Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis

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Objectives: This study explored the novel strategy of hypoxic preconditioning of bone marrow mesenchymal stem cells before transplantation into the infarcted heart to promote their survival and therapeutic potential of mesenchymal stem cell transplantation after myocardial ischemia.

Methods: Mesenchymal stem cells from green fluorescent protein transgenic mice were cultured under normoxic or hypoxic (0.5% oxygen for 24 hours) conditions. Expression of growth factors and anti-apoptotic genes were examined by immunoblot. Normoxic or hypoxic stem cells were intramyocardially injected into the peri-infarct region of rats 30 minutes after permanent myocardial infarction. Death of mesenchymal stem cells was assessed in vitro and in vivo after transplantation. Angiogenesis, infarct size, and heart function were measured 6 weeks after transplantation.

Results: Hypoxic preconditioning increased expression of pro-survival and pro-angiogenic factors including hypoxia-inducible factor 1, angiopoietin-1, vascular endothelial growth factor and its receptor, Flk-1, erythropoietin, Bcl-2, and Bcl-xL. Cell death of hypoxic stem cells and caspase-3 activation in these cells were significantly lower compared with that in normoxic stem cells both in vitro and in vivo. Transplantation of hypoxic versus normoxic mesenchymal stem cells after myocardial infarction resulted in an increase in angiogenesis, as well as enhanced morphologic and functional benefits of stem cell therapy.

Conclusions: Hypoxic preconditioning enhances the capacity of mesenchymal stem cells to repair infarcted myocardium, attributable to reduced cell death and apoptosis of implanted cells, increased angiogenesis/vascularization, and paracrine effects.

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Myocardial infarction (MI) induces the irreversible loss of cardiomyocytes, scar formation, and may ultimately result in congestive heart failure.¹ Bone marrow mesenchymal stem cells (MSCs) are multipotent adult stem cells² and able to differentiate into endothelial cells, vascular smooth muscle cells, and perhaps even cardiac-like myocytes when transplanted into the ischemic heart.³ Both animal and clinical studies have provided evidence that MSC transplantation can improve cardiac function through possible myogenesis and angiogenesis after MI.^{4,5} Some studies, however, have failed to observe the therapeutic effects of MSC transplantation^{6,7}; thus, there is a need for further investigation into the use of MSCs and the improvement of transplantation techniques after MI.

A major dilemma in stem cell therapy for ischemic heart diseases is the low survival of transplanted cells in the ischemic and peri-infarcted region.^{6,8} Most implanted cells may die within 4 days after transplantation into the ischemic heart.⁹ Endogenous and environmental factors, such as the inflammatory response, may contribute to cell death. Thus, improving grafted cell survival after transplantation is critical for enhancing the efficacy and efficiency of stem cell therapy.

Abbreviations and Acronyms

dp/dt	= rate of pressure rise
EPO	= erythropoietin
EPOR	= cognate receptor of erythropoietin
GFP	= green fluorescent protein
HIF-1 α	= hypoxia-inducible factor-1 α
H-MSC	= hypoxic mesenchymal stem cell
HP	= hypoxic preconditioning
LVEDP	= left ventricular end-diastolic pressure
LVSP	= left ventricular systolic pressure
MI	= myocardial infarction
MSC	= mesenchymal stem cell
N-MSC	= normoxic mesenchymal stem cell
PBS	= phosphate-buffered saline solution
TUNEL	= terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling
VEGF	= vascular endothelial growth factor

Angiogenesis remains one of the putative mechanisms in cardiac functional recovery after MI and stem cell transplantation.¹⁰ Stimulating angiogenesis showed therapeutic effects in ischemic heart disease.¹¹ Transplanted MSCs can stimulate angiogenesis after MI by secreting multiple angiogenic cytokines and differentiating into endothelial cells.¹² It can be reasoned that enhancing the MSC's ability to promote angiogenesis will further increase the therapeutic potential of MSC transplantation after MI.

Hypoxic preconditioning (HP) by sublethal hypoxic insult stimulates endogenous mechanisms resulting in multiple responses including protein expressions that protect against future lethal hypoxia and other insults. HP can decrease apoptosis of neurons through induction of hypoxia-inducible factor-1 α (HIF-1 α)¹³ and protect myocytes from hypoxia and reperfusion injury.¹⁴ HP stimulates myocardial angiogenesis to an extent sufficient to exert significant cardioprotection after MI.¹⁵

Cell transplantation therapy and HP have been studied as separate research topics. We¹⁶ recently demonstrated that in vitro HP of embryonic stem cells significantly increased their survival and tissue repair capabilities after transplantation into the ischemic brain. On the basis of the well-documented manifold benefits of HP, we examined the hypothesis that HP of cultured MSCs would promote their survival in vitro as well as after transplantation. Furthermore, enhanced survival and trophic support would contribute to endothelial differentiation and stimulate angiogenesis. The HP strategy for stem cell transplantation would ultimately benefit functional recovery after MI.

Materials and Methods**MSC Culture**

Bone marrow MSCs were isolated and harvested as previously described.¹⁷ In brief, MSCs were acquired from the femoral and tibial bones of green fluorescent protein (GFP) transgenic mice. MSCs

were flushed from the femurs and tibias of GFP mice using a 25-gauge needle. Mononuclear cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and plated in flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 24 hours, nonadherent cells were discarded, and adherent cells were washed three times with phosphate-buffered saline solution (PBS). Fresh complete medium was added and replaced every 4 days. Each primary culture was subcultured 1:2 when MSCs grew to approximately 80% confluence. To confirm the cellular identity of cultured cells, we subjected MSCs to fluorescence-activated cell sorting using CD90, CD34, and CD45 markers, and cultured cells were identified as CD90+ and CD34-/CD45- cells.

Sublethal Hypoxia Protocol

For HP treatment, cells were subcultured 1:2 and cultured for 3 days until confluent. Fresh complete medium was added before hypoxia. Hypoxia treatment was achieved with a well-characterized, finely controlled ProOx-C-chamber system (Biospherix, Redfield, NY) for 24 hours. The oxygen concentration in the chamber was maintained at 0.5%, with a residual gas mixture composed of 5% carbon dioxide and balanced nitrogen.

Cell Death Assays in Vitro

Cell death was assayed using trypan blue staining as described previously.¹⁸

Western Blot Analysis

Immunoblot was applied to determine the effect of HP on the expression of growth factors and antiapoptotic genes in MSCs. Cultured cells were lysed with modified radioimmunoprecipitation assay buffer (50 mmol/L HEPES, pH 7.3, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, and protease inhibitor cocktail [Roche, Nutley, NJ]) for 30 minutes, followed by centrifugation at 14,000g for 30 minutes. Protein concentration of each sample was determined with the Bicinchoninic Acid Assay (Sigma Chemical Co, St Louis, Mo). Then, 40 μ g of protein per sample was electrophoresed on a 6%- to 15%-gradient gel by sodium dodecylsulfate-polyacrylamide gel electrophoresis in a Hoefer Mini-Gel system (Amersham Biosciences, Piscataway, NJ) and transferred in a Hoefer Transfer Tank (Amersham Biosciences) to polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif). Membranes were blocked with 7% milk in Tris-buffered saline and 0.2% Tween at room temperature for 2 hours and were then incubated overnight at 4°C with specific primary antibodies. The blots were washed three times with Tris-buffered saline and 0.2% Tween and incubated with alkaline phosphatase-conjugated secondary antibodies for 2 hours at room temperature. The expression signals were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution (Sigma).

Rat Model of MI

Male Wistar rats (280–330 g) were intubated under general anesthesia with 4% chloral hydrate (4 mg/kg, administered intraperitoneally) and ventilated with room air by a small animal ventilator (Vetronics, Lafayette, Ind). MI was induced by permanent ligation of the left anterior descending coronary artery with a 6–0 silk

suture.¹⁹ Successful performance of coronary occlusion was verified by blanching of the myocardium distal to the coronary ligation. The sham-operation group received the same procedure of thoracotomy without coronary ligation. A minimum of 6 animals was in each experimental group. In our investigation, the MI-induced mortality rate was ~15%. We did not notice a significant difference between groups. The investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell Preparation and Transplantation

MSCs were labeled with Hoechst 33342 to track and count the implanted cells. In brief, sterile Hoechst 33342 was added to culture medium with a final concentration of 10 $\mu\text{mol/mL}$ for 2 hours before transplantation. Then the cells were rinsed 6 times with PBS to remove unbound Hoechst 33342, detached with 0.25% (w/v) trypsin ethylenediaminetetraacetic acid, and suspended in complete medium. Cells were centrifuged and washed with PBS, followed by the final centrifugation and suspension in serum-free medium at 1×10^6 cells per 150 μL . Before transplantation, MSCs in the HP group (H-MSCs) were treated for 24 hours with sublethal hypoxia (0.5% oxygen) and then reoxygenated in 20% oxygen for 2 hours. Half an hour after ligation of the left anterior descending coronary artery, 150 μL of medium containing 1×10^6 cells was directly injected into the ischemic peri-infarct region. MI control rats received MI alone and received the same volume of serum free/cell free medium injection. Experimental groups were divided into four groups of 10 rats each: (1) MI with transplant of normoxic MSCs (N-MSCs), (2) MI with transplant of hypoxia-pretreated MSCs (H-MSCs), (3) MI with injection of control medium, and (4) sham-operated control group.

Immunofluorescence Staining

For immunofluorescence staining, cells on dishes and in heart tissues were fixed with 10% formalin for 10 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and blocked with 1% fish gelatin (Sigma) for 1 hour at room temperature. Specimens were then incubated with primary antibodies overnight at 4°C, then washed with PBS three times, and incubated with Cy3-conjugated donkey anti-rabbit immunoglobulin G (1:500; Jackson ImmunoResearch, West Grove, Pa) or Alexa Fluor 488 anti-goat immunoglobulin G (1:200, Molecular Probes, Carlsbad, Calif) for 1 hour at room temperature. The cells were treated with Hoechst 33342 (1:20000; Molecular Probes, Carlsbad, Calif) for 5 minutes. Dishes or slides were mounted and analyzed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan).

Measurement of Hemodynamics

Cardiac hemodynamics was measured at 6 weeks after myocardial ischemia. Rats were anesthetized with 4% chloral hydrate (40 mg/kg, intraperitoneally). The carotid artery was isolated and cannulated with a microtip catheter that was connected with an MLT0699 disposable pressure transducer (ADInstrument, Colorado Springs, Colo). Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dp/dt (+dp/dt, pressure rise rate) and minimum dp/dt (−dp/dt, pressure decrease rate), and heart rate were monitored and recorded by the Powerlab/800 data acquisition system (ADInstrument).

Infarct Size Measurement

After measurement of hemodynamics, the animals were humanely sacrificed and the hearts were quickly harvested and divided into three transverse sections. Tissues from the free wall of the left ventricle including infarct and peri-infarct regions were then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc, Torrance, Calif). Frozen sections of left ventricular samples were cut at 10- μm thickness and prepared for staining. To determine infarct size, hearts at papillary muscle level were selected and Masson trichrome and hematoxylin–eosin staining were performed. Images were digitized by the NIH image analysis system (National Institutes of Health, Bethesda, Md). Infarct size was calculated by dividing the sum of the planimetric endocardial and epicardial circumferences of the infarcted area by the sum of the total epicardial and endocardial circumferences of the left ventricle.²⁰

Terminal Deoxynucleotidyl Transferase Biotin-dUPT Nick End Labeling (TUNEL) in heart sections

A TUNEL staining kit (DeadEnd Fluorometric TUNEL system, Promega, Madison, Wis) was used to visualize cell death in heart sections. After 10 minutes of fixing by 10% buffered formalin phosphate (Fisher Scientific, Pittsburgh, Pa) and pretreatment with −20°C ethanol/acetic acid (2:1) and 0.2% Triton X-100, the heart sections were incubated in an equilibration buffer as instructed by the kit. The TdT enzyme and nucleotide mix was then added at proportions specified by the kit for 75 minutes at room temperature. The slides were washed with 2× standard saline citrate washing buffer for 15 minutes and followed by three washings with PBS.

Cell and Vessel Counting

Cell count was performed using a design-based stereologic method. For counting caspase-3 positive cells in cultures, 6 fields per dish were randomly chosen under 20× magnification of a fluorescent microscope ($n = 4$). For counting caspase-3, Hoechst 33342, and GFP-positive cells and vessels in heart sections, every tenth heart section (100 μm apart) across the entire region of interest was counted, and 6 fields per heart section were randomly chosen and photographed under 40× magnification with a fluorescent microscope; this was repeated in 4 separate sections per heart.

Statistical Analysis

The Student two-tailed *t* test was used for comparison of two experimental groups. Multiple comparisons were done by 1-way analysis of variance followed by the Tukey post hoc test for multiple pairwise examinations. Data are expressed as the mean $\pm$ standard error of mean.

Results

Effect of HP on Expression of Pro-survival and Angiogenic Factors

To characterize the effect of hypoxia on MSCs, we first analyzed the protein expression levels of growth and angiogenic factors in N-MSCs and H-MSCs by Western blotting. N-MSCs displayed endogenous expression of growth and angiogenic factors including HIF-1 α , vascular endothelial growth factor (VEGF) and its receptor (Flk-1), angiopoietin-1, and erythropoietin (EPO) and its cognate receptor

(EPOR). As expected, sublethal hypoxia significantly increased the HIF-1 α protein expression level (3.4-fold) as well as Flk-1, angiopoietin-1, EPO, and EPOR in H-MSCs (Figure 1).

Consistent with a model of HP as protective to cell survival, H-MSCs displayed a significant increase in the expression of pro-survival proteins p105, the nuclear factor- κ B subunits P65 and P50, and anti-apoptotic proteins Bcl-2 and Bcl-xL. Increased nuclear fractions of P65 and P50 further suggested activation and translocation of these two nuclear factor- κ B transcription factors (Figure 2). The hypoxia treated cells also had a significantly lower level of active caspase-3 than did N-MSCs (Figure 2).

HP Promotes Cell Survival In Vitro and After Transplantation

To determine the vulnerability of N-MSCs and H-MSCs to apoptosis, we subjected cells in culture to 24 hours of normoxia or sublethal hypoxia followed by 24 hours of serum

deprivation. Cells were then stained with trypan blue and caspase-3 to assess cell death and apoptosis, respectively. The hypoxia treatment was confirmed to be sublethal with no significant increases in trypan blue (Figure 3) or caspase-3 staining (data not shown). Serum deprivation increased the ratio of trypan blue and caspase-3 staining positive cells in both N-MSCs and H-MSCs; however, the increases were much less in H-MSCs than in N-MSCs (Figure 3). The percentage of trypan blue positive cells versus total cells was $24.3\% \pm 5.4\%$ and $39.3\% \pm 6.8\%$ in H-MSCs and N-MSCs, respectively ($n = 4$, $P < .05$), and the percentage of caspase-3 positive cells was $25.6\% \pm 5.4\%$ and $36.6\% \pm 6.6\%$, respectively ($n = 4$, $P < .05$) (Figure 3).

To assess whether HP might protect MSCs in vivo, we transplanted N-MSCs and H-MSCs into the peri-infarcted region 30 minutes after MI. Twenty-four hours later, the animals were humanely sacrificed and apoptosis of implanted cells was identified by the ratio of caspase-3/Hoechst/GFP co-labeled cells versus total transplanted cells pre-labeled with Hoechst and GFP; cell death was identified by the ratio of TUNEL/Hoechst positive cells versus total transplanted cells. In the H-MSC transplantation group, the implanted cell death and apoptosis-associated caspase-3 activation were significantly suppressed (Figure 4). To test whether HP might prevent delayed MSC cell death, we assessed cell death using TUNEL staining 72 hours after transplantation. In the H-MSC transplantation group, the ratio of TUNEL positive cells was significantly reduced (Figure 4).

Figure 1. Hypoxic preconditioning up-regulated HIF-1 α and growth factor expression. HIF-1 α as well as pro-survival and pro-angiogenic factors Ang-1, VEGF, Flk-1, EPO, and EPOR were detected by Western blot. A, The expression levels of HIF-1 α , Ang-1, VEGF, Flk-1, EPO, and the EPOR in N-MSCs and H-MSCs. Mouse β -actin was used as the loading control. B, Densitometry analysis for comparisons of the relative expression levels of different factors in H-MSCs with respect to N-MSCs (dotted line). $N = 6$. * $P < .05$ compared with N-MSCs by the Student 2-tailed t test. HIF-1 α , Hypoxia-inducible factor-1 α ; N-MSC, Normoxic mesenchymal stem cell; H-MSC, hypoxic mesenchymal stem cell; Ang-1, angiopoietin-1; VEGF, vascular endothelial growth factor; EPO, erythropoietin; EPOR, cognate receptor of erythropoietin.

Enhanced Angiogenesis/Vascularization After MSC Transplantation

To assess angiogenic and vascularization activity, we stained heart sections with the endothelial cell marker CD31 and arteriole marker smooth muscle actin. Six weeks after ischemia with or without MSC transplantation, both the total vessel density/area and arteriole density/area in both MSC transplantation groups were greater than those in the ischemia-only group (Figure 5). Between the two transplantation groups, significantly greater total vessel density/area and arteriole density/area were observed in the H-MSC group than in the N-MSC group ($n = 6$ rats per group, $P < .05$) (Figure 5). To further clarify whether HP promoted endothelial cell differentiation of MSCs, we calculated the ratio of CD31/Hoechst positive cells versus total transplanted cells pre-labeled with Hoechst. A higher endothelial cell differentiation ratio was identified in the H-MSC transplantation group than in the N-MSC group ($n = 6$, $P < .05$) (Figure 5).

Possible Myogenesis From Transplanted MSCs

Myosin heavy chain staining showed that few MSCs expressed the myocyte protein myosin heavy chain in transplanted heart 6 weeks after transplantation (Figure 5). Our data, however, could not rule out the possibility that the co-labeling was due to cell fusion between exogenous and

Figure 2. Effect of hypoxic preconditioning on anti-apoptotic gene expression. Western blot analysis of several key factors in cell survival, apoptosis and angiogenesis in N-MSCs and H-MSCs. **A and B,** Expression of NF- κ B precursor P105, subunit P65, and P50 in cell lysate (**A**) and nuclear fraction (**B**). Mouse β -actin was used as the loading control. **C,** Densitometry analysis for comparisons of expression ratios of NF- κ B signals in H-MSCs with respect to the basal levels in N-MSCs (*dotted line*). **D,** Protein levels of Bcl-2, Bcl-xL, and cleaved caspase-3 detected by Western blot. **E,** Densitometry of Bcl-2, Bcl-xL, and caspase-3 expression in H-MSCs compared with that in N-MSCs (*dotted line*). * $P < .05$ compared with N-MSCs by the Student 2-tailed t test. For abbreviations, see [Figure 1](#).

host cells.^{6,7} We also did not identify a noticeable effect of HP on this potential myogenesis.

MSC Transplantation Reduces Infarct Size

Hematoxylin–eosin staining and Masson trichrome staining of cardiac tissue revealed fibrosis in the infarct region 6 weeks after ischemia. Scar formation was evident in the ischemic hearts that received a medium control injection ([Figure 6](#)). The infarct size was reduced in both groups that received MSC transplantation; however, rats receiving H-MSC transplantation showed the smallest infarct size ([Figure 6](#)).

Transplantation of MSCs improves cardiac function

Six weeks after ischemia, functional recovery of ischemic heart was measured by the LVSP, LVEDP, $+dp/dt$, and $-dp/dt$. MSC transplantation helped to prevent negative left ventricular remodeling. Rats that received N-MSCs had significantly increased LVSP and absolute values of $\pm dp/dt$ compared with the control group ([Figure 6](#)). Even greater cardiac functional benefits were found in H-MSC transplan-

tation group, showing higher LVSP, improved $\pm dp/dt$, and near normal LVEDP ([Figure 6](#)).

Discussion

The present study explored a novel preconditioning strategy to enhance the ability of MSCs to survive under pathologic conditions and promote their therapeutic effects within the ischemic heart. We have shown that sublethal hypoxia induces increases in several pro-survival and pro-angiogenic factors within the MSCs, contributing to the enhanced tolerance of H-MSCs to apoptosis and increased angiogenesis after transplantation. Uemura and associates²¹ recently reported that transplantation of hypoxic preconditioned MSCs reduced the apoptosis of cardiomyocytes. In the present investigation, we provide new evidence that transplantation of hypoxic preconditioned MSCs promotes heart functional recovery through enhancing implanted cell survival and angiogenesis after MI.

Previous studies have shown that allogeneic MSCs are immunoregulatory and do not induce immune response *in vitro*²² and *in vivo*²³; xenogeneic bone marrow stromal

Figure 3. Effect of hypoxic preconditioning on cell death and cell survival in vitro. GFP MSC nuclei were pre-stained with Hoechst 33342 (blue); the double labeling of GFP and nuclear stain facilitated cell counting of MSCs. Cell apoptosis was identified by the antibody against the cleaved/activated caspase-3 (red). A–I, Activated caspase-3 (arrow) staining in control, N-MSCs and H-MSCs subjected to 24-hour SD. Bar = 50 μ m. J and K, Summarized data of caspase-3 activation for apoptosis (J) and trypan blue staining for cell death (K). The ratio of cell apoptosis and cell death were significantly increased after SD, and hypoxic preconditioning significantly reduced apoptosis. N = 4. * P < .05 compared with N-MSCs plus SD. GFP, Green fluorescent protein; SD, serum deprivation; for other abbreviations, see Figure 1.

cells transplanted into ischemic myocardium were immunologically tolerant and feasible.²⁴ Therefore, immunosuppression was not given in the present investigation and no rejection of implanted cells was noticed. Transplanted bone marrow MSCs are sensitive to ischemic and inflammatory microenvironment as is evidenced by stem cell death that often occurs soon after transplantation.^{6,8,25} This cell death may, in turn, increase the inflammatory response and be an additional burden to the ischemic heart, hindering functional recovery. Gene modification has been investigated to enhance stem cell survival and efficiency of cell transplantation therapy. For instance, overexpression of Akt can reduce apoptosis of MSCs and then enhance the heart functional improvement.²⁶ Our previous study showed that transplantation of embryonic stem cells overexpressing Bcl-2 increased the survival and neuronal differentiation of transplanted cells, as well as functional recovery after cerebral ischemic stroke.²⁷

Whether or not permanent gene modification has a long-term risk of tumorigenesis is not clear,²⁸ which may limit its clinical utility. On the basis of this concern, increasing attention has been paid to short but comprehensive improvements of the quality of transplanted cells. The present study supports that HP may be explored as such a strategy in cell transplantation therapy for ischemic heart diseases.

Considering that up to 90% of grafted cells may die within the first few days of transplantation, the transient cytoprotective effect of HP should be sufficient to protect transplanted cells during the initial critical period after transplantation. Enhanced implanted cell survival can reduce the required number of transplanted cells, and fewer stem cells may actually differentiate better. Transplantation of MSC was performed 30 minutes after MI. In this case, some observed beneficial effects might result from myocardial rescue that prevented negative remodeling during the acute phase. Therapeutic

Figure 4. Effect of hypoxic preconditioning on implanted cell death 1 to 3 days after myocardial infarction. MSCs were labeled with Hoechst 33342 (blue) before transplantation. Cell apoptosis was identified with activated caspase-3, cell death was identified with TUNEL staining. A and B, Co-labeling of activated caspase-3 (red) and Hoechst (blue arrows) indicates apoptosis of graft cells in the ischemic heart 24 hours after myocardial infarction. C and D, Merged images of caspase-3/Hoechst/GFP that further confirmed the apoptosis of implanted cells. E and F, Co-labeling of TUNEL (red) and Hoechst (blue) 24 hours after myocardial infarction in N-MSC and H-MSC transplantation group, respectively. G and H, Co-labeling of TUNEL and Hoechst 72 hours after myocardial infarction. Bar = 20 μ m. I and J, Summarized data of caspase-3 activation for apoptosis (I) and TUNEL staining for cell death (J). Hypoxic preconditioning significantly reduced cell death and apoptosis of transplanted MSCs in the ischemic heart. * $P < .05$ compared with N-MSC. TUNEL, Terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling. For other abbreviations, see Figure 1.

Figure 5. Differentiation of transplanted cells in ischemic heart 6 weeks after MI. Immunohistochemical staining of heart sections at transplanted sites after MI and transplantation. MSCs were labeled with Hoechst 33342 before transplantation, and vascular endothelial cells were stained with CD31, arteriole marker was stained with SMA and cardiomyocyte marker was stained with myosin heavy chain. **A**, Three-dimensional image shows a vascular-like structure positively labeled with Hoechst (blue) and CD31 (red). Bar = 40 μ m. **B**, Three-dimensional image of Hoechst/MHC and GFP (green), indicating some graft cells develop into cardiac-like myocytes. Bar = 10 μ m. **C–E**, Merged images of Hoechst and CD31 (red) staining in three experimental groups; **F–H**, Merged images of Hoechst 33342 and SMA (red) staining in three experimental groups; increased numbers of CD31-positive vessels and SMA-positive arterioles were seen in the MSC transplantation groups. Bar = 40 μ m. **I–K**, Summary of total vessel density and arteriole density (**I**), area per field (**J**) and endothelial differentiation ratio (**K**) in three experimental groups. Angiogenic and/or vasculogenic activities were enhanced in the MSC transplantation rats; H-MSCs showed stronger angiogenic potency compared with N-MSCs. N = 6. * P < .05 compared with MI group. # P < .05 compared with N-MSC group. *MI*, Myocardial infarction; *SMA*, smooth muscle actin. For other abbreviations, see Figure 1.

potential of transplantation of MSCs in the chronic phase long after MI remains to be examined. MSCs may provide host tissues and themselves with trophic support. MSCs can express cytokines and growth factors that play important roles in cell survival and angiogenesis, such as VEGF, basic

fibroblast growth factor, angiopoietin-1, and EPO.^{12,29} Our study demonstrates that MSCs express these factors, which are up-regulated by HP, in line with previous studies.^{21,30} These growth factors exert both autocrine and paracrine effects. HP augmented paracrine signaling and reduced

Figure 6. MSC transplantation reduced MI-induced infarct size and improved heart function. A–C, Masson trichrome staining reveals infarct and scar formation 6 weeks after MI. N-MSC transplantation inhibited the infarct size compared with the MI-only group (B). The smallest infarct size was seen in the H-MSC transplantation group (C). D, Summary of infarct size in three experimental groups. E, LVSP decreased significantly 6 weeks after MI compared with sham group. While MSC transplantation improved LVSP, H-MSC transplantation showed stronger effect on LVSP recovery. F, LVEDP increased markedly 6 weeks after MI; N-MSC transplantation showed no significant effect while H-MSC transplantation enhanced LVEDP to a near normal level. G and H, Left ventricular +dp/dt (G) and –dp/dt (H) decreased 6 weeks after MI; both transplantation groups increased $\pm$ dp/dt, especially in the H-MSC transplantation group. N = 6. * $P < .05$ as compared with the MI-only group. # $P < .05$ as compared with N-MSC transplantation group. *MSC*, Mesenchymal stem cell (*N*, normoxic; *H*, hypoxic); *MI*, myocardial infarction; *LVSP*, left ventricular systolic pressure; *LVEDP*, left ventricular end-diastolic pressure; *dp/dt*, rate of pressure rise.

cardiomyocytes apoptosis.^{21,30} Several transcription factors may be involved in the response to hypoxia, such as activated protein-1, HIF-1 α , and nuclear factor- κ B.³¹ Activation of some growth factor pathways can activate nuclear factor- κ B and increase the survival genes such as Bcl-2 and Bcl-xL.³² Consistent with these anti-apoptotic effects, caspase-3 activation is suppressed in H-MSCs. It is likely that a combination of these multiple mechanisms is responsible for the increased viability of H-MSCs.

In this study, MI-induced animal death mostly occurred 3 days after surgery, which was not affected by H-MSC transplantation. The lack of the effect on animal mortality suggests that although the hypoxic preconditioned MSCs survives better in the ischemic heart, the increased cell survival is not sufficient to antagonize the initial overwhelming insults and functional failure in the acute phase of MI. Moreover, the H-MSC-promoted cell survival and angiogenesis are expected to primarily benefit long-term tissue repair and function recovery many days after MI, as shown in this investigation.

An earlier study showed that ex vivo HP up-regulated the synthesis of VEGF messenger RNA and stimulated endothe-

lial differentiation of bone marrow stem cells, which together contributed to improved angiogenesis in the ischemic hind limb after transplantation.³³ Our finding that HP increased vessel density, area, and the endothelial cell differentiation ratio is consistent with this previous study. The higher vessel density in H-MSC transplantation group is attributable to both enhanced paracrine effect and increased endothelial cell differentiation. Several groups have reported that MSCs can differentiate into cardiomyocytes^{9,34}; other studies question the possibility that MSCs might fuse with host cells after transplantation into the ischemic heart.^{6,7} Our study identified that, 6 weeks after transplantation, a few MSCs showed characteristic myosin protein expression. Whether transplanted MSCs may undergo cardiac myocyte differentiation is under debate. Moreover, possible fusion and uptake of Hoechst dye by endogenous cells might also affect the endothelial cell counts. In any event, transplanted MSCs helped to repair infarcted myocardium, disregarding the rare events of myogenesis or cell fusion.³⁵

In conclusion, our study demonstrates that transplantation of HP-treated MSCs shows better therapeutic effects in the ischemic heart. The functional benefit of H-MSC

transplantation might be explained by several possibilities: (1) HP enhances the autocrine and paracrine signaling of MSCs, which reduces apoptosis of transplanted cells and endogenous cardiomyocytes; (2) the increased survival of H-MSCs provides better and longer trophic support for the reparative process; (3) the increase in survival of engrafted cells contributes to increased angiogenesis. These factors collectively promote tissue repair and may provide a simple but effective new strategy for clinical MSC transplantation therapy. Of note, although HP of MSCs may show the above benefits, transplantation of H-MSCs did not improve short-term animal survival 3 days after MI; its effect on long-term mortality remains to be tested.

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