

## Original Article

# Mesenchymal stem cells preconditioned with trimetazidine promote neovascularization of hearts under hypoxia/reoxygenation injury

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**Abstract:** Background: Cell-based angiogenesis is a promising treatment for ischemic diseases; however, survival of implanted cells is impaired by the ischemic microenvironment. In this study, mesenchymal stem cells (MSCs) for cell transplantation were preconditioned with trimetazidine (TMZ). We hypothesized that TMZ enhances the survival rate of MSCs under hypoxic stimuli through up-regulation of HIF1- $\alpha$ . Methods and results: Bone marrow-derived rat mesenchymal stem cells were preconditioned with 10  $\mu$ M TMZ for 6 h. TMZ preconditioning of MSCs remarkably increased cell viability and the expression of HIF1- $\alpha$  and Bcl-2, when cells were under hypoxia/reoxygenation (H/R) stimuli. But the protective effects of TMZ were abolished after knocking down of HIF-1 $\alpha$ . Three days after implantation of the cells into the peri-ischemic zone of rat myocardial ischemia-reperfusion (I/R) injury model, survival of the TMZ-preconditioned MSCs was high. Furthermore, capillary density and cardiac function were significantly better in the rats implanted with TMZ-preconditioned MSCs 28 days after cell injection. Conclusions: TMZ preconditioning increased the survival rate of MSCs, through up-regulation of HIF1- $\alpha$ , thus contributing to neovascularization and improved cardiac function of rats subjected to myocardial I/R injury.

**Keywords:** Angiogenesis, stem cells, neovascularization, ischemia, hypoxia/reoxygenation injury, trimetazidine

## Introduction

The phenomenon of exacerbated tissue and organ damage produced by the restoration of blood flow after ischemia is known as ischemia/reperfusion (I/R) injury. Studies have demonstrated that this phenomenon takes place in a variety of tissues and organs such as the brain, heart, liver, lungs and skin. Myocardial I/R injury is a pathophysiological phenomenon commonly seen after ischemic heart disease and heart surgery [1, 2]. Reducing and eliminating this damage has become a hot topic in the field.

Mesenchymal stem cells (MSCs), capable of self-renewal and differentiating into various mesenchymal tissues, have emerged as a promising tool for I/R injury treatment. In particular, the tissue repair functions of MSCs

could act to dampen the inflammation process and promote vascular supply during ischemia and reperfusion. However, the harsh ischemic and cytokine-rich microenvironment in the ischemic myocardium, infiltrated by the inflammatory and immune cells, offers a significant challenge to the transplanted donor stem cells. Massive cell death occurs during transplantation as well as following engraftment which significantly lowers the effectiveness of the heart cell therapy. Therefore, increasing the survival ratio of cells after transplantation into the ischemic microenvironment could be a feasible strategy for enhancing the therapeutic efficacy [3]. Various approaches have been adopted to overcome this problem nevertheless with multiple limitations in each of these current approaches. Cellular preconditioning by physical, chemical [4], genetic [5-7], and pharmacological [8] manipulation of the cells has shown

promise and “prime” the cells to the “state of readiness” to withstand the rigors of lethal ischemia post-transplantation.

Trimetazidine [1-(2,3,4-trimethoxybenzyl) piperazine; TMZ] is an anti-ischemic drug that modifies metabolic function without affecting the hemodynamic determinants of myocardial oxygen consumption (e.g., heart rate, systolic blood pressure, and rate-pressure product) [9]. TMZ optimizes the cardiac metabolism by reducing fatty acid oxidation through the selective inhibition of mitochondrial 3-ketoacyl CoA thiolase. As a result, TMZ attenuates the adverse effects of free fatty acid-associated oxidative stress [10], lessens oxygen demand by decreasing oxygen consumption [11], and improves mitochondrial metabolism and cardiac performance during ischemia [12]. TMZ has also shown cytoprotective efficacy in several models of myocardial infarction [13]. The pharmacological efficacy of TMZ in augmenting myocardial stem cell therapy has been reported in two research groups [14, 15]. They demonstrated that preconditioning of MSCs by TMZ before implantation offered a significant enhancement in the functional recovery of infarcted myocardium. However, the mechanism by which TMZ rescued MSCs in I/R injury in vitro is not clarified, also the protective effect of TMZ on MSCs and the neovascularization of ischemic hearts is not deeply investigated. In our study, we found that TMZ preconditioning increased the survival ratio of MSCs, through activated HIF1- $\alpha$ , thus contributing to neovascularization and improved cardiac function of rats subjected to myocardial I/R injury.

### Materials and methods

#### *Isolation and identification of mesenchymal stem cells*

Sprague Dawley (SD) rats were purchased from the Lab Animal Center of Soochow University (SuZhou, China). The procedures followed were in accordance with the ethical standards of the Ethic Committee of Soochow University. Bone marrow derived MSCs were isolated as previously described [9]. Briefly, the SD rats were euthanatized and bone marrow from tibias and femurs was flushed with PBS. Mononuclear cells were separated by density-gradient centrifugation with Ficoll-Paque™ (Amersham Biosciences, Uppsala Sweden). Cells were resuspended in DMEM (Gibco, USA) supple-

mented with 10% fetal bovine serum (FBS) and antibiotics. Non-adherent cells were removed after 48 hours, replacing the media every two to three days. The passaged cells were cultured by using standard protocols. An MSC passage was chosen from among the 3rd to 5th passage for all experimental uses. The morphological features and characteristic surface makers detected by flow cytometry were used to identify the MSCs as reported before.

#### *Isolation and culture of cardiomyocytes*

Neonatal cardiomyocytes are generally isolated from rats that are 1-3 days old. A number of hearts can be digested simultaneously to increase the myocyte yield. Animals are decapitated, hearts removed, atria excised, and the ventricles then minced in Hanks buffer. The solution of ventricular tissue is then transferred to a spinner bottle, and a collagenase type II enzyme solution (Worthington) added. The bottle is then spun at low speed for 20 minutes, at which point the enzyme solution containing cardiomyocytes and other cell types is removed from the tissue chunks and set aside. A new enzyme solution is then added to the tissue, and the procedure repeated 8 times. The collected enzyme-solution is next centrifuged, the supernatant discarded, and the cardiomyocyte fraction re-suspended in 10% FBS/L-DMEM. Further purification of cardiomyocytes is attained using differential adhesion method. Cells are then cultured in 10% FBS/L-DMEM and media changed every two to three days.

#### *In vitro hypoxia/reoxygenation treatment of cardiomyocytes and MSCs*

For hypoxic culture, cells were cultured in serum-free DMEM in a gas mixture composed of 94% N<sub>2</sub>, 5% CO<sub>2</sub>, and 1% O<sub>2</sub> for three hours and then transferred to normoxic culture for two hours. The H/R conditioned media was collected and the enzymatic activities of LDH, MDA and SOD in the H/R conditioned media were detected as instructed by the protocols of the manufactures. MSCs were preconditioned with 10  $\mu$ M TMZ for 6 h and then cultured in the H/R conditioned media for 12 h. Non-treated MSCs and normal conditioned media were used as the controls.

#### *Cell viability (mitochondrial activity) by CCK-8 assay*

The effect of TMZ on the mitochondrial activity of MSCs was determined by CCK-8 assay using

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a cell counting kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan). Ten thousand cells were cultured in each well of a 96-well plate for the assay. After TMZ treatment and co-culture with H/R conditioned media, the culture media of MSCs was removed and 100  $\mu$ L of fresh medium containing 10  $\mu$ L CCK-8 was added to each well with triplicate experiments for each set of conditions. The cells were then incubated at 37°C for 2 h. The absorbance at 450 nm was measured with a plate reader (Multiskan GO Microplate Spectrophotometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

### *HIF-1 $\alpha$ siRNA transfection and TMZ treatment of MSCs*

HIF-1 $\alpha$  siRNA and scrambled HIF-1 $\alpha$  siRNA sequences were synthesized from GenePharma (Suzhou, China). Cells were plated in 60-mm dishes 12 hours before transfection. The transfection of MSCs with HIF-1 $\alpha$  siRNA (200 pmol for each dish) was performed with Lipofectamine 2000 (Invitrogen) with a standard protocol. Medium was changed 6 hours after transfection. QRT-PCR was utilized to detect knockdown efficiency 24 h and 48 h after transfection. MSCs were further preconditioned with 10  $\mu$ M TMZ for 6 h and then cultured in the H/R conditioned media for 12 h. Non-treated MSCs and normal conditioned media were used as the controls.

### *Quantitative RT-PCR*

Total RNA was isolated from cell samples using RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using PrimeScript RT reagent Kit (TAKARA BIO Inc., Japan). For quantitative RT-PCR, the converted cDNA samples (2  $\mu$ l) were amplified in triplicate in a final volume of 10  $\mu$ l using SYBR Green Master Mix reagent (Applied Biosystems) and gene-specific primers with StepOne plus (Applied Biosystems, CA, USA). Melting curve analysis was performed with Dissociation Curves software (Applied Biosystems) and the mean cycle threshold (Ct) values were used to calculate gene expression levels with normalization to GAPDH. Forward (F) and reverse (R) primer sequences were as follows: Bcl-2 (F) 5'-GAACTGGGGGAGGATTGTGG-3' and (R) 5'-GGGGTGACATCTCCCTGTTG-3'; Bax (F) 5'-CTC-AAGGCCCTGTGCACTAA-3' and (R) 5'-TAGGAA-AGGAGGCCATCCCA-3'; HIF-1 $\alpha$  (F) 5'-CCGGCTCAAAGAAAACAGTCC-3' and (R) 5'-ATACCAAC-AGGATAGGCAGAACATT-3'; GAPDH (F) 5'-CAAGG-

TCATCCATGACAACCTTTG-3 and (R) 5'-GTCCACC-ACCCTGTTGCTGTAG-3'.

### *Western blot*

RIPA buffer which contains protease inhibitors (cOmplete, ULTRA, Mini, EDTA-free, EASYpack Roche, Germany) was applied to extract protein from cells samples and heart tissues and BCA method was used to detect protein concentration. Equal amount of proteins (20  $\mu$ g) were separated with 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Roche Germany). Membrane was put into 5% skimmed milk for blocking. The primary antibodies (HIF-1 $\alpha$ , 1:1000; Bcl-2, 1:1000; Bax, 1:1000; Abcam, Cambridge, MA, USA) was then added at 4°C overnight. The secondary antibodies were anti-rabbit and anti-mouse HRP-linked which was purchased from Beyotime (Nantong, Jiangsu, China). The blots were developed using ECL reagent (Biological Industries). The  $\beta$ -actin antibody was used to confirm equal amount of protein loading in each lane. The integrated density of the band was quantified by ImageJ software.

### *Ischemia-reperfusion in rats and echocardiographic measurements*

This study was carried out in a strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (NIH 1996). The eight-week-old Sprague Dawley (SD) rats were anesthetized abdominally by 2% pentobarbital sodium (40 mg/kg). A left thoracotomy was performed, and the left anterior descending coronary artery (LAD) was ligated with 6-0 silk suture  $\approx$ 4 mm from its origin with a slipknot. A successful performance of coronary occlusion was confirmed by regional cyanosis of the myocardial surface distal to the suture, accompanied by S-T segment elevation of more than 0.2 mv on the electrocardiogram (ECG). After 60 minutes, the LAD ligature was released and reperfusion was visually confirmed. For short-term tracking of transplanted cells, MSCs were labeled by CM-Dil (Introvigen, C7000, USA) before transplantation. To track the injected cells in vivo for 28 days, MSCs were transduced with GFP lentivirus at an MOI of 100 (LV3:H1/GFP&Puro, Suzhou, GenePharma). Rats were randomly divided into four groups for cell therapy, including 1) Sham with no I/R (Sham), 2) PBS-injected control group

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who had I/R (PBS), 3) MSCs-injected group after exposure to I/R (MSCs), and 4) TMZ-preconditioned-MSCs-injected group after exposure to I/R (TMZ-MSCs). Three hours after surgery, 2 ml of blood was collected from femoral vein of each rat and the enzymatic activities of LDH, MDA and SOD in the blood serum were detected. Transthoracic M-mode echocardiography measurements were conducted at baseline and at 4 weeks after MSC transplantation using a GE Vivid 7 ultrasound imaging system equipped with a 15-MHz linear array transducer. Rats were anesthetized with 2% isoflurane in air for the duration of the procedure. Heart size and shape were calculated using the M-mode and two dimensional short-axis image plane of the LV. Measurements were averaged from three cardiac cycles. The data were used to estimate percentage LV ejection fraction (EF) and fractional shortening (FS).

### *Tissue harvesting*

For cell survival studies, rat I/R hearts were harvested 24 hours after cell injection and prepared for frozen tissue sectioning after fixation with 4% PFA/PBS. The survival of implanted cells was identified by the number of CM-Dil-positive cells in the frozen sections (6  $\mu$ m in thickness) under fluorescence microscope, and fluorescence intensity was used to reflect the transplanted cell numbers. For cell differentiation studies, rats were anesthetized 28 days after surgery. Griffonia (Bandeiraea) Simplicifolia lectin 1 (Vector, 0.2 mg per rat) was then injected systemically by direct cardiac puncture. Ten minutes later, the animals were euthanized, and hearts were harvested and prepared for paraffin tissue sectioning after fixation with 4% PFA/PBS.

### *Immunofluorescence staining*

Sections were blocked in antibody dilution buffer 2% BSA/PBS for 1 hour at RT. After removal of the blocking solution, antibody to Griffonia (Bandeiraea) Simplicifolia lectin 1 (Vector, 1:100) was added and sections were kept at 4°C overnight. After washing three times with PBS for 5 min each, sections were incubated with Alexa Fluor 546 rabbit anti-goat IgG (MP/Invitrogen, 1:1000) for 30 min at RT. To detect arterioles, the sections were stained with anti- $\alpha$  smooth muscle actin (SMA) antibody (Dako, 1:250), then washed with PBS and stained with

Alexa Fluor 594 donkey anti-mouse IgG2a (MP/Invitrogen, 1:1000) at RT for one hour. After secondary antibodies were removed and sections were washed with PBS for three times, DAPI solution (Sigma, 1:5000) was added and nuclei were stained for 10 min at RT. Five non-overlapping 400 $\times$  magnification fields from four sections of each heart were randomly selected. The capillary density was averaged and expressed as the number of capillaries per unit area, and the data were finally statistically analyzed with SPSS.

### *TTC staining*

The hearts were perfused with 5% solution of Phthalo blue dye in normal saline over 3 minutes. The right ventricle of each heart was excised. The hearts were then frozen at -20°C for 20 minutes, followed by transverse sectioning into 2-mm slices. Sections were then incubated in 15 ml of 1.5% TTC for 20 minutes at 37°C. The sections were fixed in 10% formaldehyde. Twenty-four hours later, the slices were weighed and photographed. Color digital images of each transverse slice were obtained using a digital camera (Canon 640A). The blue regions represent non-ischemic normal tissue, red regions represent risk area (ischemic but non-infarcted), and unstained pale white regions represent infarct tissue. On each slice, the fraction of the LV area representing infarct-related tissue (average of 2 images) was multiplied by the weight of the section to determine the absolute weight of infarct-related tissue.

### *Statistical analyses*

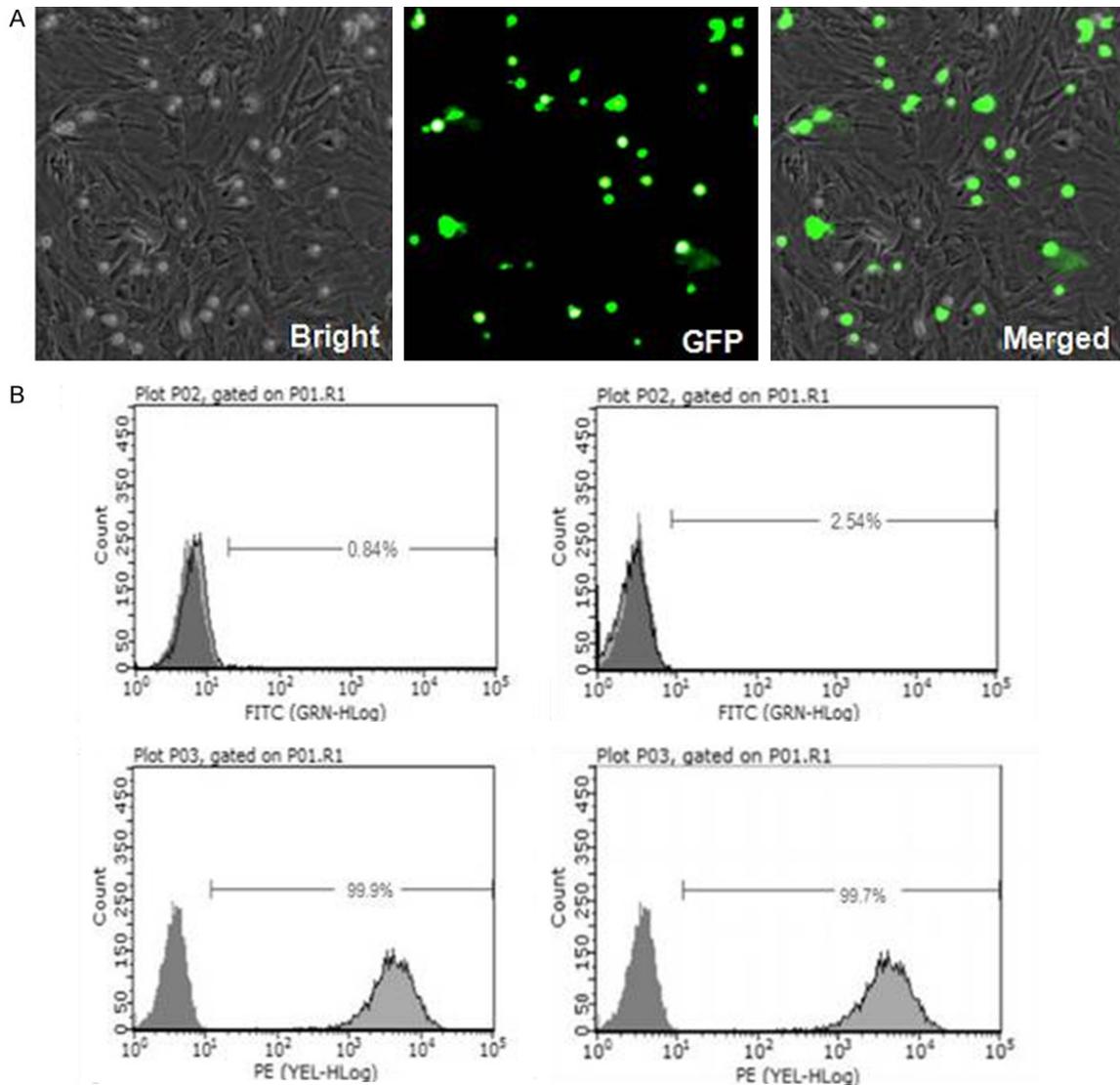
All the values are presented as mean  $\pm$  standard deviation. Results were compared by one-way analysis of variance (ANOVA) and least significant difference t test. Statistical analysis was performed using SPSS 19 software. A value of  $P < 0.05$  was considered to be statistically significant.

## **Results**

### *The characteristics of MSCs*

Density gradient centrifugation culture method was used in the isolation and cultivation of MSCs. The morphological features were observed and the characteristic surface markers were detected by flow cytometry. MSCs

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**Figure 1.** GFP lentivirus transduction of MSCs and expression of MSCs surface markers measured by FACS analysis. A. Growth morphology of GFP lentivirus-transduced MSCs. B. Representative plots of CD90, CD29, CD34 and CD45, respectively. Data are expressed as relative mean fluorescence intensity  $\pm$  SEM.

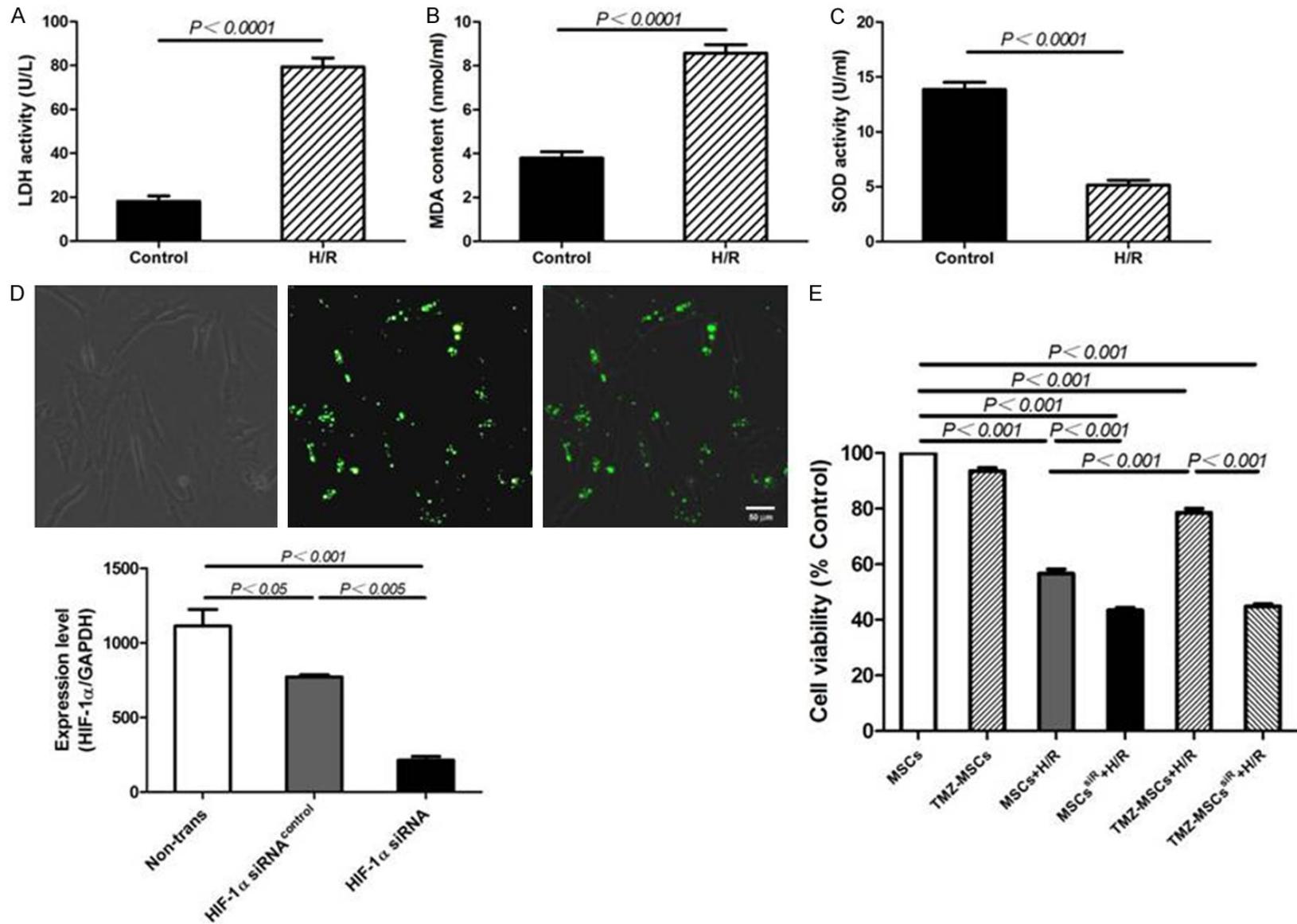
used for in vivo study were transduced with GFP lentivirus and about 70 to 80% of MSCs were GFP positive (**Figure 1A**). The MSCs were uniformly positive for CD90 (99.9%), CD29 (99.7%), CD44 (99.6%) and CD105 (99.0%), and negative for CD34 (0.84%) and CD45 (2.54%) (**Figure 1B**).

*TMZ preconditioning increases cell viability and up-regulates expression of survival genes in MSCs*

After hypoxic culture of neonatal cardiomyocytes for three hours and normoxic culture for

two hours, the H/R conditioned media were collected and the enzymatic activities of LDH and SOD and the MDA content in the media were detected. In the H/R conditioned media, the enzymatic activities of LDH were significantly increased compared with those in the control media (H/R:  $79.3 \pm 4.1$  vs control:  $18.2 \pm 2.4$ ,  $P < 0.0001$ ) (**Figure 2A**), and similar tendency was observed for the MDA content (H/R:  $8.6 \pm 1.1$  vs control:  $3.8 \pm 0.8$ ,  $P < 0.0001$ ) (**Figure 2B**), while the activity of SOD was decreased compared with that in the control media (H/R:  $5.1 \pm 1.3$  vs control:  $13.9 \pm 1.8$ ,  $P < 0.0001$ ) (**Figure 2C**). MSCs were transfected

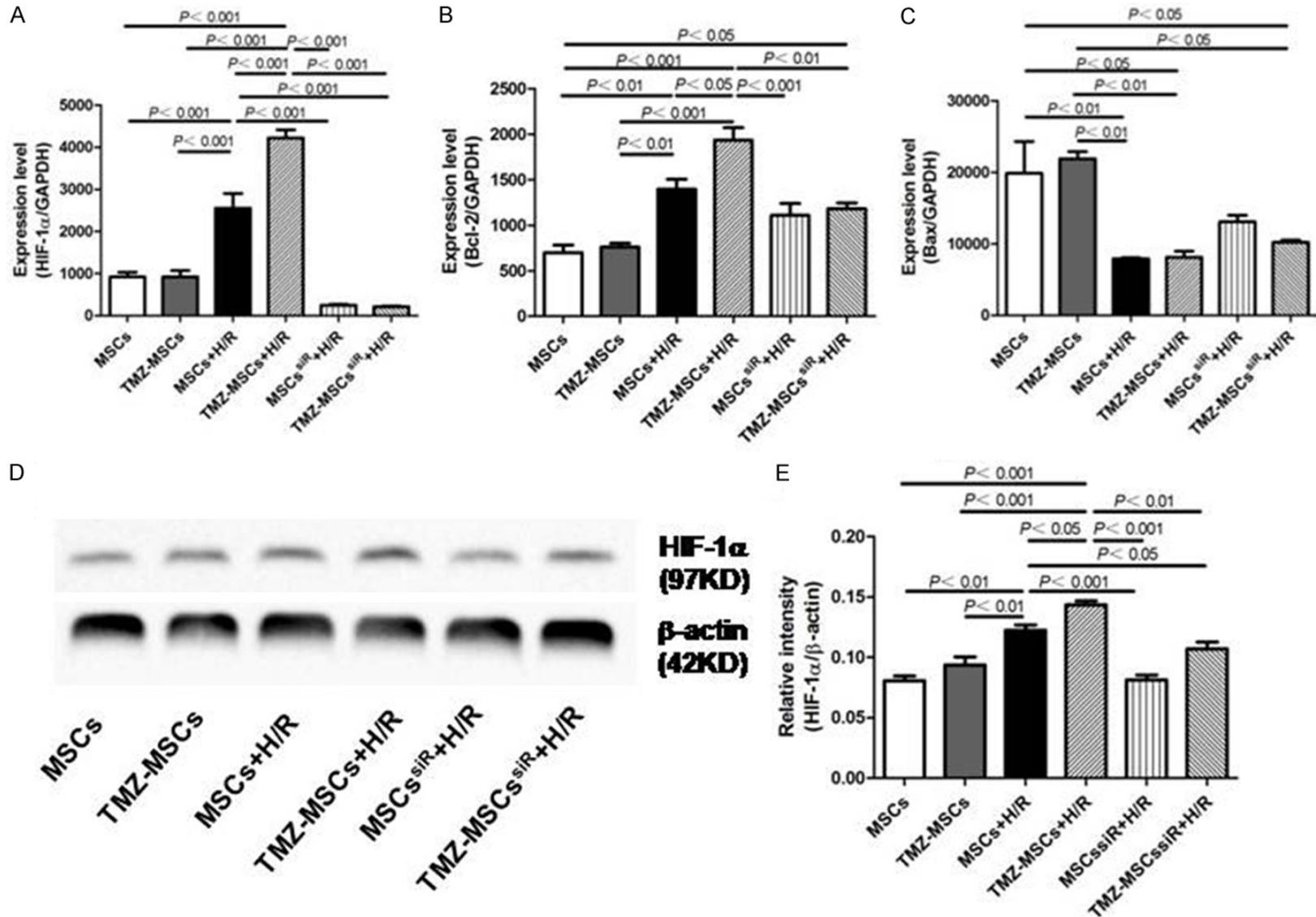
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**Figure 2.** LDH release, MDA content and SOD activity of cardiomyocytes exposed to H/R, HIF-1α siRNA transfection of MSCs and the effect of TMZ on the viability of MSCs cultured in the H/R conditioned media of cardiomyocytes. LDH release (A), MDA content (B) and SOD activity (C) were evaluated in the conditioned media of cardiomyocytes after exposure to hypoxia for 3 hours and normoxia for 2 hours. \* $P < 0.001$  vs control group. (D) The transfection efficiency of HIF-1α siRNA. The

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expression level of HIF-1 $\alpha$  was inhibited to 20% by siRNA knocking down (0.19 $\pm$ 0.04,  $P < 0.001$ ). (E) The cell viability in the control group was normalized to 100%. The values represent the mean (% of control)  $\pm$  SEM of each group in this experiment. MSCs vs MSCs+H/R, MSCs<sup>siR</sup>+H/R, TMZ-MSCs+H/R and TMZ-MSCs<sup>siR</sup>+H/R,  $P < 0.001$ ; MSCs+H/R vs MSCs<sup>siR</sup>+H/R and TMZ-MSCs+H/R,  $P < 0.001$ ; TMZ-MSCs+H/R vs TMZ-MSCs<sup>siR</sup>+H/R,  $P < 0.001$ .



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**Figure 3.** Assessment of survival genes in MSCs cultured in the H/R conditioned media of cardiomyocytes by real time qPCR and Western blot. Following transfection with HIF-1 $\alpha$  siRNA for 48 h, MSCs were preconditioned with 10  $\mu$ M TMZ for 6 h and then cultured in the H/R conditioned media for 12 h. Non-treated MSCs and normal conditioned media were used as the controls. A-C. The mRNA expression levels of HIF-1 $\alpha$ , Bcl-2 and Bax in MSCs were assessed by real-time quantitative PCR. The mRNA expressions were normalized to GAPDH (n=3). The values represent the mean (% of control)  $\pm$  SEM of each group in this experiment. All assays were triplicated and demonstrated similar results. MSCs vs MSCs+H/R and TMZ-MSCs+H/R,  $P<0.001$ ; TMZ-MSCs vs MSCs+H/R and TMZ-MSCs+H/R,  $P<0.001$ ; MSCs+H/R vs MSCs<sup>siR</sup>+H/R, TMZ-MSCs+H/R and TMZ-MSCs<sup>siR</sup>+H/R,  $P<0.001$ ; TMZ-MSCs+H/R vs MSCs<sup>siR</sup>+H/R and TMZ-MSCs<sup>siR</sup>+H/R,  $P<0.001$ . D and E. The protein levels of HIF-1 $\alpha$  were assessed by western blot. The protein intensities were normalized to b-actin (n=3). MSCs vs MSCs+H/R and TMZ-MSCs+H/R,  $P<0.01$ ; TMZ-MSCs vs MSCs+H/R and TMZ-MSCs+H/R,  $P<0.001$ ; MSCs+H/R vs MSCs<sup>siR</sup>+H/R, TMZ-MSCs+H/R vs TMZ-MSCs<sup>siR</sup>+H/R,  $P<0.001$ ; TMZ-MSCs+H/R vs MSCs+H/R,  $P<0.05$ .

with HIF-1 $\alpha$  siRNA for 48 h (Suzhou, GenePharma). For TMZ preconditioning, MSCs were treated with 10  $\mu$ M of TMZ for 6 h, which was determined from a preliminary dose/time study. MSCs with or without TMZ treatment were cultured in the conditioned media of cardiomyocytes for 10 hours. Six groups were set: MSCs cultured in the control media, TMZ-MSCs cultured in the control media, MSCs cultured in the H/R media, MSCs<sup>siR</sup> cultured in the H/R media, TMZ-preconditioned-MSCs cultured in the H/R media and TMZ-preconditioned-MSCs<sup>siR</sup> cultured in the H/R media. CCK-8 assay was then performed to detect the cell viability of the six groups of cells. MSCs cultured in the H/R media had a significantly decreased survival rate compared with MSCs cultured in the control media (MSCs+H/R: 56.7 $\pm$ 4.4% vs TMZ-MSCs+control: 93.5 $\pm$ 3.4% and MSCs+control: 100%,  $P<0.001$ ) (**Figure 2E**), while this phenomenon was dramatically rescued when MSCs were pre-treated with TMZ (TMZ-MSCs+H/R: 78.5 $\pm$ 4.3% vs MSCs+H/R: 56.7 $\pm$ 4.4%,  $P<0.001$ ) (**Figure 2E**). But, if we inhibited HIF-1 $\alpha$  prior to TMZ preconditioning with the knocking down efficiency of 30% (**Figure 2D**), the protective effect of TMZ on MSCs in the H/R media was abolished (MSCs<sup>siR</sup>+H/R: 43.3 $\pm$ 2.6% vs MSCs+H/R: 56.7 $\pm$ 4.4%,  $P<0.001$ ; TMZ-MSCs<sup>siR</sup>+H/R: 44.8 $\pm$ 2.4% vs TMZ-MSCs+H/R: 78.5 $\pm$ 4.3%,  $P<0.001$ ) (**Figure 2E**). We further investigated the expression levels of some key survival proteins, including Bcl-2 and Bax by qPCR. HIF-1 $\alpha$  expression was markedly increased in cells cultured in the H/R media ( $P<0.001$ ). And pretreatment with TMZ further resulted in a significant increase in HIF-1 $\alpha$  expression compared with MSCs cultured in the H/R media ( $P<0.001$ ) (**Figure 3A**). But the increase was obviously inhibited by HIF-1 $\alpha$  siRNA transfection ( $P<0.001$ ). The same tendency was confirmed by western blot (**Figure 3D**). Moreover, in cells exposed to TMZ before culture in the H/R media, there was an enhance-

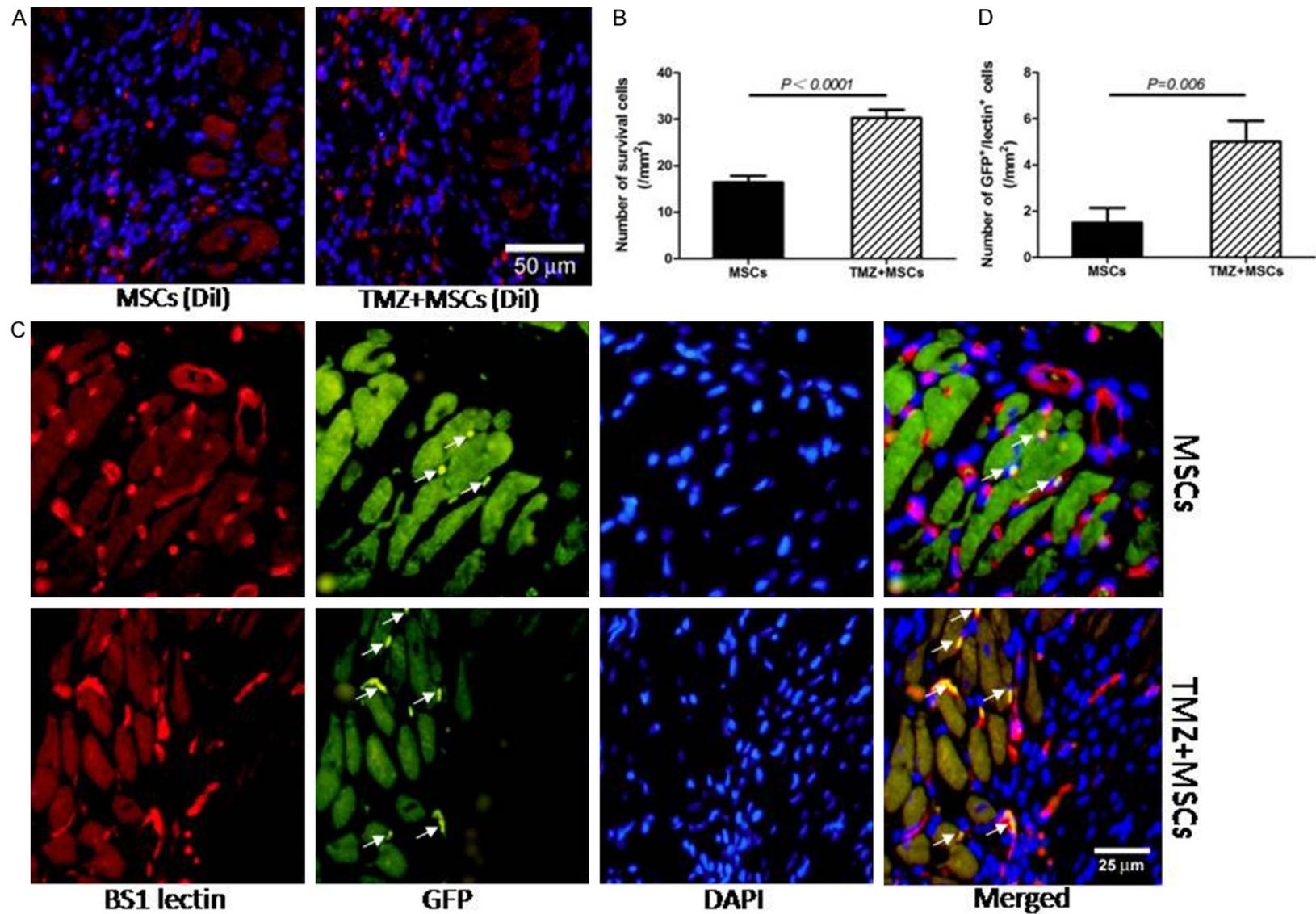
ment of Bcl-2 expression and decreased Bax expression comparing to MSCs cultured in the control media ( $P<0.05$ ) (**Figure 3B, 3C**). After knocking down of HIF-1 $\alpha$ , the expression level of Bcl-2 went up a little and the expression level of Bax went down a little in MSCs cultured in the H/R media in comparison with MSCs in the control media (**Figure 3B, 3C**), suggesting that other anti-apoptotic pathways may exist in the absence of HIF-1 $\alpha$ -dependent pathway when cells are subjected to hypoxic stimulation. However, we could not detect the significant variation of Bcl-2 and Bax at the protein levels.

*Enhanced cell survival, cell differentiation, capillary density and reduced infarction in the ischemia/reperfusion hearts due to TMZ preconditioning of MSCs*

For short-term tracking of transplanted cells, MSCs were labeled by CM-Dil and then injected around the I/R zone during the surgery. The enzymatic activities of LDH, MDA and SOD in the blood of rats subjected to I/R surgery and cell transplantation were shown in [Supplemental Figure 1](#). Heart samples were harvested three days after cell injection and examined for the presence of fluorescent-labeled cells. Dil-positive cells were observed in the ischemic myocardium of cell-transplanted groups (**Figure 4A**). And MSCs survived more by TMZ preconditioning (MSCs: 16.4 $\pm$ 4.2/mm<sup>2</sup> vs TMZ+MSCs: 30.3 $\pm$ 4.9/mm<sup>2</sup>;  $P<0.0001$ ) (**Figure 4B**).

To track the injected cells in vivo for 28 days, MSCs were transduced with GFP lentivirus at an MOI of 100. Four weeks after cell injection, the animals were anesthetized. Griffonia (Bandeiraea) Simplicifolia lectin 1 was injected systemically by direct cardiac puncture. Ten minutes later, hearts were harvested and prepared for paraffin tissue sectioning. GFP-positive cells were observed in the ischemic border zone and co-localized with vessels stained with anti-BS1 lectin (**Figure 4C**), which

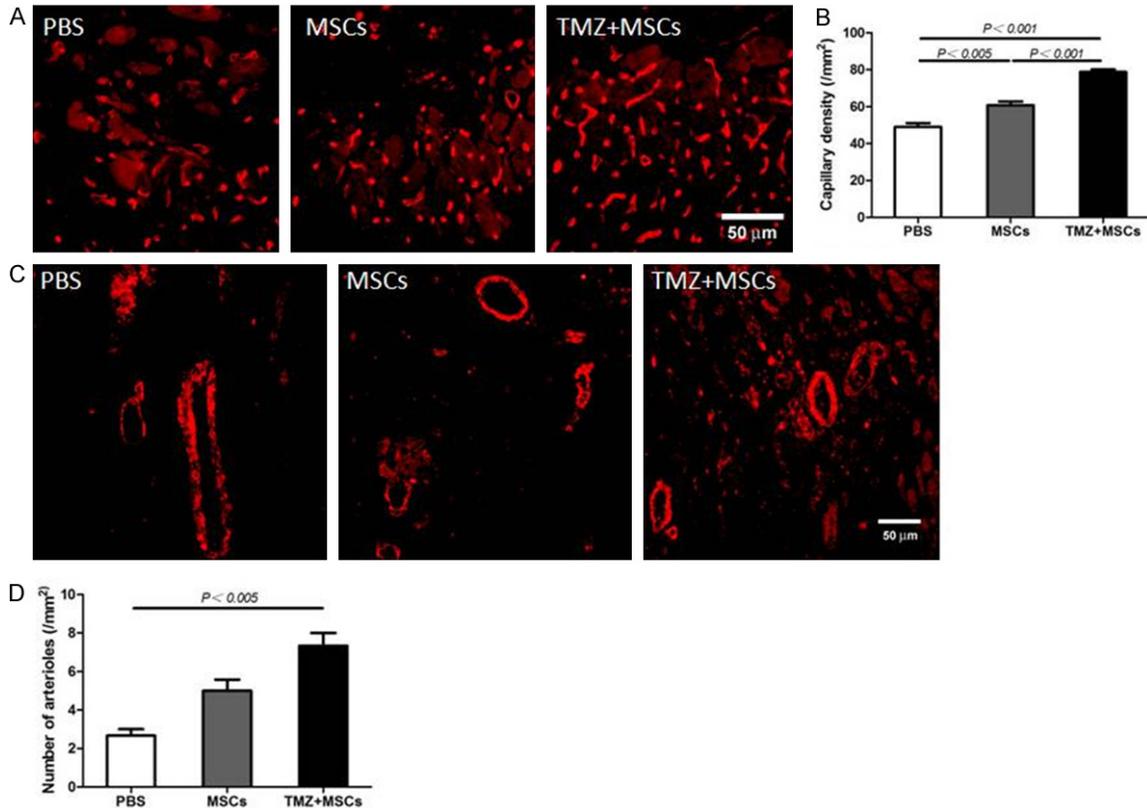
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**Figure 4.** Assessment of cell recruitment and cell differentiation in the ischemia/reperfusion hearts. MSCs were preconditioned with 10  $\mu$ M TMZ for 6 h and stained with Dil. Dil-labeled cells were injected into the peri-infarct zone during the I/R surgery, and heart samples were examined histologically three days after cell injection. A. Dil positive cells (red) detected in ischemic myocardium under a fluorescence microscope. B. Number of Dil positive cells was counted in the ischemic

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border zone (bilateral sides of peri-infarct area) and averaged ( $n=3$ ). MSCs vs TMZ+MSCs,  $P<0.0001$ . MSCs were transduced with GFP lentivirus, and then preconditioned with  $10\ \mu\text{M}$  TMZ for 6 h. The cells were injected into the peri-infarct zone during the I/R surgery, and heart samples were examined histologically 28 days after cell injection. C. Capillaries were visualized by immunofluorescent staining for BS1 lectin (red). Cells co-localizing with capillaries were marked by arrows and shown in yellow. D. The numbers of cells co-localized with capillaries were counted under a fluorescence microscope separately and averaged. Arrows indicate the double positive cells. MSCs vs TMZ+MSCs,  $P=0.006$ .



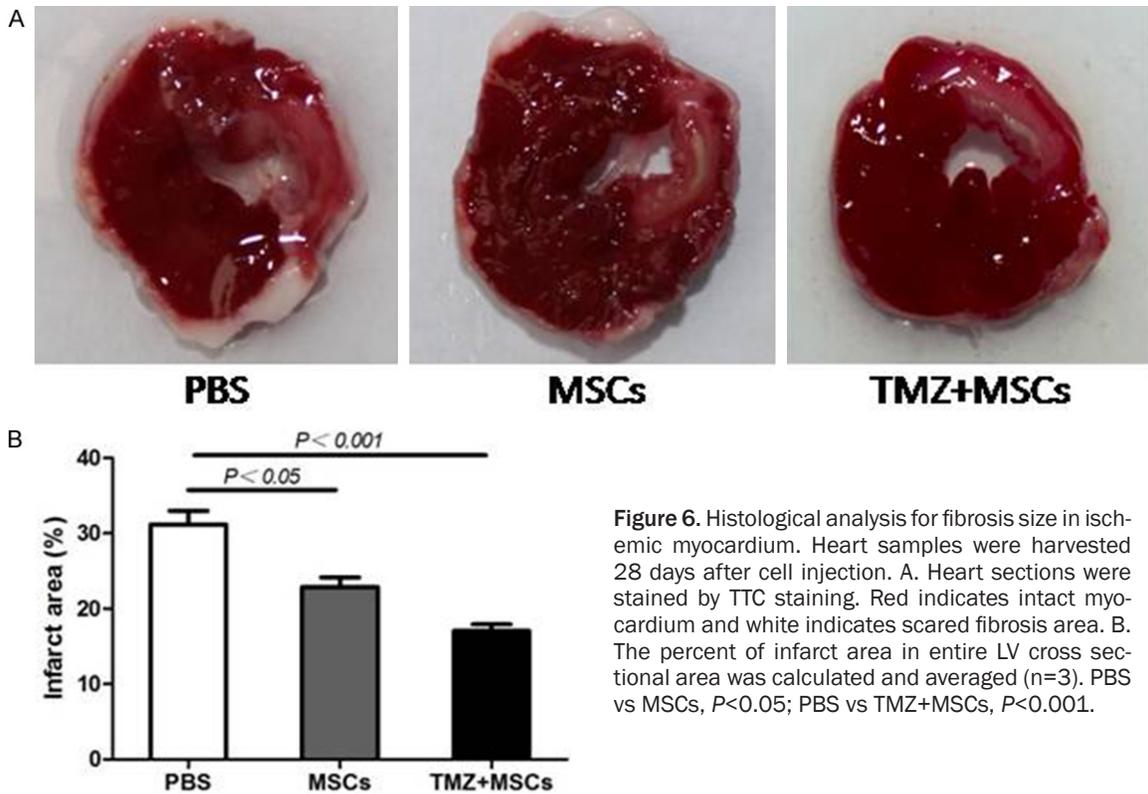
**Figure 5.** Histological analysis for capillary density and arterioles in ischemic myocardium. Heart samples were harvested following BS1 lectin systemic perfusion 28 days after cell injection. A. Capillaries were visualized as tubular structure perfused by BS1 lectin. B. The numbers of capillaries were counted in bilateral sides of the peri-infarct zone on LV cross sections and averaged in each group ( $n=3$ ), respectively. PBS vs MSCs and TMZ+MSCs,  $P<0.005$ ; MSCs vs TMZ+MSCs,  $P<0.001$ . C. Arterioles were visualized following  $\alpha$ -smooth muscle actin staining. D. The numbers of arterioles were counted in bilateral sides of the peri-infarct zone on LV cross sections and averaged in each group ( $n=3$ ), respectively. PBS vs TMZ+MSCs,  $P<0.005$ .

indicated the endothelial differentiation of MSCs. Hearts injected with MSCs by TMZ preconditioning had higher number of GFP<sup>+</sup>/BS1 lectin<sup>+</sup> cells than those injected with non-treated MSCs (TMZ+MSCs:  $5 \pm 1.8$  vs MSCs:  $1.5 \pm 1.3$ ,  $P<0.05$ ) (Figure 4D). The staining of in vivo-perfused BS1 lectin and  $\alpha$ -smooth muscle actin reflects angiogenesis in functional vessels in the peri-infarct myocardium four weeks after I/R in all groups (Figure 5). The averaged capillary density in the ischemic border zones of LV, an index of neovascularization, was significantly greater in the TMZ-preconditioned-MSCs-injected group compared to the MSCs-injected

group (TMZ+MSCs:  $78 \pm 3$  vs MSCs:  $61 \pm 4$  and PBS:  $49 \pm 4$ ,  $P<0.001$ ) (Figure 5B). Although the averaged number of arterioles was higher in the group with TMZ-preconditioned-MSCs injection, no significant difference could be found between the groups with TMZ-preconditioned-MSCs injection and MSCs injection (TMZ+MSCs:  $7 \pm 1$  vs MSCs:  $5 \pm 1$  and PBS:  $2.7 \pm 0.5$ ,  $P<0.005$ ) (Figure 5D).

### *Reduced fibrosis in the ischemia/reperfusion hearts due to TMZ preconditioning of MSCs*

Heart sections stained with TTC showed extensive infarction in the PBS-injected hearts



**Figure 6.** Histological analysis for fibrosis size in ischemic myocardium. Heart samples were harvested 28 days after cell injection. A. Heart sections were stained by TTC staining. Red indicates intact myocardium and white indicates scarred fibrosis area. B. The percent of infarct area in entire LV cross sectional area was calculated and averaged (n=3). PBS vs MSCs,  $P < 0.05$ ; PBS vs TMZ+MSCs,  $P < 0.001$ .

(**Figure 6A**). The extent of infarction was significantly reduced in the MSCs group compared with the PBS group (MSCs:  $22.9 \pm 2.2\%$  vs PBS:  $31.2 \pm 3.2\%$ ,  $P < 0.05$ ) (**Figure 6B**). The hearts that received MSCs pretreated with TMZ showed significantly further reduction of infarction compared with the PBS group (TMZ+MSCs:  $17.0 \pm 1.5\%$  vs PBS:  $31.2 \pm 3.2\%$ ,  $P < 0.001$ ) (**Figure 6B**).

*Over-expression of survival proteins in the infarct hearts transplanted with preconditioned MSCs*

To clarify whether MSCs-transplantation promoted survival of the infarcted myocardium, we dissected the LV area of the infarct hearts and performed western blot analysis. In western blot analysis, HIF-1 $\alpha$  and Bcl-2 dramatically over-expressed in hearts implanted with TMZ-treated MSCs compared with those implanted with MSCs and PBS ( $P < 0.001$ ), whereas the expression level of Bax was decreased due to the injection of TMZ-MSCs ( $P < 0.05$ ) (**Figure 7A-D**).

*Preconditioned MSCs effectively preserve LV function after ischemia/reperfusion*

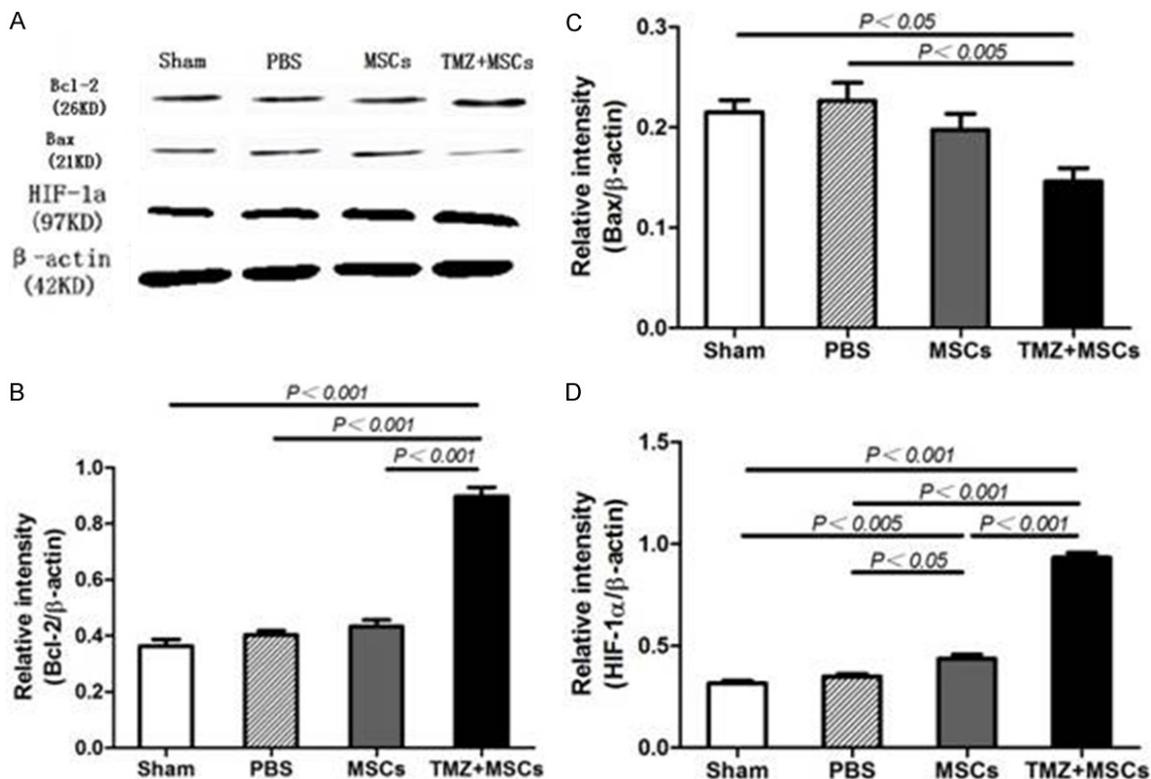
Echocardiography performed four weeks after ischemia/reperfusion revealed significantly

higher values of LVEF and FS in the MSCs group as compared to the PBS group (**Figure 8**) and the preconditioned MSCs further attenuated LV remodeling. LVEF was  $54.7 \pm 2.3\%$  in the PBS group,  $63.1 \pm 2.0\%$  in the MSCs group and  $70.7 \pm 2.8\%$  in the TMZ+MSCs group (n=9,  $P < 0.001$  vs PBS and MSCs groups) (**Figure 8B, 8C**). Similarly, FS was  $26.2 \pm 1.5\%$  in the PBS group,  $31.0 \pm 2.7\%$  in the MSCs group and  $35.2 \pm 1.7\%$  in the TMZ+MSCs group (n=9,  $P < 0.05$  vs PBS and MSCs groups) (**Figure 8B, 8C**).

**Discussion**

The low survival ratio of transplanted stem cells becomes a hurdle for their clinical use. Besides the time point and route of transplantation, cell death in the acute phase is assumed to be the critical factor that restricts the protective effects of MSCs. Genetic modification and preconditioning of MSCs are the most popular and effective ways to promote the survival and keep the function of MSCs. Whether permanent genetic modification could cause tumor or not remains an unsolved question. On the contrary, preconditioning of stem cells could enhance the transplantation efficiency in a fast, simple and effective way. And pharmacological preconditioning of stem cells becomes a new

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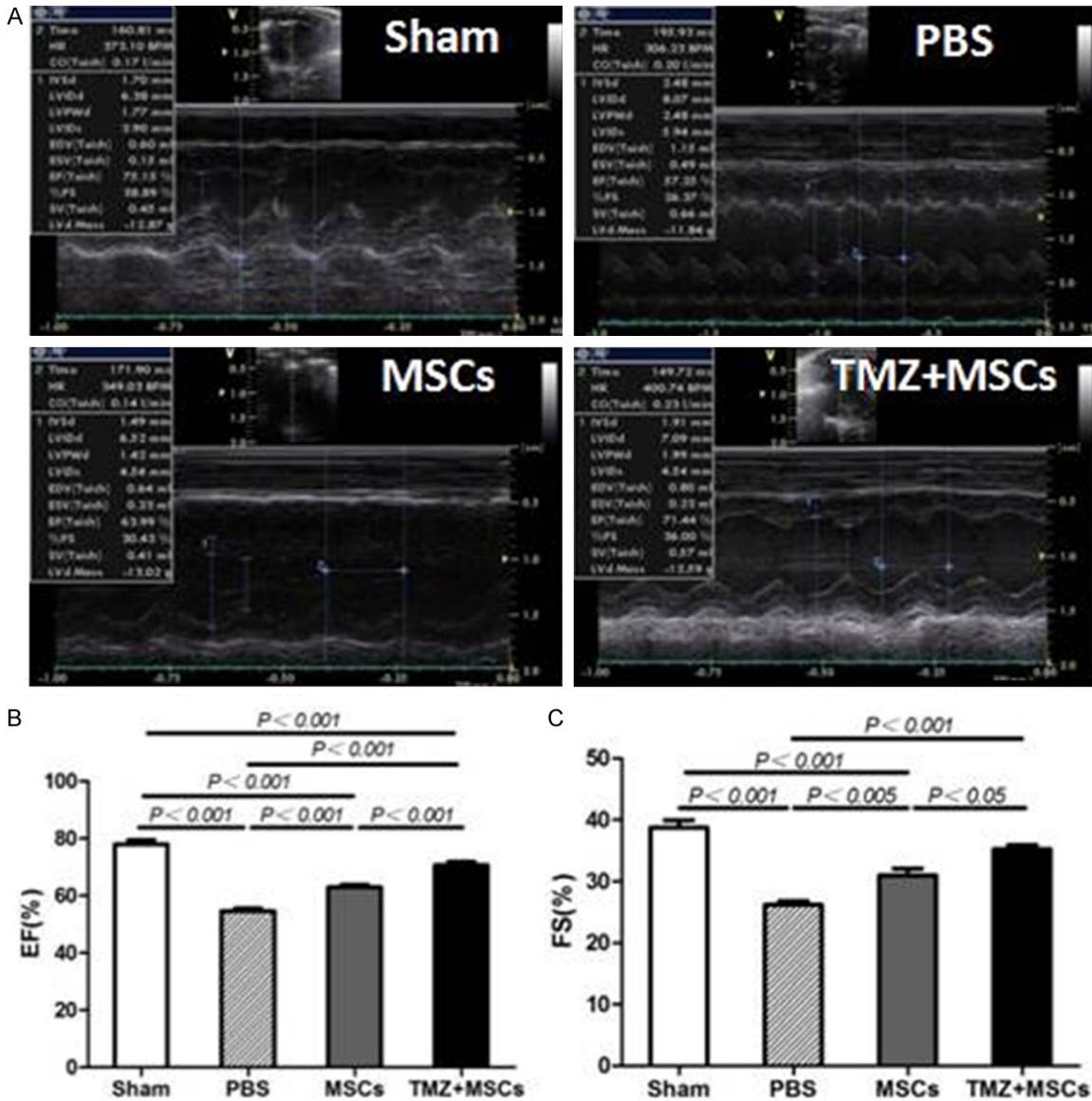


**Figure 7.** Analysis of hypoxia and survival proteins expressed by the ischemia/reperfusion hearts. The LV area of the infarct hearts was dissected 28 days after cell transplantation and western blot analysis was performed. A. Representative blots of HIF-1 $\alpha$ , Bcl-2 and Bax are shown. B-D. Quantitative analysis of HIF-1 $\alpha$ , Bcl-2 and Bax using data obtained from three different blots and expressed as mean  $\pm$  S.D. HIF-1 $\alpha$ , MSCs vs Sham and PBS,  $P < 0.05$ ; TMZ+MSCs vs Sham, PBS and MSCs,  $P < 0.001$ . Bcl-2, TMZ+MSCs vs Sham, PBS and MSCs,  $P < 0.001$ . Bax, TMZ+MSCs vs Sham and PBS,  $P < 0.05$ .

promising method for functional maintenance. Trimetazidine (TMZ) is a partial inhibitor of lipid oxidation. It has been proposed as a metabolic regulator for several cardiovascular pathologies. TMZ has also shown cytoprotective efficacy in several models of myocardial infarction [13]. The pharmacological efficacy of TMZ in augmenting myocardial stem cell therapy has been reported in two research groups [14, 15]. They demonstrated that preconditioning of MSCs by TMZ before implantation offered a significant enhancement in the functional recovery of infarcted myocardium. However, the mechanism by which TMZ rescued MSCs in I/R injury in vitro is not clarified, also the protective effect of TMZ on MSCs and the neovascularization of ischemic hearts is not deeply investigated. Therefore, we evaluated the protective role of TMZ precondition on stem cells for transplantation.

Hypoxia-inducible factor-1 (HIF-1), the most important factor involved in the cellular

response to hypoxia, has been extensively studied. HIF-1 is specifically activated in hypoxia and induces target genes involved in cell proliferation, vascular development [16], vascular tone, and energy metabolism [17]. The broad impact of HIF-1 on cell biology is that, among all hypoxia target genes, over 70 have so far been identified as being regulated by HIF-1 [18]. HIF-1 directly activates the transcription of the VEGF gene by binding to a hypoxia response element located 50 to the gene [19]. Reports by Kim *et al.* and Papandreou *et al.* demonstrate that HIF-1 performs an active suppression of mitochondrial pyruvate catabolism and O<sub>2</sub> consumption in hypoxic cells [20, 21], while TMZ could optimize the cell metabolism by reducing fatty acid oxidation through the selective inhibition of mitochondrial 3-ketoacyl CoA thiolase. Therefore, it is very likely that TMZ plays the role in a HIF-1 $\alpha$ -dependent way. We proposed that TMZ could recover the function of MSCs exposed to ischemia/reperfusion injury through up-regulation of HIF-1 $\alpha$ .



**Figure 8.** Recovery of cardiac function at 4 weeks after MSCs transplantation in the ischemia/reperfusion hearts. Transthoracic echocardiography was performed in the Sham group, the PBS-injected group, I/R hearts treated with MSCs (MSCs), and I/R hearts treated with MSCs preconditioned with TMZ (TMZ+MSCs). Representative recordings of M-mode echocardiogram (A), LV ejection fraction (EF) (B), and fraction shortening (FS) (C) are shown. I/R hearts treated with TMZ-preconditioned cells had greater functional recovery than those treated with non-preconditioned cells. EF, PBS vs Sham,  $P < 0.001$ ; MSCs vs Sham and PBS,  $P < 0.001$ ; TMZ+MSCs vs Sham, PBS and MSCs,  $P < 0.001$ . FS, PBS vs Sham,  $P < 0.001$ ; MSCs vs Sham and PBS,  $P < 0.005$ ; TMZ+MSCs vs PBS and MSCs,  $P < 0.05$ .

In our study, we used  $10 \mu\text{M}$  of TMZ to treat MSCs for 6 hours. We found that short term exposure of MSCs to TMZ can significantly enhance cell viability in the H/R conditioned media. But, if we knocked down HIF-1 $\alpha$  prior to TMZ preconditioning, surprisingly, this effect could be abolished (Figure 2D). The Bcl-2 family can be divided into three classes: BH3-only proteins that are activated by various forms of

cellular stress, Bax and Bak proteins that mediate mitochondrial membrane permeabilization, and inhibitory proteins such as Bcl-2 and Bcl-XL [22]. Certain members of the BCL-2 protein family, such as Bcl-2, Bcl-xl and Mcl-1 are anti-apoptotic, whilst others like Bax are pro-apoptotic. Bcl-2 is specifically considered as an important anti-apoptotic protein and is thus classified as an oncogene. Apoptosis regulator

BAX promotes apoptosis by binding to and antagonizing the Bcl-2 protein [23]. Therefore, we further checked Bcl-2 and Bax expression in MSCs exposed to the H/R media by qPCR. There was a concomitant increase of Bcl-2 expression and a decrease of Bax expression when cells were cultured in the H/R media following TMZ preconditioning. But after knocking down of HIF-1 $\alpha$ , the expression level of Bcl-2 also went up a little and the expression level of Bax went down a little in MSCs cultured in the H/R media in comparison with MSCs in the control media, suggesting that other anti-apoptotic pathways may exist in the absence of HIF-1 $\alpha$ -dependent pathway when cells are subjected to hypoxic stimulation. The exact interaction among HIF-1 $\alpha$ , Bcl-2 and Bax needs further study. As for the in vivo study, TMZ preconditioning of MSCs could enhance cell survival and capillary density following transplantation into the ischemic myocardium, thus contributing to the reduced fibrosis and the preserved LV function after ischemia/reperfusion.

In conclusion, TMZ-treated MSCs transplantation exhibited a significant improvement of cardiac function with reduced infarct size following ischemia/reperfusion compared with non-treated MSCs transplantation. The favorable effect of TMZ-treatment to MSCs could be attributed to increased cell survival in ischemic myocardium. This simple and effective treatment to MSCs might be a promising strategy for autologous cell therapy in patients with ischemic heart diseases.

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### Disclosure of conflict of interest

None.

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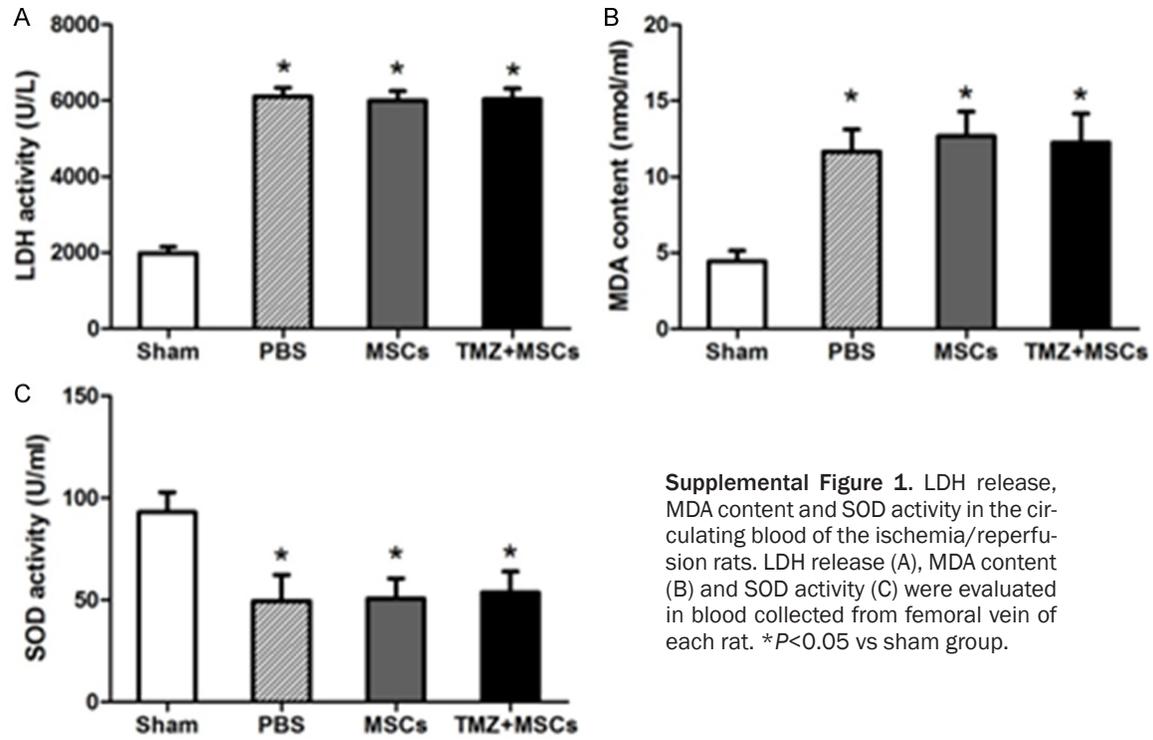
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**Supplemental Figure 1.** LDH release, MDA content and SOD activity in the circulating blood of the ischemia/reperfusion rats. LDH release (A), MDA content (B) and SOD activity (C) were evaluated in blood collected from femoral vein of each rat. \* $P < 0.05$  vs sham group.