The antiapoptotic effect of mesenchymal stem cell transplantation on ischemic myocardium is enhanced by anoxic preconditioning

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BACKGROUND: Cardiomyocyte apoptosis takes place at an early stage after myocardial infarction (MI). Therapy with mesenchymal stem cells (MSCs) is reported to reduce apoptosis.

OBJECTIVES: To determine whether anoxic preconditioning (AP) could enhance the antiapoptotic effect of MSCs.

METHODS: Cultured cardiomyocytes were treated with Dulbecco's modified Eagle's medium (as a control), MSCs or AP-MSCs, and were exposed to hypoxia/reoxygenation. Apoptotic cardiomyocytes were stained with Annexin V fluorescein isothiocyanate (BioVision, USA), visualized by fluorescence microscopy and analyzed by flow cytometry. In vivo, MI was produced in Sprague-Dawley rats by permanent ligation of the left anterior descending coronary artery and the left ventricles were randomly injected with Dulbecco's modified Eagle's medium, MSCs or AP-MSCs one week after MI. The cardiomyocyte apoptotic rate in peri-infarcted areas was assessed by terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labelling assay one week after transplantation. Cardiac function was assessed by echocardiography four weeks after transplantation. Infarct size was measured by hematoxylin and eosin staining one and four weeks after transplantation. The expression of Bcl-2, Bax protein and cleaved cysteine-aspartic acid protease-3 was analyzed by Western blot techniques.

RESULTS: Cardiomyocyte apoptosis (both induced by hypoxia/reoxygenation and MI) was significantly reduced by treating with MSCs and AP-MSCs, the Bcl-2 to Bax protein ratio was increased and cleaved cysteine-aspartic acid protease-3 was decreased. AP-MSCs were superior to MSCs.

CONCLUSIONS: MSCs protected the infarcted heart by preventing cardiomyocyte apoptosis and AP enhanced the cardioprotective effects of MSCs.

Key Words: Apoptosis; Hypoxia; Myocardial infarction

L'effet antiapoptotique de la greffe de cellules souches mésenchymateuses sur le myocarde ischémique est accru par un préconditionnement anoxique

HISTORIQUE : L'apoptose cardiomyocytaire se produit peu après un infarctus du myocarde (IM). Une thérapie aux cellules souches mésenchymateuses (CSM) réduit l'apoptose.

OBJECTIFS : Déterminer si un préconditionnement anoxique (PA) améliore l'effet antiapoptotique des CSM.

MÉTHODOLOGIE : Les auteurs ont traité des cultures de cardiomyocytes au moyen du milieu Eagle modifié par Dulbecco (culture témoin), de CSM ou de PA-CSM, puis les ont exposées à une hypoxie et une réoxygénation. Ils ont coloré les cardiomyocytes apoptotiques avec de l'isothiocyanate de fluorescéine Annexin V (BioVision, États-Unis), les ont visualisées par microscopie par fluorescence et analysées par cytométrie de flux. *In vivo*, ils ont produit un IM chez des rats Sprague-Dawley au moyen d'une ligature permanente de la branche antérieure de l'artère coronaire gauche, puis ont injecté au hasard le milieu Eagle modifié par Dulbecco, les CSM ou le PA-CSM dans les ventricules gauches, une semaine après l'IM. Ils ont évalué le taux d'apoptose cardiomyocytaire des zones péri-infarctées par marquage des brins d'ADN avec la désoxyribonucléotidyl-transférase terminale 2'-désoxyuridine 5'-triphosphate une semaine après la greffe. Ils ont évalué la fonction cardiaque par échocardiographie quatre semaines après la greffe. Ils ont mesuré la dimension de l'infarctus par hématoxyline et par coloration à l'éosine une semaine et quatre semaines après la greffe. Ils ont analysé l'expression de Bcl-2, de la protéine Bax et de la protéase-3 de la cystéine et de l'acide aspartique clivée au moyen des techniques de transfert Western.

RÉSULTATS : L'apoptose cardiomyocytaire (induite par hypoxie et réoxygénation et IM) diminuait considérablement grâce à un traitement aux CSM et au PA-CSM, le ratio entre le Bcl-2 et la protéine Bax augmentait et la protéase-3 de la cystéine et de l'acide aspartique clivée diminuait. Le PA-CSM donnait de meilleurs résultats que les CSM.

CONCLUSIONS : Les CSM protégeaient le cœur infarcté en prévenant l'apoptose cardiomyocytaire, et le PA accroissait les effets cardioprotecteurs des CSM.

Myocardial infarction (MI), leading to irreversible loss of cardiomyocytes and scar formation, is the primary cause of congestive heart failure (1). The identification of stem cells capable of contributing to tissue regeneration has raised the possibility of stem cell therapy

for the repair of damaged myocardium. Mesenchymal stem cells (MSCs) are considered to be an effective therapeutic approach for MI, based on basic investigations (2,3) and clinical trials (4,5). Underlying mechanisms may include direct regeneration of lost cardiomyocytes or

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other cell types constituting the cardiac tissue (6-8), although there is much debate regarding the frequency of these phenomena.

Recently, it has been reported that the cardioprotective effect of MSCs is related to a paracrine effect, which may be enhanced by hypoxia (9-11). Because bone marrow is a hypoxic place of residence of MSCs, we hypothesized that treating MSCs with anoxic preconditioning (AP) before transplantation would increase the cardioprotective effect of MSCs.

METHODS

Neonatal and adult male Sprague-Dawley (SD) rats (80 g and 250 g, respectively) were obtained from the Medical Institute Animal Center of Zhejiang University (Hangzhou, Zhejiang, China). The experiments were approved by the Animal Care and Use Committee of the Zhejiang Province Medical Institute and were in compliance with the "Guide for the Care and Use of Laboratory Animals" (12), published by the National Institutes of Health (USA).

Cell culture

MSCs were obtained from the femora and tibiae of male SD rats, using a modified method as previously described (7,8). The bones were dissected free, and the proximal and distal ends were removed to reveal the marrow cavity, which was aspirated with 10 mL of Dulbecco's modified Eagle's medium (DMEM) via a 21 G needle. The aspirant was layered over Percoll solution (1.073 g/mL; GE Healthcare Bio-Sciences AB, Sweden) and centrifuged at 900 g for 25 min at room temperature. The mononuclear cells were recovered at the interface, resuspended in growth medium (DMEM, 10% fetal bovine serum, 100 U/mL penicillin G and 100 U/mL streptomycin; Gibco, USA), and then plated on 50 cm² flasks and left for 24 h. The flasks were then washed with phosphate-buffered saline to leave an adherent layer of cells containing MSCs. The cultures were maintained at 37°C in a 5% CO₂ incubator; the medium was changed every three to four days. With three to five passages, MSCs negative for CD45 and positive for CD44 and CD90 were used for the experiments. For *in vitro* experiments, MSCs were seeded in the 3 µm Transwell insert (Corning, USA). For transplantation, MSCs were labelled with 4,6-diamidino-2-phenylindole (DAPI; Sigma, USA) as previously described (13,14) to track engrafted cells. Briefly, MSCs were incubated in growth medium containing DAPI (50 µg/mL) for 3 h. Then, the cells were rinsed six times in phosphate-buffered saline to remove unbound DAPI, detached with 0.25% (weight/volume) trypsin and suspended in DMEM for cell transplantation.

Primary cardiomyocyte cultures were prepared by the method originally described by Simpson and Savion (15), with minor modifications. Briefly, the hearts from neonatal male rats were minced and dissociated with 0.125% trypsin (Gibco) and 0.1% collagenase type II (Worthington, USA). After incubation of dispersed cells on a 25 cm² flask for 60 min in a normoxic incubator (95% air and 5% CO₂), unattached viable cells were collected and seeded into a 25 cm² flask or Transwell lower plates (Corning) and incubated. The cells were then incubated with DMEM supplemented with 20% fetal calf serum plus 0.1 mmol/L 5-bromo-2-deoxyuridine (Sigma) for 72 h to prevent low-level nonmyocardial cell proliferation, and then replaced with DMEM plus 20% calf serum.

AP

For AP of MSCs (AP-MSCs), MSCs were incubated in a modular incubator chamber (Billups-Rothenberg, USA) for 3 h in serum-free DMEM; normal air was replaced by 95% nitrogen and 5% CO₂. AP-MSCs for transplantation were incubated in serum-free DMEM containing DAPI (50 µg/mL) in the modular incubator chamber.

Apoptosis of AP-MSCs was evaluated by a chromatin dye (5 µg/mL; Hoechst 33342; Sigma). Cells were fixed for 1 h in 4% paraformaldehyde at room temperature, and then exposed to the dye for 30 min in the dark. All samples were observed by using a fluorescence microscope. Apoptotic cells were characterized by morphological alteration such as condensed nuclei and cell shrinkage.

Hypoxia/reoxygenation

Cardiomyocytes in the lower plates of the Transwells were randomly divided into three groups: the DMEM (control) group, the MSCs group and the AP-MSCs group. For coculture experiments, MSCs or AP-MSCs along with cardiomyocytes were cultured in two individual chambers separated by a semipermeable membrane (3 µm hole). This system allowed for the sharing of the culture medium between two chambers but prevented cell contact. Each was treated with hypoxia/reoxygenation (H/R). To mimic hypoxia, the cardiomyocytes were incubated at 37°C in the anoxic chamber for 24 h; normal air was replaced by 95% nitrogen/5% CO₂. Then, cardiomyocytes were moved into a normoxic incubator (95% air/5% CO₂) for 3 h to mimic the reoxygenation process. The control group was incubated in DMEM supplemented with 20% fetal calf serum under standard cell culture conditions (95% air/5% CO₂).

Apoptosis of cardiomyocytes was determined using an Annexin V fluorescein isothiocyanate apoptosis detection kit (BioVision, USA) according to the manufacturer's instructions. Cells were visualized directly on glass slides under a fluorescence microscope and analyzed by flow cytometry.

MI model and transplantation

Adult male SD rats (250 g) were included in the studies and anesthetized with 4% chloral hydrate (4 mg/kg, administered intraperitoneally), then mechanically ventilated using a small-animal ventilator (Zhejiang University apparatus). An MI was created by permanently ligating the left anterior descending coronary artery with a 7-0 silk suture. Successful coronary occlusion was verified by blanching of the distal myocardium. The sham-operated group was subjected to thoracotomy without coronary ligation. The chest was closed, and rats were weaned from the ventilator and allowed to recover for one week. Then, the MI rats were randomly divided into three groups (16 rats per group): control animals that received a DMEM injection, cell-treated animals that received MSCs (5×10⁶) and cell-treated animals that received AP-MSCs (5×10⁶). The cells suspended in 150 µL DMEM were prepared individually and injected into peri-infarcted regions of anterior left ventricles at three to five locations. The sham group received the same injected volume of DMEM as transplant subjects. The rats were allowed to live for another one or four weeks.

Infarct size measurement

The rats (eight in each group) were euthanized and the hearts were removed one week after transplantation. The excised heart was cut into three transverse sections and embedded in paraffin. Sections (5 µm thick) were cut and mounted on slides; heart sections at the papillary muscle level were selected to determine infarct size. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin (13,16). Images were digitized using a computerized image analysis system (NIH Image; National Institutes of Health, USA). 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, and expressing the result as a percentage (13,16).

Terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labelling analysis

Apoptotic cardiomyocytes in the border zone of the ischemic region were evaluated by terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labelling (TUNEL) assay with an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instructions. The percentage of TUNEL-positive cells was assessed in five randomly selected fields in each heart section. A total of 16 sections were analyzed, each from the four groups (ie, sham plus three MI groups).

Western blotting analysis

Protein (40 µg to 150 µg) prepared from the cardiomyocytes or infarcted heart was loaded per lane and electrophoresed in sodium

dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride Immobilon-P membrane (Bio-Rad, USA) using a transblot apparatus (Bio-Rad). The membranes were blocked in 10 mmol/L Tris-HCL (pH 8.0), 150 mmol/L sodium chloride and 0.05% Tris-buffered saline Tween 20 (TBST) with 5% (weight/volume) nonfat milk at room temperature, followed by overnight incubation at 4°C with primary antibodies diluted in TBST (1:1000 for Bcl-2, Bax, cleaved caspase-3 and beta-actin; Cell Signal, USA). After washing with TBST, the membranes were incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody diluted (1:5000) in TBST, and the labelled proteins were detected by using the enhanced chemiluminescence reagents and exposed to the film (Kodak, USA).

Measurement of cardiac function

Cardiac function was assessed by transthoracic echocardiography (Vivid 7; GE Healthcare, USA) in MI rats four weeks after transplantation. Briefly, a two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles, and M-mode tracings were recorded and analyzed to evaluate cardiac function.

Identification of implanted cells

Animals were sacrificed after echocardiography. The hearts were quickly harvested and tissues from the free wall of the left ventricle including the infarct and peri-infarct regions were then embedded in optimal cutting temperature tissue-freezing medium. Frozen sections (6 µm thick) of left ventricular samples were made for identification of implanted cells. Subsets at the papillary muscle level were stained with hematoxylin and eosin for measurement of infarct size, as described above.

Statistical analyses

Data are expressed as mean ± SEM. Statistical significance between groups was assessed by one-way ANOVA followed by the Student-Newman-Keuls test, using SPSS 11.5 (SPSS Inc, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Morphology of AP-MSCs

MSCs were attached to culture dishes and the majority displayed a spindle-like shape (Figure 1A). AP-MSCs changed little with respect to morphology (Figure 1B) or with respect to CD markers; hypoxia for up to 6 h did not cause irreversible damage (Figure 1C).

AP-MSCs prevented apoptosis induced by H/R

The apoptotic rate of normal cultured cardiomyocytes was low ($2.16\% \pm 0.78\%$; Figures 2A and 2E) but increased after H/R ($21.5\% \pm 2.71\%$, $P < 0.05$ versus normal; Figures 2B and 2E). Coculturing with MSCs reduced that rate ($17.47\% \pm 1.62\%$, $P < 0.05$ versus normal and DMEM; Figures 2C and 2E), and coculturing with AP-MSCs reduced the rate further ($11.01\% \pm 2.10\%$, $P < 0.05$ versus normal, DMEM and MSCs; Figures 2D and 2E).

The activation of caspase-3 in cardiomyocytes during H/R was examined next. It was found that coculture with MSCs or AP-MSCs prevented the activation of caspase-3 ($P < 0.05$ versus DMEM, Figure 3A); the effect was more obvious in the AP-MSCs group ($P < 0.05$ versus MSCs, Figure 3A).

Further examination of the expression of antiapoptotic protein Bcl-2 and proapoptotic protein Bax revealed that H/R reduced the Bcl-2 to Bax ratio in cardiomyocytes. Coculture with MSCs or AP-MSCs prevented this reduction ($P < 0.05$ versus DMEM, Figure 3B), which was more obvious in the AP-MSC group ($P < 0.05$ versus MSCs, Figure 3B).

AP-MSCs prevented cardiomyocyte apoptosis after MI

In vivo experiments were performed to estimate the antiapoptotic effect of AP-MSCs. TUNEL was performed one week after transplantation (Figures 4A to 4D) and the results showed that the number of TUNEL-positive cells was significantly reduced by treating with MSCs or AP-MSCs (MSC group, $11.18\% \pm 0.88\%$; AP-MSC group, $9.03\% \pm 0.42\%$;

DMEM group, $14.54\% \pm 0.60\%$; $P < 0.05$, Figure 4E). AP-MSCs had the predominant effect ($P < 0.05$ versus MSCs; Figure 4E).

Further examination of the expression of cleaved caspase-3, Bcl-2 and Bax in infarcted hearts revealed that MSCs and AP-MSCs prevented the activation of caspase-3 ($P < 0.05$ versus DMEM; Figure 5A) and increased the Bcl-2 to Bax ratio ($P < 0.05$ versus DMEM; Figure 5B). AP-MSCs were predominant ($P < 0.05$ versus MSCs; Figure 5).

Assessments of cardiac function

Fractional shortening and ejection fraction significantly increased, and left ventricular diastolic dimensions and systolic dimensions significantly decreased four weeks after cell transplantation ($P < 0.05$ versus DMEM; Table 1). AP-MSCs were predominant ($P < 0.05$ versus MSCs; Table 1).

AP-MSC transplantation reduced infarct size

Infarct size was measured one week and four weeks following transplantation (Figure 6). Infarct size decreased in the AP-MSCs group one week after transplantation (AP-MSCs group, $37.46\% \pm 1.79\%$; DMEM group, $44.60\% \pm 2.41\%$; and MSCs group, $39.22\% \pm 1.71\%$; $P < 0.05$). Infarct size decreased in the cell transplantation group four weeks after transplantation (MSCs group, $38.76\% \pm 1.42\%$; AP-MSCs group, $32.8\% \pm 1.67\%$; versus DMEM group, $47.30\% \pm 2.63\%$; $P < 0.05$) and there was a trend toward smaller infarct size in the AP-MSC group compared with the MSC group ($P < 0.05$).

Identification of engrafted MSCs

The cells before transplantation were labelled (approximately 100%) with DAPI (Figure 7A). Figure 7B shows engrafted cells in ischemic myocardium, demonstrating that the implanted cells do survive in the peri-infarct region for at least four weeks after transplantation.

DISCUSSION

MSCs are multipotent adult stem cells and, as such, their potential plasticity and self-renewal capacity offer a huge potential for clinical tissue regeneration. There appears to be a general agreement that MSC therapy has the potential to improve cardiac function in the injured heart. The mechanism underlying this therapeutic effect has not been clearly defined, with an intense debate over differentiation versus fusion; it appears to be far more complex than previously anticipated. Our experiments were not designed to address whether MSCs differentiated into cardiomyocytes. Recently, paracrine effects have been emphasized. It has been reported (17,18) that MSCs provide protection by paracrine mechanisms involving release of a wide array of cytokines (such as vascular endothelial growth factor, hepatocyte growth factor and insulin-like growth factor-1) that exert their effects on surrounding cells.

In our in vitro study, we used a coculture system (Transwell), which allowed for the sharing of the culture medium between two chambers while preventing cell contact. We found that coculture with MSCs reduced cardiomyocyte apoptosis induced by H/R; this prevention was more obvious when cocultured with AP-MSCs. Thus, we conclude that MSCs exerted an antiapoptotic effect partially by a paracrine effect and that AP enhanced this effect.

MI leads to cardiomyocyte loss, resulting in reduced cardiac function and heart failure. In addition to necrosis, apoptosis also plays a role in the process of tissue damage after MI (19). However, studies are also conflicting with respect to the area of the heart in which apoptosis is found. In humans, apoptosis seems to occur primarily in the border zone of the ischemic region (19,20) and, according to some studies, in the regions remote from ischemia (20). In our study models, we found that the apoptosis was more obvious in the peri-infarct area than in the remote area, so we compared the apoptosis that occurred in the border zone of the ischemic region and further focused on the antiapoptotic effect of MSCs on MI. We found that MSCs prevented cardiomyocyte apoptosis and further improved cardiac function. In accordance with the results of our in vitro study, AP enhanced the cardioprotective effect of MSCs. The underlying reasons may be that

Figure 1 Characteristics of mesenchymal stem cells (MSCs) and anoxic preconditioned MSCs (AP-MSCs). Phase-contrast micrographs of the third passage of MSCs (A) and AP-MSCs (B). C The apoptotic curve of MSCs exposed to hypoxia for up to 12 h, evaluated by Hoescht 33342 stain in five randomly selected fields under fluorescence microscopy. Results are representative of five independent experiments. Data are shown as means and SEMs. * $P < 0.01$ versus 0 h

Figure 2 Annexin V fluorescein isothiocyanate (FITC) staining of cardiomyocytes exposed to hypoxia/reoxygenation (H/R) in vitro. The early-stage apoptotic cells (FITC-labelled) appeared green under fluorescence microscopy in normal cells (A), Dulbecco's modified Eagle's medium (DMEM) (B), mesenchymal stem cells (MSCs) (C) and anoxic preconditioned MSCs (AP-MSCs) (D). The apoptotic rate in the four groups was measured by fluorescence-activated cytometry (E). Results are representative of five independent experiments. Data are shown as means and SEMs. * $P < 0.05$ versus normal; † $P < 0.05$ versus DMEM; ‡ $P < 0.05$ versus MSCs

Figure 3 Western blot analysis of the expression of cleaved caspase-3, Bcl-2 and Bax in whole-protein extracts from cultured cardiomyocytes. Beta (β)-actin was the internal control. Results are representative of three independent experiments. Data are shown as means and SEMs. * $P < 0.05$ versus normal; † $P < 0.05$ versus Dulbecco's modified Eagle's medium (DMEM); ‡ $P < 0.05$ versus mesenchymal stem cells (MSCs). AP-MSCs Anoxic preconditioned MSCs; H/R Hypoxia/reoxygenation

AP enhanced the survival rate of grafted MSCs in ischemic myocardium (although the apoptotic rate before transplantation is similar) or that AP enhanced the paracrine effect of MSCs (21), and the secreted cytokines may contribute to a profound antiapoptotic effect and reduction of infarct size.

Caspases are a family of cysteine proteases that are activated during apoptosis (22,23). Caspase-3 (21) is the ultimate apoptotic proenzyme in most types of cells. Proteins of the Bcl-2 family play a key role in controlling the activation of caspases (24). The Bcl-2 protein family falls into two groups that generally either repress apoptosis (Bcl-2 and Bcl-xL) or promote apoptosis (Bax, Bak and Bad). Bcl-2 interacts with the proapoptotic Bax protein at an upstream check point to regulate the apoptosis by activation of caspase-3. Ischemia-induced apoptosis was accompanied by a decrease in Bcl-2 protein and an increase in the expression of Bax. We found that MSCs could increase the Bcl-2 to Bax ratio and inhibit the activation of caspase-3, thus preventing cardiomyocyte apoptosis; these phenomena were more obvious in the AP-MSCs group.

CONCLUSION

The results of the present study indicate that MSCs exert antiapoptotic effects, partially by paracrine action. Anoxic preconditioning

Figure 4 Terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labelling (TUNEL) assay of left ventricles one week after transplantation. TUNEL staining showed the apoptotic cardiomyocytes (arrows) in the peri-infarcted area (**A** Sham; **B** Dulbecco's modified Eagle's medium [DMEM]; **C** Mesenchymal stem cells [MSCs]; **D** Anoxic preconditioned MSCs [AP-MSCs]). The percentage of TUNEL-positive cells in the four groups is shown (**E**). Data are presented as means and SEMs (eight rats per group). * $P < 0.05$ versus sham; † $P < 0.05$ versus DMEM; ‡ $P < 0.05$ versus MSCs.

Figure 5 Western blot analysis of the expression of cleaved caspase-3, Bcl-2 and Bax in whole-protein extracts from infarcted tissues one week after transplantation. Beta (β)-actin was the internal control. Results are representative of three independent experiments. Data are shown as means and SEMs. * $P < 0.05$ versus sham; † $P < 0.05$ versus Dulbecco's modified Eagle's medium (DMEM); ‡ $P < 0.05$ versus mesenchymal stem cells (MSCs). AP-MSCs Anoxic preconditioned MSCs.

may be an effective and convenient way to enhance the cardioprotective effects of MSCs.

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TABLE 1
Cardiac function assessments four weeks after transplantation

	Sham	DMEM	MSCs	AP-MSCs
EF, %	83.66±1.57	47.82±2.28*	72.57±2.43*†	79.84±1.82†‡
FS, %	47.64±1.55	20.94±1.20*	37.12±2.00*†	43.32±1.82†‡
LVDd, cm	0.48±0.03	0.75±0.06*	0.57±0.02†	0.49±0.03†
LVDs, cm	0.25±0.02	0.60±0.05*	0.36±0.02†	0.28±0.02†
LVPWd, cm	0.19±0.02	0.19±0.02	0.20±0.01	0.18±0.04
LVPWs, cm	0.28±0.01	0.26±0.02	0.27±0.01	0.27±0.01

Data are shown as mean ± SEM (eight rats per group). * $P < 0.05$ versus sham; † $P < 0.05$ versus Dulbecco's modified Eagle's medium (DMEM); ‡ $P < 0.05$ versus mesenchymal stem cells (MSCs). AP-MSCs Anoxic preconditioned MSCs; EF Ejection fraction; FS Fractional shortening; LVDd Left ventricular diastolic dimension; LVDs Left ventricular systolic dimension; LVPWd Left ventricular posterior wall diastolic thickness; LVPWs Left ventricular posterior wall systolic thickness.

Figure 6 Infarct size measurement at one and four weeks after transplantation. Data are shown as means and SEMs (eight rats per group). * $P < 0.05$ versus Dulbecco's modified Eagle's medium (DMEM); † $P < 0.05$ versus mesenchymal stem cells (MSCs). AP-MSCs Anoxic preconditioned MSCs.

Figure 7) Engrafted cells labelled with 4,6-diamidino-2-phenylindole (DAPI). DAPI-labelled cells appear blue under fluorescence microscopy. Culture cells before transplantation were all labelled with DAPI (A). DAPI-positive cells existed around the infarct border zone in rats four weeks after cell transplantation (B)

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