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ORIGINAL ARTICLE

Basic Study

Heat shock pretreatment improves stem cell repair following ischemia-reperfusion injury *via* autophagy

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Abstract

AIM: To investigate whether heat shock pretreatment (HSP) improves mesenchymal stem cell (MSC) repair *via* autophagy following hepatic ischemia-reperfusion injury (HIRI).

METHODS: Apoptosis of MSCs was induced by 250 mM hydrogen peroxide (H_2O_2) for 6 h. HSP was carried out using a 42 ℃ water bath for 1, 2 or 3 h. Apoptosis of MSCs was analyzed by flow cytometry, and Western blot was used to detect Bcl-2, Bax and cytochrome C expression. Autophagy of MSCs was analyzed by flow cytometry and transmission electron microscopy, and the expression of beclin Ⅰ and LC3-Ⅱ was detected by Western blot. MSCs were labeled *in vivo* with the fluorescent dye, CM-DiI, and subsequently transplanted into the portal veins of rats that had undergone HIRI. Liver levels of proliferating cell nuclear antigen (PCNA) were quantified by fluorescent microscopy. Serum aminotransferase activity and the extent of HIRI were also assessed at each time point.

RESULTS: HSP for 2 h reduced apoptosis of MSCs induced by H_2O_2 as seen by a decrease in apoptotic rate, a decrease in Bax and cytochrome C expression and an increase in Bcl-2 expression ($P < 0.001$). In addition, HSP for 2 h induced autophagy of MSCs exposed to H_2O_2 as shown by an increase in acidic vesicular organelle-positive cells, beclin 1 and LC3-Ⅱ expression, and autophagosome formation ($P < 0.05$). Treatment with 3-methyladenine attenuated HSP-induced autophagy and abolished the protective effects of HSP on the apoptosis of MSCs. Rapamycin failed to have additional effects on either autophagy or apoptosis compared with HSP alone. The phosphorylation of p38MAPK was significantly elevated and the phosphorylation of mTOR was downregulated in heat shock pretreated MSCs. Treatment with the p38MAPK inhibitor, SB203580, reduced HSP-induced autophagy in MSCs. *In vivo* studies showed that the transplantation of HSP-MSCs resulted in lower serum aminotransferase levels, lower Suzuki scores, improved histopathology and an increase in PCNA-positive cells ($P < 0.05$).

CONCLUSION: HSP effectively induces autophagy following exposure to H_2O_2 *via* the p38MAPK/mTOR pathway, which leads to enhanced MSC survival and improved MSC repair following HIRI in rats.

Key words: Hepatic ischemia-reperfusion injury; Heat shock pretreatment; Mesenchymal stem cells; Autophagy; Transplantation

Core tip: We investigated the interaction between autophagy and apoptosis in mesenchymal stem cells (MSCs) exposed to H_2O_2. We found that heat shock pretreatment (HSP)-induced autophagy served as a protective mechanism. HSP for 2 h improved the therapeutic potential of MSCs in the treatment of ischemia-reperfusion (I/R) injury in rats and enhanced autophagy *via* the p38MAPK/mTOR pathway, which is involved in the protective effects of HSP on H_2O_2-induced MSC apoptosis. Systemic administration led to an increase in HSP-MSCs homing to I/R liver cells compared with MSCs, resulting in a significant improvement in liver function, accelerated mitogenic response and alleviation of histopathological damage.

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INTRODUCTION

During surgical trauma, particularly liver transplantation, hepatic ischemia-reperfusion injury (HIRI) may occur, which is associated with a significant reduction in liver function[1,2]. Effective treatment strategies aimed at reducing HIRI may therefore offer major benefits in hepatic surgery and liver transplantation. A previous study demonstrated the specific involvement of bone marrow mesenchymal stem cells (MSCs) in the repair of HIRI[3]. However, due to local hypoxia, inflammation, and especially oxidative stress in the targeted tissue, the transplanted MSCs did not withstand the difficult microenvironment caused by ischemia-reperfusion (I/R) injury. Thus, low cell survival reduced the therapeutic effect[4]. It was also reported that < 1% of transplanted MSCs survived to the fourth day in an immunodeficient mouse heart model[5]. The poor MSC survival rate was also observed after transplantation into lungs and kidneys with I/R injury[6,7]. Therefore, it is imperative to protect MSCs from oxidative stress and other pro-apoptotic factors to improve their therapeutic effect.

Heat shock pretreatment (HSP) is known to activate certain types of self-protective proteins and protects cells *in vitro* from various environmental insults[8-10]. Several reports have shown that HSP of transplanted cells enhanced their survival in a heart model both *in vivo* and *in vitro*[11,12]. Thus, we hypothesized that HSP of MSCs could enhance their survival following transplantation into the liver after I/R injury. Recently, the induction of autophagy was shown to be a novel method of protecting MSCs from apoptosis[13,14]. Several reports have shown that heat shock treatment can activate autophagy in multiple cell types[15,16]. However, it is unknown whether autophagy can be activated by heat shock treatment or how it affects MSCs.

Autophagy is an essential cellular mechanism that occurs in eukaryotic cells[17,18]. In recent years, it has been found that autophagy plays a vital role in cell apoptosis and its role depends on cell type and cellular conditions. Autophagy can lead to pro-survival pathways, while inappropriate autophagy can induce cell death[19]. Under ischemia or hypoxia/serum deprivation (H/SD), autophagy can protect MSCs from apoptosis by eliminating reactive oxygen species and damaged organelles to provide energy[13,20]. Moreover, H/SD-induced autophagy has also been demonstrated to induce apoptosis in some cell types. Autophagy can also directly promote type Ⅱ programmed cell death[21]. However, the functional role of autophagy in oxidative stress-induced apoptosis in MSCs has not been fully elucidated.

Mitogen-activated protein kinase (p38MAPK) is a positive regulator of autophagy and is regulated by heat shock treatment to improve cardiac cell survival[8]. p38MAPK can be activated in response to exogenous stress such as hypoxia, starvation and heat shock, which in turn activates mitogen-activated protein kinase kinases (MKK)-3/4/6 and their effector kinases to stimulate autophagy[22]. However, little is known about the function of the p38MAPK pathway

in regulating the activation of autophagy in MSCs following heat shock treatment.

The aim of this study was to examine the function of autophagy in MSC apoptosis induced by oxidative stress injury. Further, we investigated whether HSP activates autophagy *via* the p38MAPK/mTOR pathway to protect MSCs against apoptosis.

MATERIALS AND METHODS

Animals

Thirty-two male Sprague-Dawley rats (about 220 g; 10 wk old) from the Animal Center of the Second Affiliated Hospital, Harbin Medical University were used in this study. The rats were cared for in accordance with the guidelines published by the US National Institutes of Health. All study procedures were approved by the Harbin Medical University Institutional Animal Care and Use Committee. The study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press.

Cell culture and treatment

MSCs were collected as previously described[3], and density centrifugation was performed to isolate MSCs[23]. The femurs and tibias from male Wistar rats aged 4 wk were flushed, and bone marrow cells were collected and then fractionated in Lymphoprep™ density solution. Following centrifugation at 800 × *g* for 20 min, the cells at the interface were collected and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, United States) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37 ℃ with 95% humidity and 5% CO_2. After 48 h, the culture medium was changed to remove non-adherent cells. After the fourth passage, MSCs were washed with phosphate buffered saline (PBS), exposed to HSP for different time periods (1, 2 or 3 h) in a 42 ℃ water bath and then incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2 for 24 h (HSP-MSC group). Control cells were cultured under normal conditions without HSP (MSC group). To simulate tissue I/R microenvironment *in vitro*, MSCs were treated with 250 mM H_2O_2 (Sigma-Aldrich, United States) for 6 h[24,25]. The autophagy inhibitor, 3-methyladenine (3-MA; 5 mM; Sigma-Aldrich, United States), the autophagy promoter, rapamycin (10 nM; Cell Signaling Technology, United States), or the p38MAPK inhibitor, SB203580 (5 mM; Beyotime, China), was added to further examine the role of autophagy on MSC apoptosis.

Evaluation of autophagy and apoptosis by flow cytometry

Cell apoptosis was examined using the Annexin V-FITC/PI Kit (Becton-Dickinson, United States). Briefly, MSCs were collected in 200 mL medium. Following resuspension, approximately 10 mL of Annexin V solution were added and incubated for 15 min at room temperature in the dark. Then, 300 mL medium buffer and 5 mL propidium iodide (PI) were added and the cell suspension was incubated for 15 min at room temperature in the dark. The cell suspension was then immediately analyzed by flow cytometry (Becton-Dickinson, United States). Cell Quest software was used to analyze 10^4 cells.

Cell autophagy was examined by detecting acidic vesicular organelles (AVO) using acridine orange (AO) stain (Solarbio, China) according to published protocols[26]. Briefly, cells were stained with 1 mg/mL AO for 15 min and collected in PBS. In AO-stained cells, the cytoplasm fluoresces bright green, whereas AVOs, including lysosomes and autolysosomes, fluoresce bright red. The green (510-530 nm) and red (650 nm) fluorescence emission from 10^4 cells illuminated with blue (488 nm) excitation light was measured by flow cytometry using Cell Quest software.

Transmission electron microscopy

MSCs were harvested and fixed with 2.5% glutaraldehyde at 4 ℃ for 2 h. Cells were then suspended in PBS containing 1% osmic acid at 4 ℃ for 2 h, Following dehydrating and embedding[13], ultrathin sections were prepared on uncoated copper grids using an Ultrotome (Leica, Reichert Ultracuts) and stained with uranyl acetate and lead citrate. Images were captured using a transmission electron microscope (JEM1230; JEOL).

Western blot

Protein lysates were separated using SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, United States). Membranes were probed with the appropriate primary antibodies (Supplemental Table 1). Alexa Fluor® 680 donkey anti-mouse IgG (H + L) or Alexa Fluor® 680 donkey anti-rabbit IgG (H + L) were used as secondary antibodies (1:5000; Invitrogen, United States). Fluorophores were detected using the Odyssey™ Infrared Imaging System (Li-Cor, Lincoln, NE, United States).

Labeling of MSCs

The transplanted MSCs were labeled with 10 μmol/L CM-Dil (Invitrogen, United States) according to published protocols[27].

Model of HIRI and cell transplantation

HIRI in the rat model was performed as previously described[3]. Briefly, a midline laparotomy was performed following anesthesia administration with intraperitoneal sodium pentobarbital (60 mg/kg). The left lateral and medial lobes of the liver were then clamped at their bases using an atraumatic clip. Ischemia was induced in 70% of the segmental liver and prevented ischemia in the mesenteric veins[28]. Throughout the administration of anesthesia, body

temperature was monitored by a rectal probe and maintained at 37 ℃ by a heating lamp. The clamp was removed after 60 min, and 1 × 10^6 CM-Dil-labeled MSCs or HSP-MSCs suspended in 200 μL PBS were immediately transplanted into the portal vein using a 30-gauge needle, in the MSC group and HSP-MSC group, respectively. The control group underwent laparotomy only and received 200 μL PBS. The 32 rats were randomly divided into 4 groups. At 24 h after transplantation, 2 mL blood was harvested from the inferior vena cava before the animals were sacrificed by cervical spine dislocation. Livers were harvested immediately.

Immunofluorescence microscopy

The chest was opened following tracheal intubation and the rats were perfused with 4% paraformaldehyde (Sigma-Aldrich, United States) in 0.01 M PBS following an overdose of anesthesia (sodium pentobarbital; 100 mg/kg, intraperitoneal) for 2 min[29]. Harvested livers were cryopreserved in 30% sucrose at 4 ℃ overnight, embedded in optimal cutting temperature (OCT) compound, and cut into 4 μm-thick sections using a cryostat. The sections were rinsed twice with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed three times with PBS. After permeabilization with 0.2% Triton X-100, the sections were blocked at 4 ℃ overnight in 1% BSA/0.05% Triton X-100. Sections were then incubated with an antibody against PCNA (1:200) at 37 ℃ for 2 h. After washing three times with PBS, the sections were incubated with Alexa Fluor® 488-conjugated Affinipure goat anti-rabbit IgG (H + L) secondary antibody (1:200; ZSGB-Bio, China) for 1 h at room temperature. After extensive washing, the sections were examined under a fluorescence microscope[30].

Measurement of liver function

To evaluate the severity of HIRI, the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by an automatic analyzer (Hitachi, Japan) as described previously[31].

Immunohistochemical staining

Tissue sections of 1.5 cm × 1.5 cm × 2 mm were subjected to immunohistochemical staining to assess PCNA 24 h after cell transplantation. Immunohistochemical staining of sections for PCNA expression was performed by a standard streptavidin-biotin peroxidase complex method[32]. Tissue sections (4 mm) were deparaffinized and rehydrated by standard protocols, autoclaved at 95 ℃ for 20 min, and cooled to 30 ℃. Normal rabbit serum (10%) was used to block non-specific binding sites. Sections were then incubated with anti-PCNA primary antibody (1:100) in PBS containing 1% bovine serum albumin at 4 ℃ overnight. The sections were washed in PBS, incubated with biotinylated anti-rabbit IgG for 30 min at room temperature, and then a streptavidin-biotin peroxidase complex solution (Nichirei, Japan). The chromogen, 3, 3'-diaminobenzidine tetra-hydrochloride, was used as a 0.02% solution containing 0.005% H_2O_2 in 50 mmol/L ammonium acetate-citrate acid buffer (pH 6.0). Sections were counterstained with Mayer's hematoxylin and mounted. Negative controls were established by replacing the primary antibody with normal rabbit serum. No staining was detected in the negative controls.

Histological analysis

The degree of HIRI was assessed by histological analysis as previously described[3].

Statistical analysis

The data were expressed as the mean ± SD, and representative results were from at least three independent experiments. For quantitative continuous data, the differences between two groups were examined and the data were analyzed using *t*-tests. When multiple comparisons were possible, ANOVA coupled with Tukey's post-hoc test correction was used. $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using SPSS version 21 (SPSS Inc., Chicago, IL, United States) or the GraphPad Prism 5.0 software package (GraphPad Software, Inc., La Jolla, CA, United States).

RESULTS

Heat shock pretreatment protected MSCs exposed to H_2O_2 against apoptosis

The apoptotic rate and levels of the pro-apoptotic proteins, Bax and cytochrome C, were all reduced. The anti-apoptotic protein, Bcl-2, was increased in the HSP_{1h} and HSP_{2h} groups compared to the control and H_2O_2 group (Figure 1; $P < 0.01$). However, in the HSP_{3h} group, the apoptotic rate and expression of Bax and cytochrome C were increased, while Bcl-2 expression was reduced (Figure 1; $P < 0.01$). These results suggest that 2 h of HSP protected MSCs from H_2O_2-induced apoptosis.

HSP induced time-dependent autophagy in MSCs

To examine whether HSP activated autophagy in MSCs, the cells were pretreated with heat shock for 1, 2 or 3 h, and then exposed to H_2O_2 for 6 h. The number of AVO-positive cells identified by flow cytometry was increased in the HSP group compared with the control group (Figure 2A; $P < 0.05$). Different durations of HSP led to a time-dependent increase in the action of autophagy in MSCs exposed to H_2O_2, which peaked in the HSP_{3h} group ($P < 0.01$). HSP-MSCs showed a significant time-dependent increase in the expression of LC3B-Ⅱ and the autophagic marker, beclin 1, compared to the control group (Figure 2D). Autophagosomes observed in HSP-MSCs exposed to

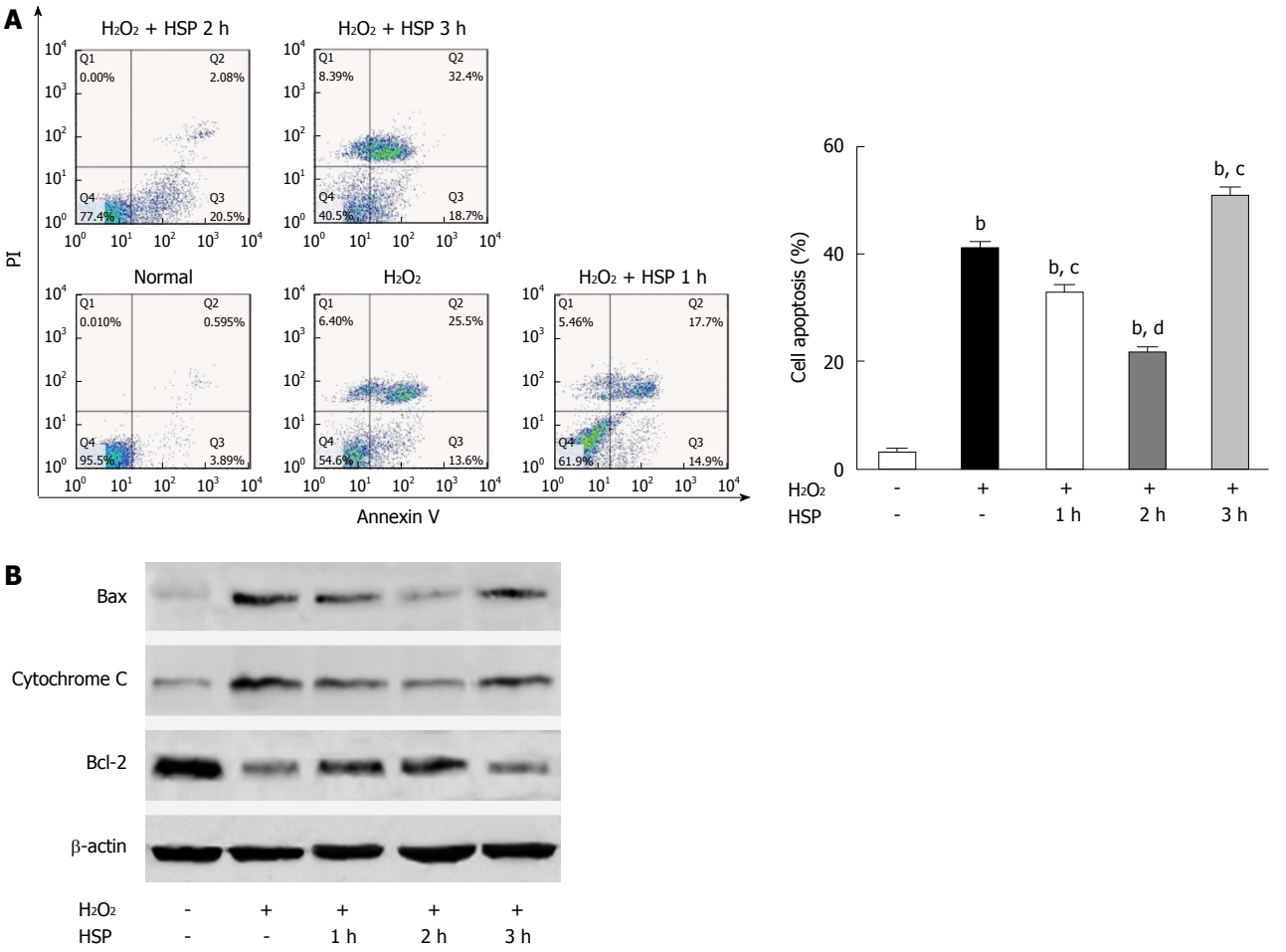


Figure 1 Heat shock pretreatment protected mesenchymal stem cells from apoptosis induced by H_2O_2. Apoptosis was analyzed using flow cytometry (A) and Western blot (B). The apoptotic rate and pro-apoptotic proteins Bax and cytochrome C were reduced, while the anti-apoptotic protein Bcl-2 was increased in the HSP_{1h} and HSP_{2h} group, particularly in the HSP_{2h} group ($P < 0.01$). However, the apoptotic rate, and both Bax and cytochrome C were increased, while Bcl-2 was reduced in the HSP_{3h} group. The data represent the results of three separate experiments. $^bP < 0.01$ compared with the normal control group; $^cP < 0.05$, $^dP < 0.01$ compared with the H_2O_2 group. HSP: Heat shock pretreatment.

H_2O_2 are shown in Figure 3. These results suggest that HSP promoted autophagic activity in MSCs exposed to H_2O_2 in a time-dependent manner.

HSP protects MSCs against H_2O_2-induced apoptosis by activating autophagy

We found that HSP_{2h} achieved the greatest protective effect against H_2O_2-induced apoptosis using flow cytometry and Western blot (Figure 1). To determine the role of autophagy in MSCs, we exposed cells to HSP for 2 h with 3-MA or rapamycin and H_2O_2 treatment for 6 h, and assessed autophagy and the apoptotic rate. Following 6 h of H_2O_2 treatment, 3-MA attenuated both the activation of autophagy and the anti-apoptotic capacity in MSCs treated with heat shock for 2 h, as shown by fewer AVO-positive MSCs (Figure 2C), lower expression of LC3-Ⅱ and beclin 1 (Figure 4B) and fewer autophagosomes in MSCs ($P < 0.01$; Figure 3). In addition, a higher apoptotic rate (Figure 4A), increased expression of Bax and cytochrome C, and decreased expression of Bcl-2 (Figure 4B) were found compared with the control group ($P < 0.01$) and the HSP_{2h} group ($P < 0.05$). In addition, rapamycin failed to have any effect on autophagic activity and the apoptotic rate in MSCs pretreated with heat shock for 2 h. These results indicated that activation of autophagy by HSP for 2 h may serve as a protective mechanism against apoptosis in MSCs exposed to H_2O_2.

The p38MAPK/mTOR pathway is involved in HSP-induced autophagy

To investigate whether HSP induced autophagy by activating the p38MAPK pathway, the p38MAPK inhibitor, SB203580, was used and the levels of autophagy were evaluated in HSP-MSCs exposed to H_2O_2. The results revealed that the expression of p38MAPK and mTOR did not significantly change in any of the groups. However, the phosphorylation of p38MAPK was upregulated and the phosphorylation of mTOR was downregulated in the HSP_{2h} group compared with the control group (Figure 5). SB203580 reduced autophagy in the HSP_{2h} group, as shown by a decrease in the number of AVO-positive MSCs ($P < 0.05$) (Figure 2C), expression of LC3-Ⅱ and beclin 1

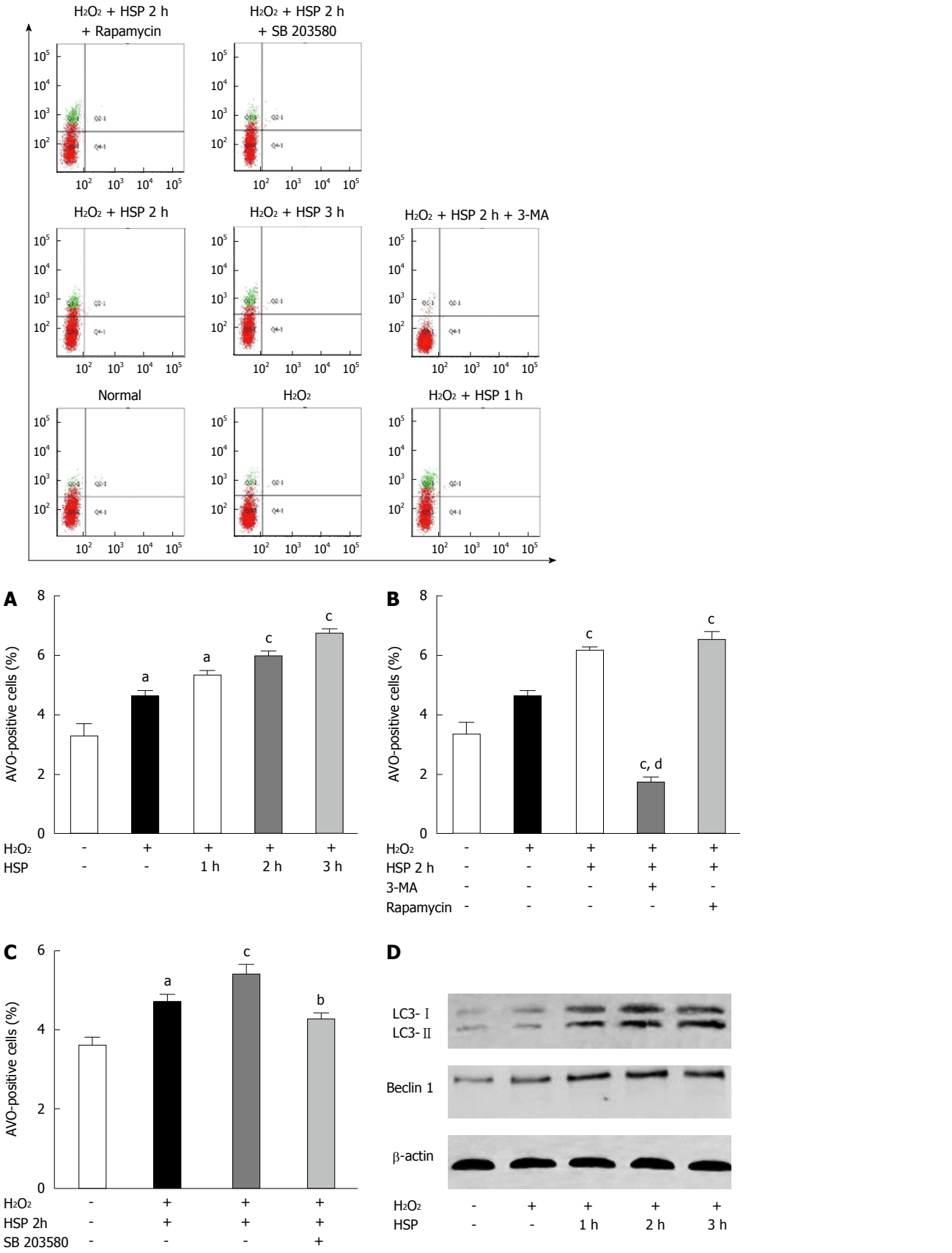


Figure 2 Autophagy was determined by acidic vesicular organelle-positive mesenchymal stem cells (labeled in the circle) using a fluorescent dye (AO) and flow cytometry. A: Different periods of heat shock pretreatment (HSP) ranging from 1 to 3 h led to a time-dependent increase in autophagy in mesenchymal stem cells (MSCs) exposed to H_2O_2, which peaked in the HSP_{3h} group; B: 3-MA attenuated, whereas rapamycin failed to further increase HSP-induced autophagy; C: SB203580 significantly suppressed HSP-induced autophagy in MSCs exposed to H_2O_2; D: Western blot showed a significant time-dependent increase in expression of the autophagic marker LC3B-Ⅱ and beclin 1 in MSCs. The data represent the results of three separate experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with the normal control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with the HSP_{2h} control group.

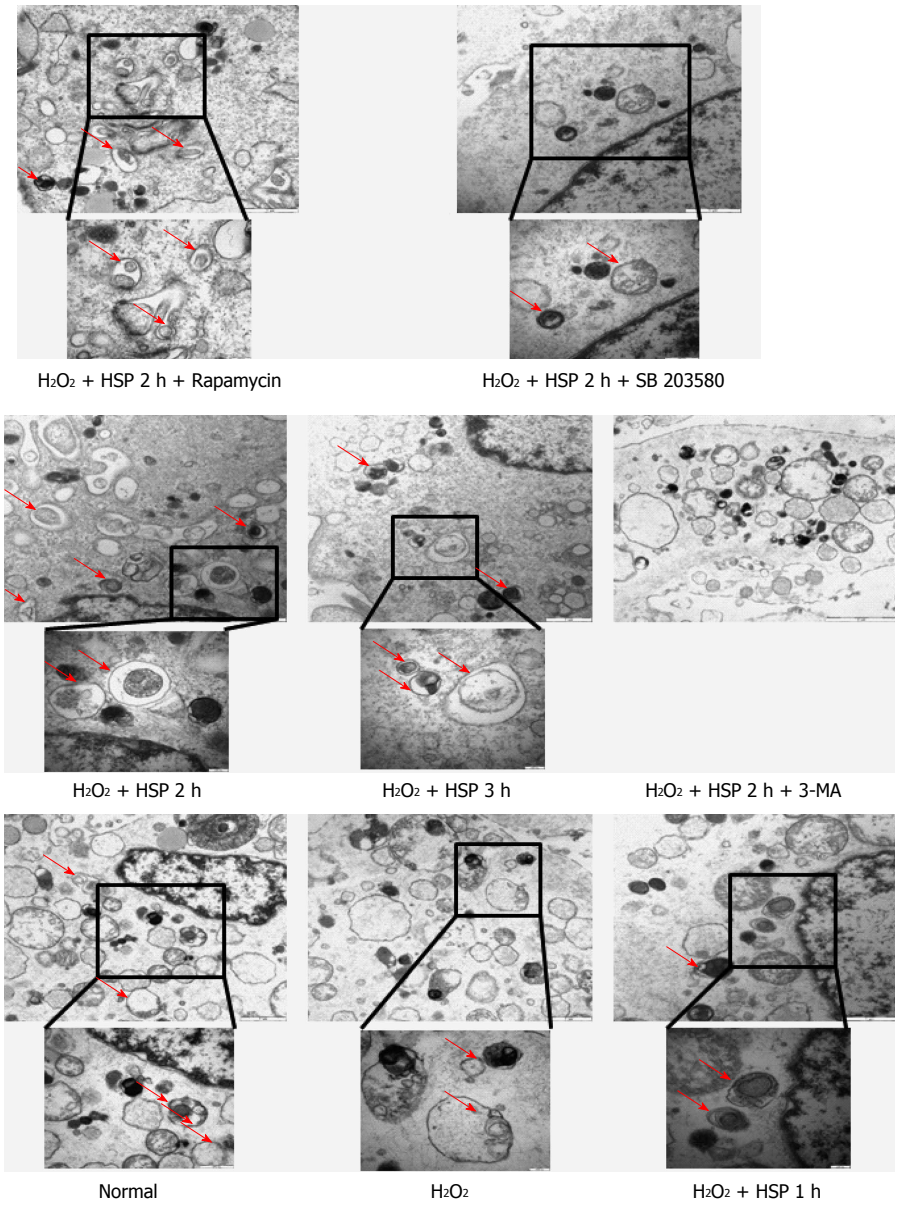


Figure 3 Representative electron micrographs demonstrating autophagic vacuole formation in each group. The arrows indicate the double-membrane vacuoles digesting organelles or cytosolic contents.

(Figure 5) and autophagosome formation (Figure 3). Furthermore, treatment with SB203580 abrogated the effects of p38MAPK phosphorylation, but failed to have any effect on the phosphorylation of mTOR. These data suggested that the p38MAPK/mTOR signaling pathway had a stimulatory role in the effects of HSP on MSC autophagy under H_2O_2 conditions.

HSP increased the homing and survival rate of transplanted MSCs to I/R livers in vivo

We then investigated the survival rate and homing of transplanted MSCs to livers. Representative fluorescence microscopic images of MSCs after transplantation are shown in Figure 6. CM-Dil-labeled cells were detected only in sections that received transplanted MSCs. The total number of double-positive MSCs labeled by CM-Dil and PCNA in the HSP-MSC-treated group was higher than that in the MSC-treated group ($P < 0.05$). CM-Dil-labeled MSCs also showed PCNA reactivity.

HSP improved the therapeutic potential of MSCs in the treatment of HIRI in rats

Twenty-four hours after MSC transplantation, liver function was assessed by serum AST and ALT levels. Compared with the control group, transplantation of MSCs improved liver function in rats. However, HSP-MSC-treated rats had lower AST and ALT levels compared with MSC-treated animals (Figure 7A; $P < 0.05$). A histological score was then assigned to the liver and the expression of PCNA was examined 24 h after transplantation. As expected, all I/R-induced livers showed sinusoidal congestion, cytoplasmic vacuolization and focal necrosis, which are indicative

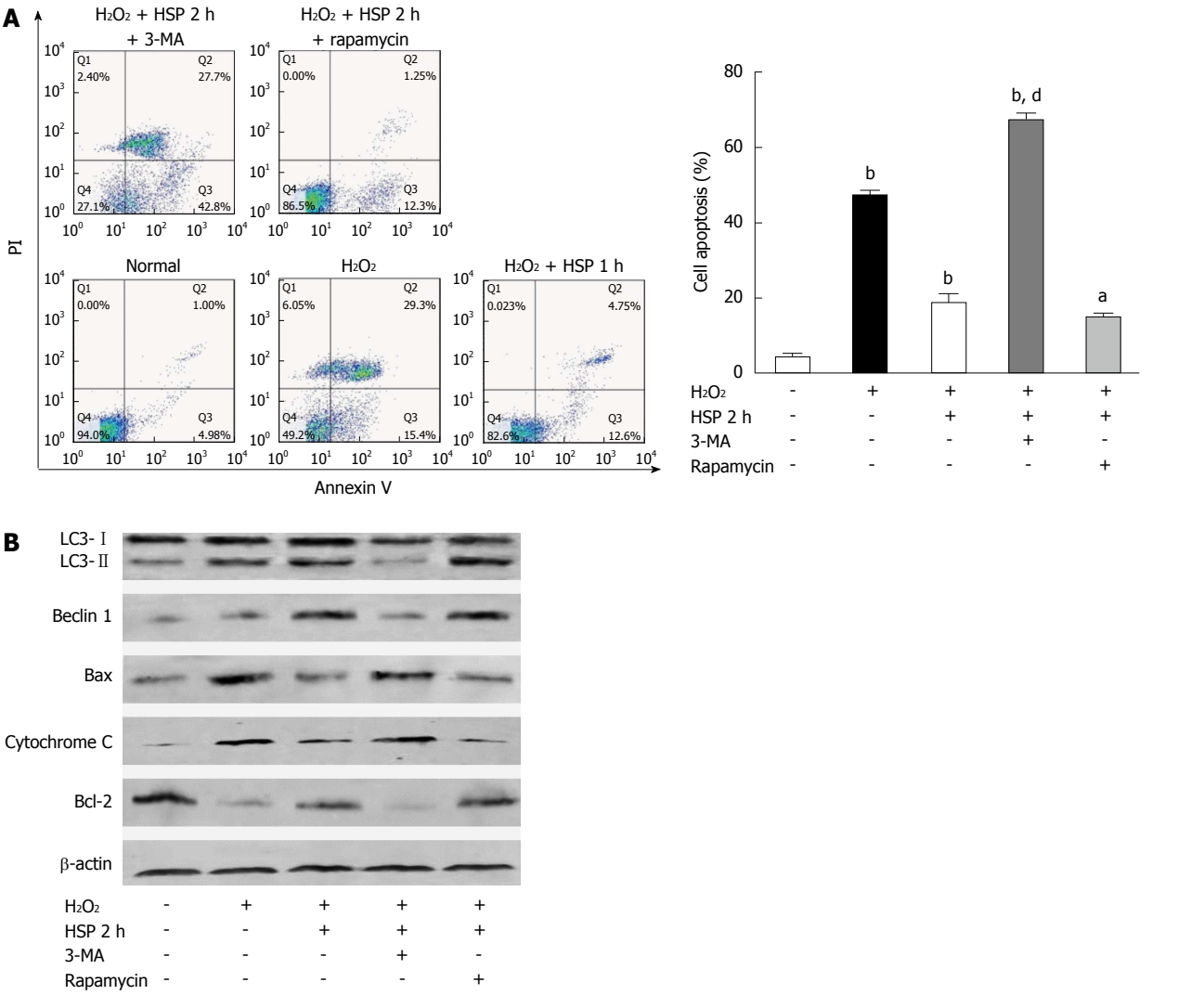


Figure 4 Inhibition of autophagy abrogated the effects of heat shock pretreatment on apoptosis reduction in mesenchymal stem cells exposed to H_2O_2 treatment. A: Flow cytometry indicated that 3-MA significantly increased the apoptotic rate in heat shock pretreatment (HSP)-treated mesenchymal stem cells; B: Western blot showed that 3-MA significantly increased Bax and cytochrome C expression, and decreased Bcl-2, LC3-Ⅱ and beclin 1 expression. The data represent the results of three separate experiments. $^aP < 0.05$, $^bP < 0.01$ compared with the control group; $^dP < 0.01$ compared with the HSP_{2h} group.

of severe damage. When compared with the I/R control group and the MSC-treated group, the HSP-MSC-treated group showed significantly improved histopathology and lower Suzuki scores 24 h after transplantation (Figure 7B). Moreover, compared with the I/R control group and PBS-treated rats, the livers from HSP-MSC-treated and MSC-treated rats showed a significantly increased number of PCNA-positive cells. Interestingly, the number of PCNA-positive cells in livers from HSP-MSC-treated rats was significantly increased compared with MSC-treated rats (Figure 7C; $P < 0.05$).

DISCUSSION

In the present study, we investigated the interaction between autophagy and apoptosis and the protective mechanism of autophagy activation by HSP in MSCs exposed to H_2O_2. Our results show that HSP for 2 h improves the therapeutic potential of MSCs in the treatment of HIRI in rats and enhances autophagy *via* the p38MAPK/mTOR pathway, which partly acted in the protective role of HSP on MSC apoptosis induced by H_2O_2. When administered systemically, more viable HSP-MSCs homed to the I/R liver compared with MSCs, which led to a significant improvement in liver function, an accelerated mitogenic response and alleviation of histopathological damage in the rat model.

In a previous study, we found that transplanted MSCs attenuated HIRI by suppressing oxidative stress and inhibiting apoptosis in rats[3]. However, the I/R microenvironment is detrimental to graft cells and induces cell death, thereby attenuating the therapeutic effect of stem cell transplantation[5-7]. Implanted MSCs must have a long life to ensure long-term MSC-based therapy in I/R tissues. It has been reported that short-term HSP can significantly improve

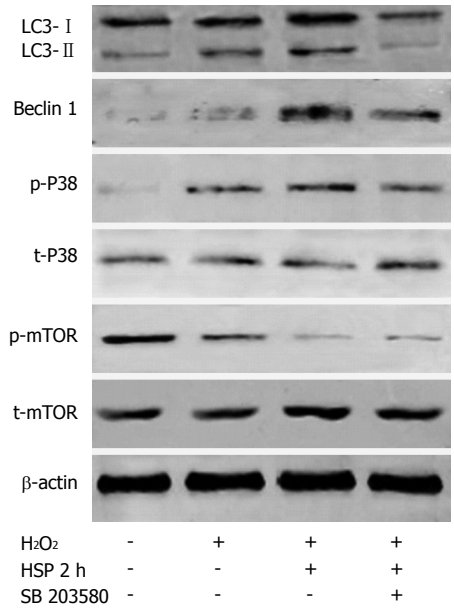


Figure 5 The p38MAPK/mTOR pathway was involved in autophagy activation by heat shock pretreatment in mesenchymal stem cells. Western blot showed that p-p38MAPK was upregulated and p-mTOR was downregulated by heat shock pretreatment (HSP) in mesenchymal stem cells. The p38MAPK inhibitor, SB203580, counteracted the effects of HSP on LC3-Ⅱ, beclin 1 and p-p38MAPK expression. The data represent the results of three separate experiments.

the viability of transplanted cells and thus enhance their tissue repairing capabilities in I/R tissue[10,11]. As H_2O_2 was previously shown to be a critical mediator of I/R-induced cell death[24,32], we induced a HIRI microenvironment by treating MSCs with H_2O_2 to investigate the function of HSP *in vitro*. We found that HSP for 2 h resulted in the most significant anti-apoptotic effects in MSCs exposed to H_2O_2 compared to the other groups. In addition, H_2O_2-induced apoptosis of MSCs was aggravated in the HSP_{3h} group (Figure 1). More importantly, exposure to HSP for 2 h before transplantation enhanced the survival rate and therapeutic outcome of MSCs *in vivo*. These data suggest that HSP at 42 ℃ for 2 h was the optimal period for improving the effect of MSCs transplantation in the repair of HIRI in rats. The HSP procedure is a simple method to improve implanted cell survival with little risk and can be performed not only in the liver, but also other organs.

Autophagy has been implicated in many processes, including cell differentiation, growth, development and survival[33]. Autophagy can be activated by various stresses involved in mediating cell survival or death[25]. In the present study, we found that HSP ranging from 1 to 3 h leads to a time-dependent increase in the action of autophagy in MSCs exposed to H_2O_2 (Figure 2A and D; Figure 3). In addition to the anti-apoptotic effect of HSP in MSCs, these findings suggest that autophagy induced by HSP for 2 h results in the most significant anti-apoptotic effect in MSCs exposed to H_2O_2. We therefore performed HSP for 2 h to examine the effect of H_2O_2-induced apoptosis and the protective effect of autophagy against apoptosis in MSCs. The protective effect of autophagy against apoptosis has previously been reported in models of I/R injury[34], including a model using H_2O_2. One well-established view is that appropriate autophagy is essential for cell survival[35]. More recently, Herberg *et al*[20] reported that the SDF-1/CXCR4 axis plays a key role in mediating MSC survival exposed to H_2O_2 by activating autophagy. Consistent with these results, our data show that the autophagy inhibitor, 3-MA, abrogates the anti-apoptotic effect observed in the HSP_{2h} group, and the autophagy inducer, rapamycin, does not reduce apoptosis of MSCs exposed to H_2O_2. These data suggest that moderate activation of autophagy mediated by HSP for 2 h may play a critical role in HSP to improve the survival of MSCs exposed to H_2O_2. It is known that autophagy is considered a double-edged sword in terms of cell survival. Moreover, we found that the activation of autophagy by HSP in MSCs is not paralleled by a corresponding increase in tolerance to H_2O_2-induced apoptosis. HSP for 1 and 2 h induced autophagy, which was an anti-apoptosis mechanism rather than a pro-apoptosis pathway in MSCs exposed to H_2O_2. Prolonged or excessive autophagy, which was mediated by HSP for 3 h, may digest essential components and lead to cell death. Thus, activation of autophagy may be a new mechanism in the process of HSP protecting MSCs from H_2O_2-induced apoptosis.

p38MAPK appears to have a dual role in that it has a positive or negative role in autophagy depending on conditions, cell type or type of cell stress[36-39]. In the present study, we assessed p38MAPK/mTOR pathway activation levels to determine the mechanisms underlying HSP-induced autophagy in MSCs exposed to H_2O_2. Interestingly, we found that HSP for 2 h increases p38MAPK activation and correspondingly alleviates mTOR activation. Moreover, p38MAPK inhibition abrogates autophagy induced by HSP for 2 h, but does not significantly impair mTOR suppression. In addition, our results indicate that treatment with rapamycin does not further induce autophagy of MSCs compared with HSP alone in the presence of H_2O_2, indicating that HSP may be involved in the same mechanism as rapamycin to activate autophagy in MSCs. These data suggest that the p38MAPK/mTOR signaling pathway may be involved in the mechanism of HSP-induced autophagy in MSCs exposed to H_2O_2.

To confirm the observations in the *in vitro* assay, we investigated the protective effect of HSP on MSCs *in vivo*. We determined the extent of MSCs localized in I/R livers of the recipient group by counting the number of CM-Dil fluorescent-labeled cells. It is well established that PCNA, which is synthesized in the cell nucleus, is a nuclear antigen related to the cell life cycle. PCNA is expressed in the G1 and S phases, and performs the essential function of providing replicative DNA polymerases in eukaryotic cells. The

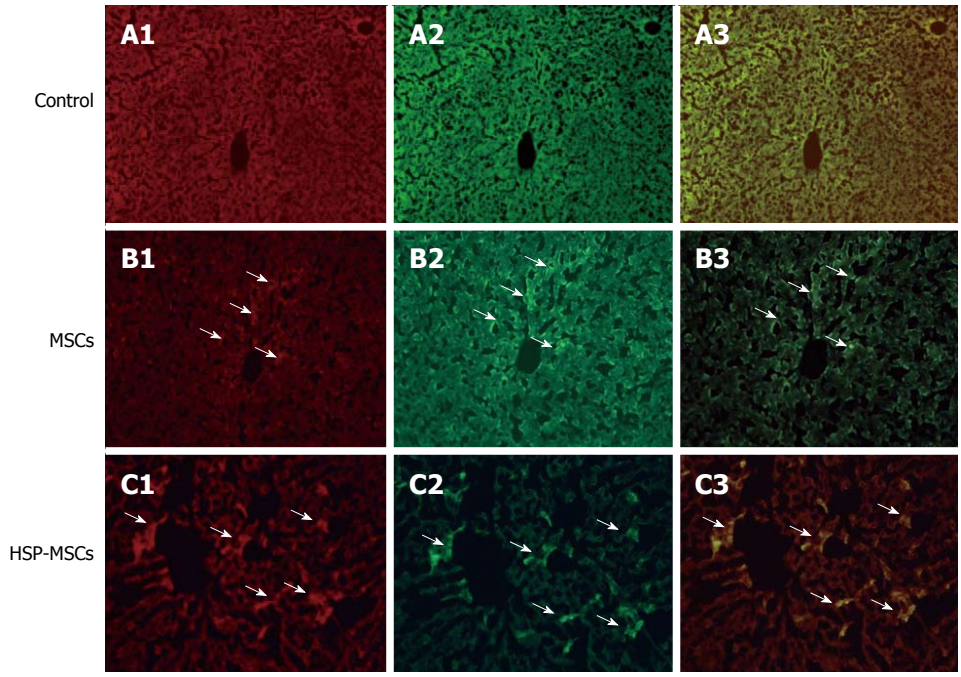
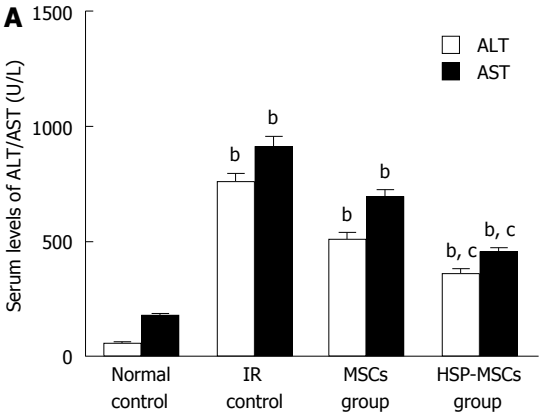
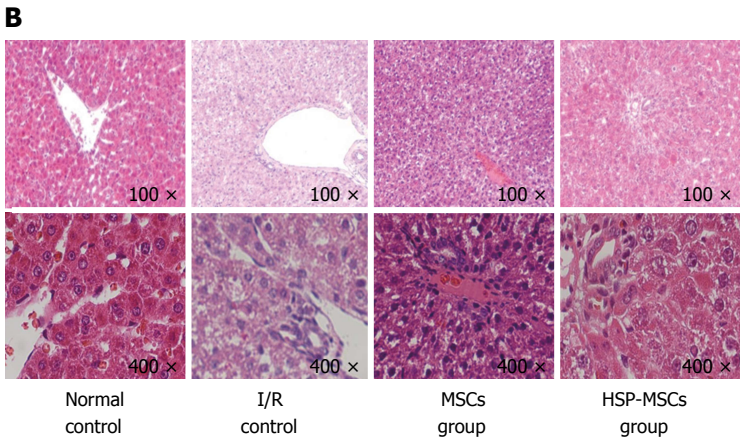
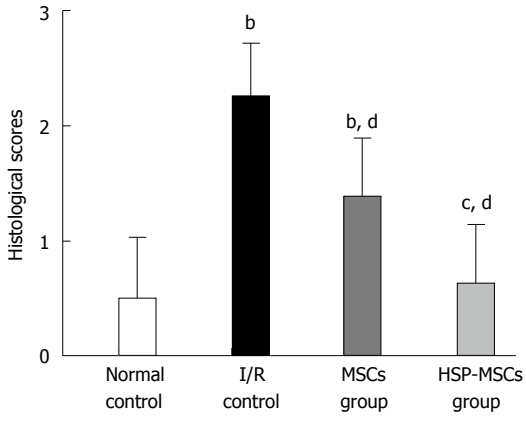


Figure 6 Heat shock pretreatment increases the homing and survival rate of transplanted mesenchymal stem cells in I/R livers *in vivo*. CM-Dil-labeled positive cells (red color, B1, C1), PCNA-conjugated with FITC (green color, B2, C2) and their co-localization (yellow color, B3, C3) were detected by immunofluorescence microscopy, respectively (magnification × 100). The total number of double-positive cells labeled by CM-Dil and PCNA in the heat shock pretreatment-mesenchymal stem cell (MSC)-treated group was higher than that in the MSC-treated group. The arrows indicate positive stained cells by CM-Dil, PCNA or their co-localization, respectively. The data represent the results of three separate experiments.







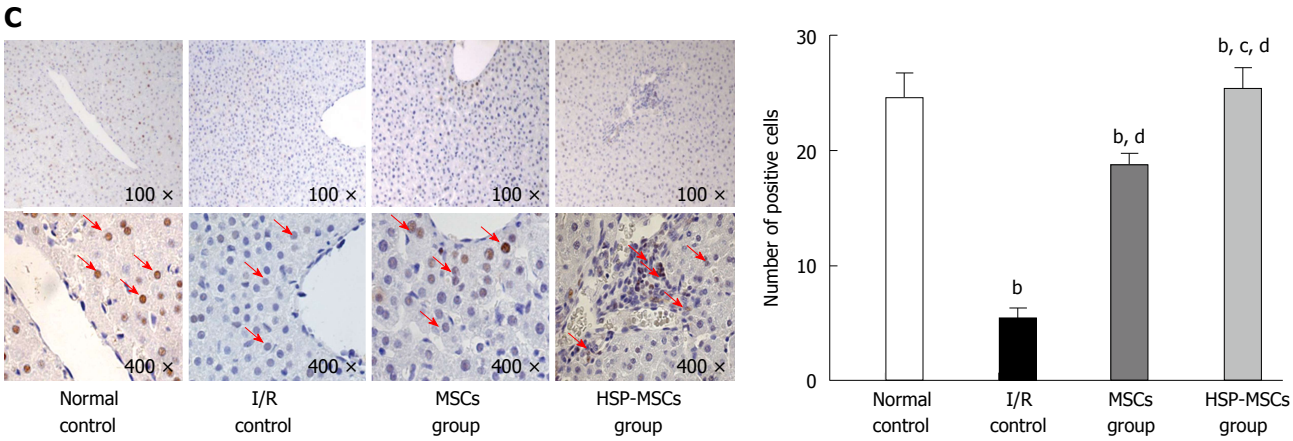


Figure 7 Heat shock pretreatment improves the therapeutic potential of mesenchymal stem cells in the treatment of hepatic ischemia-reperfusion injury *in vivo*. A: Serum aminotransferase levels were measured using an automatic analyzer following treatment; B: Histopathological analyses of livers from the normal control, I/R-control, mesenchymal stem cells (MSCs) and HSP-MSC groups. Liver tissue sections were stained with HE and scored according to the Suzuki Scoring System. Original magnification, × 100 and × 400, respectively, for each slide; C: Expression of PCNA by immunohistochemistry in liver tissues (magnification × 100 and × 400). The arrows indicate positive stained cells by PCNA. The data represent the results of three separate experiments. $^{b}P < 0.01$ compared with the normal control group; $^{d}P < 0.01$ compared with the I/R control group; $^{c}P < 0.05$ compared with the MSC group.

level of PCNA in resting cells is low, but is substantially increased in multiplying and transformed cells[40,41]. As shown in Figure 6, the HSP-MSCs group show more double-positive cells labeled by CM-Dil and PCNA than the MSCs group, which indicates that more HSP-MSCs subsequently underwent cell division and that HSP enhances the survival rate of transplanted MSCs in the liver. Furthermore, a marked decrease in serum aminotransferase levels, improved histopathology, lower Suzuki scores and an increased number of PCNA-positive cells in response to transplantation of HSP-MSCs were observed compared with the MSC group and the control group (Figure 7). These results indicate that HSP increases the homing and survival rate of transplanted MSCs, and thus improves the therapeutic potential of MSCs in the treatment of HIRI *in vivo*.

In summary, we found, for the first time, that HSP effectively enhances the homing and survival rate of MSCs, and thereby improves the therapeutic outcome of MSCs in the treatment of HIRI. The activation of autophagy *via* the p38MAPK/mTOR pathway may be a novel mechanism of HSP to improve the survival of MSCs exposed to H_2O_2. Activation of autophagy by HSP may be an attractive method of preventing apoptosis of MSCs and promoting their application in cellular therapies in regenerative medicine.

COMMENTS

Background

Mesenchymal stem cells (MSCs) exert a protective effect in hepatic ischemia-reperfusion injury (HIRI). However, due to local hypoxia, inflammation, and particularly oxidative stress in the targeted tissue, the transplanted MSCs do not withstand the difficult microenvironment due to ischemia-reperfusion (I/R) injury and low cell survival reduces the therapeutic effect. Autophagy is a complex "self-eating" process and can reduce apoptosis of MSCs exposed to H_2O_2. Heat shock pretreatment (HSP) is known to protect cells from various environmental insults and has been shown to induce autophagy in some cell lines. Previous studies show that HSP can regulate mitogen-activated protein kinase (p38MAPK), a positive modulator of autophagy in MSCs. Therefore, the authors designed this study to determine the role of HSP in autophagy activation *via* the p38MAPK/mTOR pathway to protect MSCs against apoptosis induced by oxidative stress injury.

Research frontiers

Autophagy is an evolutionarily conserved process that occurs in all eukaryotic cells. Evidence suggests that under hypoxia/serum deprivation (H/SD) conditions, autophagy can protect MSCs by providing energy or eliminating reactive oxygen species and damaged organelles, and can reduce apoptosis. In addition, several reports show that HSP increases survival rate following cell transplantation in the heart. However, it is unknown whether autophagy can be activated by HSP or its effect and exact mechanism in MSCs.

Innovations and breakthroughs

This study shows that activation of autophagy was a protective mechanism of HSP in MSCs. The results show that HSP for 2 h improves the therapeutic potential of MSCs in the treatment of HIRI in rats and enhances autophagy *via* the p38MAPK/mTOR pathway, which mediates, at least partly, the protective effects of HSP on MSC apoptosis exposed to H_2O_2. When administered systemically, more viable HSP-MSCs home to the I/R liver compared with MSCs, which leads to a significant improvement in liver function, an accelerated mitogenic response and the alleviation of histopathological damage in the rat model.

Applications

This study indicates that HSP effectively enhances MSCs homing and survival rate, and thus improves the therapeutic outcome of MSCs in the treatment of HIRI in rats. The activation of autophagy *via* the p38MAPK/mTOR pathway may be a novel mechanism of HSP to enhance the survival of MSCs exposed to H_2O_2. The regulation of autophagy by HSP may be an attractive strategy in preventing apoptosis of MSCs, thus promoting their application in cellular therapies in regenerative medicine.

Terminology

HIRI is an inevitable event and occurs in a number of clinical settings, including liver surgery, hemorrhagic shock with subsequent fluid resuscitation, sepsis, hepatic artery ligation, trauma, and some vascular lesions, and especially in

liver transplantation. Autophagy is an evolutionarily conserved process that occurs in all eukaryotic cells and is considered a double-edged sword in relation to cell survival. Heat shock pretreatment involves short-term exposure to mild hyperthermia that can significantly enhance cell tolerance and viability.

Peer-review

The work presented here is an interesting contribution that demonstrates the interaction of autophagy with apoptosis on MSCs under H_2O_2 conditions, and the activation of autophagy as a protective mechanism of HSP on MSCs.

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