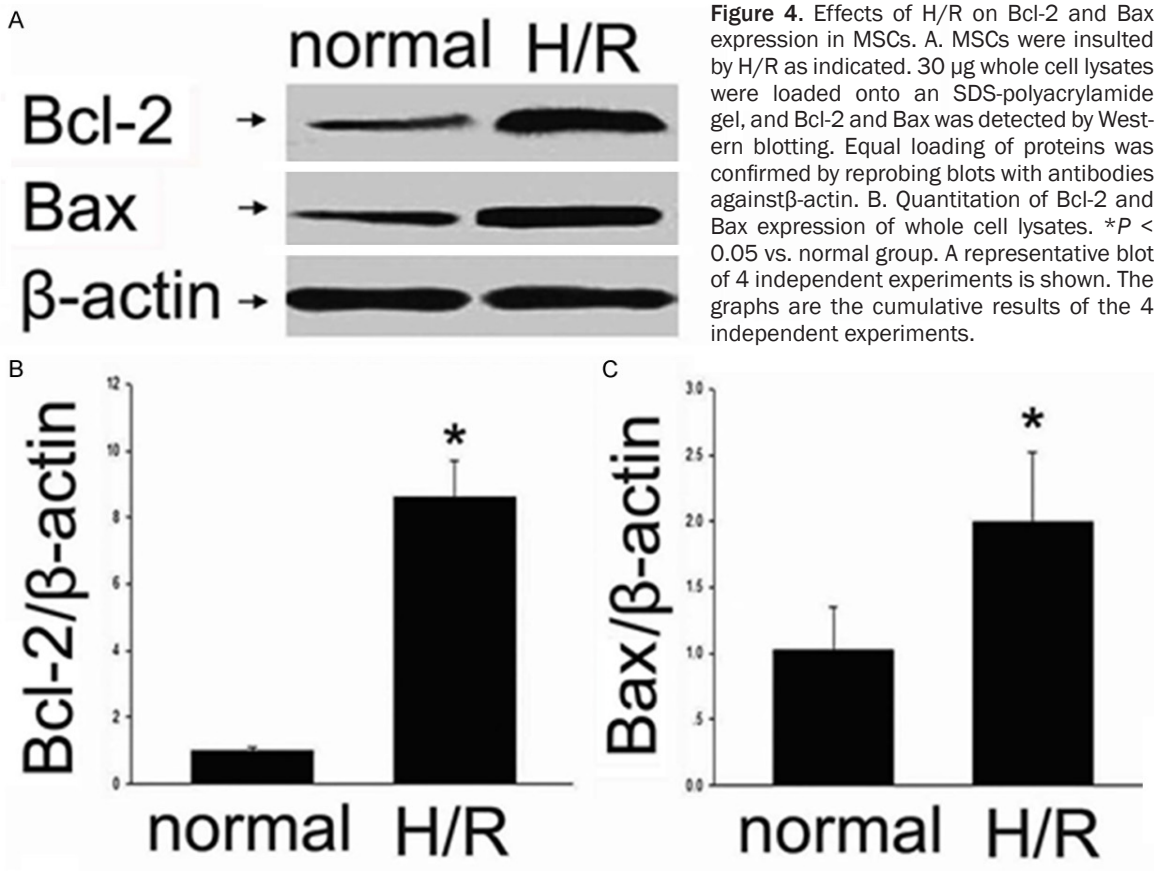

Figure 4. Effects of H/R on Bcl-2 and Bax expression in MSCs. A. MSCs were insulted by H/R as indicated. 30 μg whole cell lysates were loaded onto an SDS-polyacrylamide gel, and Bcl-2 and Bax was detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies againstβ-actin. B. Quantitation of Bcl-2 and Bax expression of whole cell lysates. *P < 0.05 vs. normal group. A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

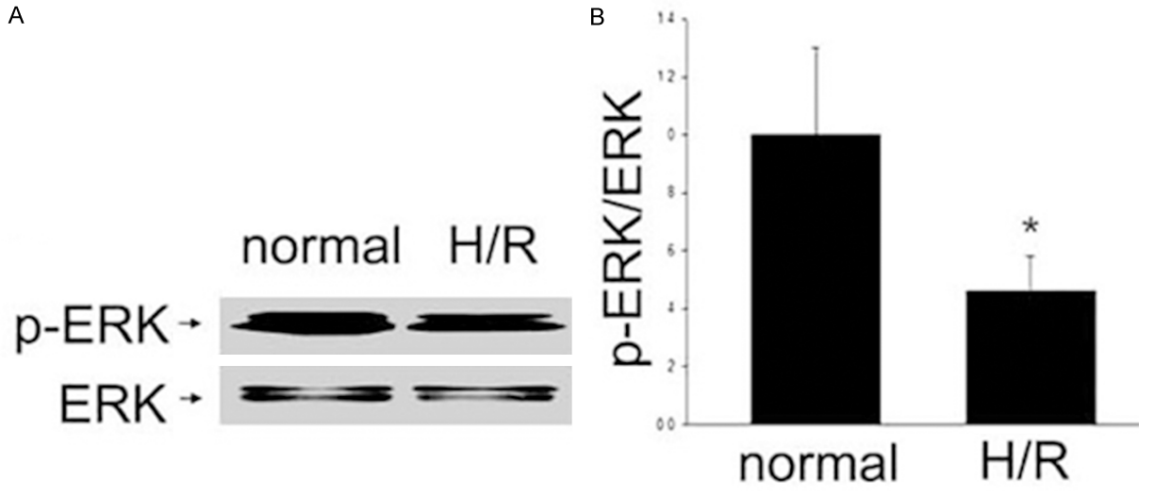

Figure 5. Effects of H/R on extracellular signal-regulated protein kinase (ERK-1/2) in MSCs. A. MSCs were insulted by H/R stimulation. Phosphorylation of ERK1/2 was determined by Western blot analysis, using antibody specific to activated ERK-1/2. B. Quantitation of ERK1/2 activity in MSCs *P < 0.05 vs. normal group. A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

Effects of H/R on HIF-1α and VEGF expressions in MSCs

HIF and VEGF might be involved in apoptosis in MSCs when MSCs were in response to hypoxia, which were the survival factors in some cell lines. To further determine the underlying mechanism of the effects of H/R on MSCs, we next tested the HIF-1α and VEGF expressions. Interestingly, after 6 h hypoxia and 12 h reoxy-

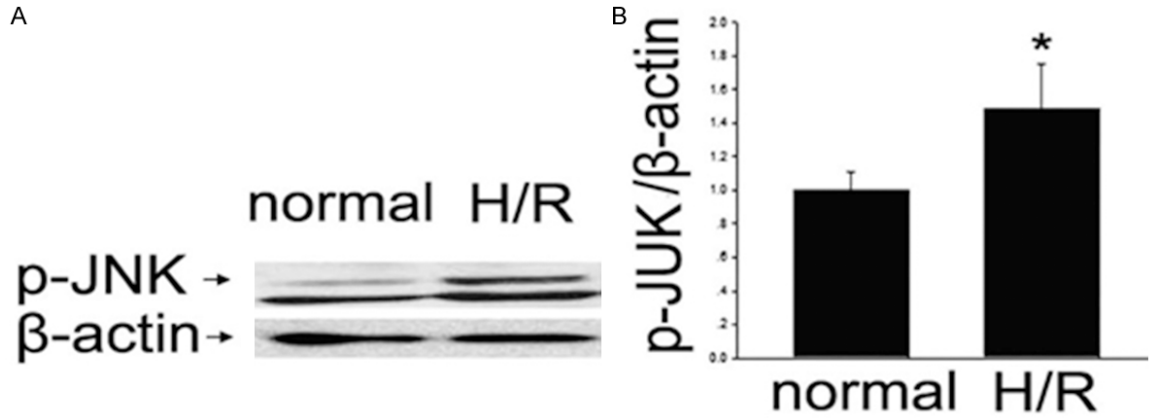

Figure 6. Effects of H/R on p-JUK expression in MSCs. A. MSCs were insulted by H/R stimulation. The expression of p-JUK was determined by Western blot analysis, using antibody specific to p-JUK. B. Quantitation of p-JUK expression in MSCs. *$P < 0.05$ vs. normal group. A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

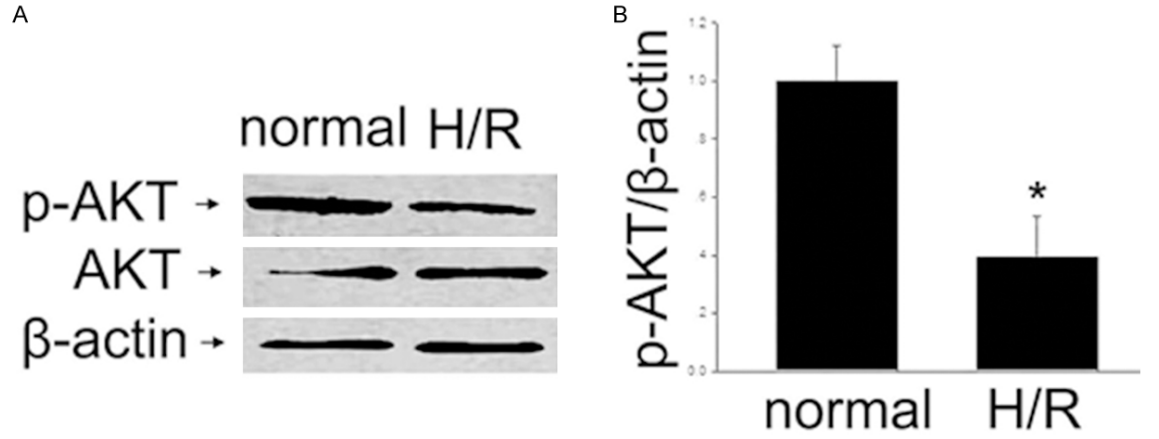

Figure 7. Effects of H/R on p-AKT expression in MSCs. A. MSCs were insulted by H/R stimulation. The expression of p-AKT was determined by Western blot analysis, using antibody specific to p-AKT. B. Quantitation of p-AKT expression in MSCs. *$P < 0.05$ vs normal group. A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

genation, the expression of HIF-1α did not significantly change in the MSCs subjected to H/R (**Figure 8A**). While the VEGF content in our model was increased in the MSCs after H/R by 1.89 ± 0.15-fold versus normal cells ($P < 0.05$, HR *vs.* normal cells). This data suggested that VEGF in MSCs expression was upregulated in the H/R-induced apoptosis (**Figure 8B**).

Discussion

MSCs could differentiate into cardiomyocytes, increase capillary density, secrete some cytokines and restore heart function. Moreover, MSCs are readily accessible with no immunological complications [22]. Hence, MSCs transplantation is a promising therapy in ischemia heart disease and heart failure. However, the poor survival rate and low transplantation efficiency of the donor cells in the infarcted myocardium become the challenge of the therapeutic efficacy [10]. The majority of transplanted cells underwent apoptosis in the ischemic heart because of poor blood supply, ischemia/reperfusion and inflammation. We think that studies on how MSCs respond to proapoptotic microenviroment and its possible mechanisms are helpful for the transplantation therapy. Previous studies had showed that survival growth factor withdrawal and hypoxia caused transplanted MSCs apoptosis that resulted in the caspase-dependent manner, which was

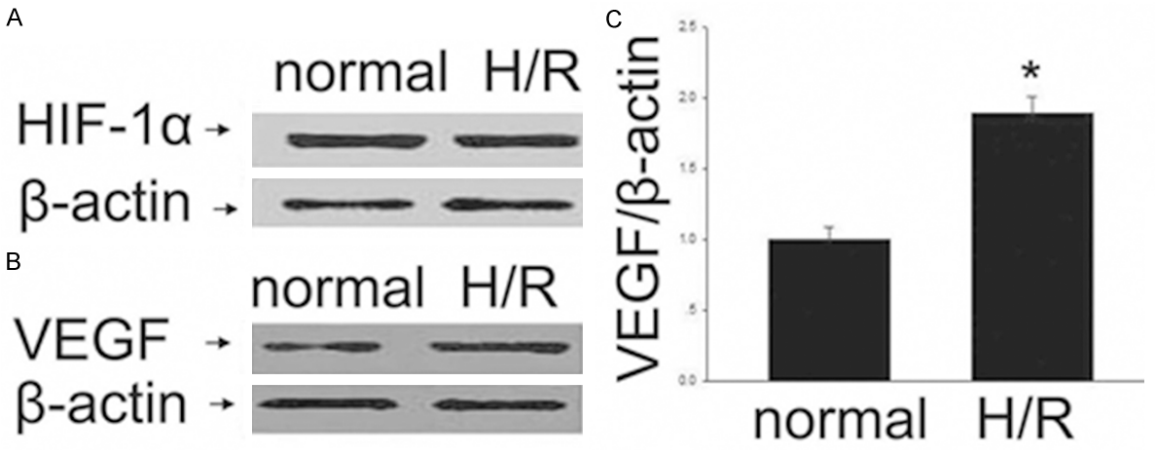

Figure 8. Effects of H/R on HIF-1α and VEGF expression in MSCs. A. MSCs were insulted by H/R stimulation. HIF-1α was determined by Western blot analysis, using antibody specific to HIF-1α. B. VEGF was determined by Western blot analysis, using antibody specific to VEGF. C. Quantitation of VEGF expression in MSCs. *$P < 0.05$ vs. normal group. A representative blot of 4 independent experiments is shown. The graphs are the cumulative results of the 4 independent experiments.

regulated by translocation of Bax. However, the response process was so complicated that we need further studies to explore the process and mechanism. Up to now, whether other pathways involved in apoptosis of MSCs have not been identified. On the other hand, ischemia/reperfusion was a widespread phenomenon in heart disease and affected patients' prognosis in clinic. Therefore, in this study, we used H/R and survival growth factor withdrawal to mimic ischemia/reperfusion for exploring the characteristics and mechanisms of the MSCs apoptosis.

Our experiments showed that when MSCs insulted by H/R, apoptosis arose in a time-dependent manner, as detected by TUNEL. In the same time, as Hoechst 33342 stain showed chromatin condensation and decrease in cell size, which were the characteristics of apoptosis in MSCs. The proapoptosic effects of H/R were confirmed by MTT. In addition, when MSCs put in a H/R microenviroment, cell viability reduced rapidly in a time-dependent manner. MTT data was in accordance with the TUNEL and Hoechst 33342 analyses.

Next, we detected the possible pathways of apoptosis in our model. The intrinsic pathway of apoptosis was involved in integrity of the mitochondrial membrane regulated by the activity of the Bcl-2 family proteins. Mitochondrion is not only an energy production organelle but also an important apoptosis regulator. Firstly, MSCs developed a diffuse green staining pattern, representative of reduced ΔΨm, when insulted by H/R. The release of proapoptotic factors from mitochondria into cytoplasm, such as cytochrome c, promoted the activation of caspase family in some cell lines [14]. For the underlying mechanism of apoptosis in MSCs, we detected cytochrome c translocation from mitochondria into cytoplasm. The results showed that H/R led to cytochrome c released from mitochondria to cytoplasm in MSCs. The Bcl-2 protein played a critical role in the regulation of mitochondrial dysfunction and apoptosis, which could stabilize MPT, thereby inhibiting the release of the proapoptotic factors. In this study, when MSCs were put in the H/R microenvironment, Bcl-2 and Bax expressions both increased. We presumed that MSCs might elevate Bcl-2 for self-care in H/R. These changes were in accordance with previous studies in human endothelial cells and cardiomyocytes [4, 23]. As mentioned above, the mitochondria pathway was involved in apoptosis in MSCs induced by H/R.

For further illustration of the apoptosis pathways, we detected ERK and JNK expressions. The ERK and JNK signaling pathways are widely involved in various cellular functions, including growth, differentiation, inflammation and apoptosis. ERK and JNK could be activated by hypoxia and might play an important role in response to hypoxia [24, 25]. In the present study, we observed a remarkable decrease of

phosphorylated ERK level under H/R conditions. However, when insulted by H/R, the expression of phosphorylated JNK level increased. We presumed that phosphorylated ERK and JNK played different roles in apoptosis of MSCs. Results from our study suggested that ERK and JNK phosphorylation were involved in the proapoptosisc effects of H/R. ERK and JNK regulation seemed to be a correlation apoptosis with oxidative stress and inflammation in some cell lines [26].

Recently, much attention has been paid on the role of PI 3-kinase as a kind of survival signals. However, the function of Akt in apoptosis seemed to depend on cell types. For example, an inhibition of PI 3-kinase which was not involved in cerebellar granule neurons maintained in serum and K^+-rich medium [27]. Meantime, the overexpression of Akt was sufficient to inhibit MSCs apoptosis [28]. Akt-overexpressing MSCs implantation significantly preserved cardiac function in an ischemic/reperfusion model of pigs [29, 30]. In our study, a remarkable decrease of phosphorylated Akt level responded to H/R conditions. The results suggested that Akt pathway was involved in MSCs apoptosis process.

VEGF production might be induced in MSCs through injury by H/R, especially in hypoxia stage. When a cell is deficient in oxygen, it produces HIF, a transcription factor. HIF stimulates the release of VEGF [31, 32]. However, in our study, HIF-1α showed no difference in MSCs after 6 h hypoxia and 12 h reoxygenation between normal group and H/R group. We presumed that HIF-1α degraded in reoxygenation stage, as VEGF increased in H/R group in our model. VEGF was an angiogenic peptide which was released in response to hypoxia [33]. The angiogenic action of VEGF involved an anti-apoptotic effect that promoted cell survival [34].

It should be indicated that the possible cross-talk among these pathways, such as Bcl-2 overexpression, could enhance VEGF secretion. VEGF increased the expression of Bcl-2 in turn, Bcl-2 had been shown to induce VEGF expression in different cell lines [35, 36]. Akt and ERK pathways activated by VEGF play opposite roles in neuronal apoptosis [36].

Some limitations of this study should also be acknowledged. We did not use corresponding blockers to confirm each pathway, although defining the functional importance of every pathway will be a complex work. To our knowledge, the pathways of mitochondria, ERK, JNK, Akt and VEGF all played an important role in the process of apoptosis in MSCs. In further study, we will try to seek for harmless and useful agents to intervene in MSCs apoptosis progress for transplantation in vivo.

In conclusion, the results suggested that H/R insult led to apoptosis and cell viability lost in a time dependent manner. Multiple pathways were involved in apoptosis in MSCs, including cytochrome c released from mitochondria to cytosol, ΔΨm loss, p-ERK and p-Akt downregulation, Bcl-2, p-JUN and VEGF upregulation. These multiple pathways will be greatly helpful in understanding and explaining MSCs apoptosis process and assessing the possible target for intervene in MSCs apoptosis. Further studies should be performed on the intervention in MSCs apoptosis by using corresponding agents. In addition, more attentions should be paid to the screening for more powerful and less harmful agents in cell transplantation.

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Disclosure of conflict of interest

None.

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