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Shock Wave-Pretreated Bone Marrow Cells Further Improve Left Ventricular Function After Myocardial Infarction in Rabbits

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Background: We tested whether shock wave (SW) offers additional benefits in improving left ventricular (LV) function after acute myocardial infarction (AMI) in rabbits receiving SW-treated autologous bone marrow-derived mononuclear cells (BMDMNCs) transplantation.

Methods and Results: Saline (750 μL; group 2), BMDMNCs (1.0×10^7; group 3), or preimplant SW-treated BMDMNCs (group 4) were implanted into the infarct area of male rabbits 15 minutes after left coronary artery ligation, whereas eight rabbits without AMI served as controls (group 1; $n = 8$ per group). The results showed that in infarct area of LV, protein expressions of Cx43 and cytochrome C in mitochondria and endothelial nitric oxide synthase mRNA expression were lower in group 2 than in other groups, and decreased in group 3 as compared with groups 1 and 4 (all p values < 0.01). Conversely, mRNA expressions of endothelin-1 and matrix metalloproteinase-9, mitochondrial oxidative stress, and total fibrotic area were higher in group 2 than in other groups (all p values < 0.05). Furthermore, 6-month LV function by 2-D echo/angiogram showed significant impairment in group 2 than in other groups and in group 3 than in groups 1 and 4 (all p values < 0.005).

Conclusions: Application of SW-treated autologous BMDMNCs is superior to BMDMNCs alone for preserving LV function after AMI.

INTRODUCTION

Acute myocardial infarction (AMI) remains the leading cause of death in patients hospitalized for cardiovascular disease.[1,2] Numerous clinical studies have demonstrated that the achievement of brisk thrombolysis in myocardial infarction grade 3 flow immediately after thrombolytic therapy or primary

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percutaneous coronary intervention in AMI is effective in minimizing the effect of ischemic insult to myocardium, preserving left ventricular (LV) function, and improving overall survival.[3-6] However, there is limited benefit of reperfusion therapy if medical attention is delayed.[7,8] Additionally, despite application of reperfusion therapy,[3-6] nonviable cardiomyocytes after MI cannot be regenerated. Therefore, LV dilatation and remodeling from poor regional and global contractile function mainly account for poor clinical outcomes.[9-13] Therefore, restoring lost myocardium is desirable in treating AMI.

Increasing evidence shows that bone marrow stem cell (SC) therapy seems highly advantageous in improving ischemia-induced or infarct-related cardiac dysfunction.[14-16] Clinical observational studies have further established that the improvement in LV function is mainly because of angiogenesis after SC transplantation.[17,18] Moreover, in vitro studies indicate that extracorporeal shock wave therapy not only can upregulate the expression of vascular endothelial growth factor in cultured human umbilical vein endothelial cells[19,20] and in rat bone marrow cells,[21]but it can also promote bone marrow cells to develop endothelial phenotype.[21] Accordingly, this study tested the hypothesis that autologous transplantation of shock wave (SW)-treated culturing bone marrow-derived mononuclear cells (BMDMNCs) into infarct LV myocardium is superior to BMDMNC therapy alone in improving LV function in a rabbit model of AMI.

METHODS

Ethics

All experimental procedures had been approved by the Institute of Animal Care and Use Committee at our hospital and performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, revised 1996).

Animals, Protocol, and Procedure

Pathogen-free, male New Zealand rabbits, weighing 2.5-2.8 kg were used in this study. Rabbits were anesthetized by mixture of intraperitoneal ketamin (25 mg/kg) and rompon (12 mg/kg). After being shaved on the chest, each rabbit was placed in supine position on a warming pad at 37°C. Under sterile conditions, the heart was exposed through a sternotomy without intubation.

AMI was induced in 28 rabbits by left coronary artery ligation (LCAL) just below the first diagonal branch with 5-0 prolene suture. Regional myocardial ischemia was verified by noting a rapid change from reddish to whitish-dark color of anterior surface of the LV and rapid development of akinesia and dilatation in the at-risk area. These rabbits were assigned to saline-treated group (group 2, $n = 8$), BMDMNC-treated group (group 3, $n = 10$), and preimplantation SW-treated BMDMNCs (group 4, $n = 10$). Eight rabbits receiving sternotomy without LCAL served as sham controls (group 1).

Preparation of BMDMNCs for Transplantation

Groups 3 and 4 rats were anesthetized by intraperitoneal ketamin (25 mg/kg) and rompon (12.0 mg/kg) 7 days before LCAL. After being shaved on both thighs, the rabbit was placed on a warming pad at 37 °C. Local anesthesia with 1 mL of 2% xylocaine was injected into the area before separating the ligament from the patella. An electric rotablator with diameter of 0.2 mm was used to screw straight into the femoral bone from the distal end of femoral fossa. A sterile 22-gauge needle syringe was then used to aspirate the bone marrow from the orifice created.

Bone marrow cells from each rabbit were buffered in 10 mL RPMI 1640 medium (Gibco, Carlsbad, CA) and then digested for 40 minutes with 0.01% collagenase B and DNase1, and filtered through a 30-μM nylon mesh. The BMDMNCs were then isolated by Ficoll-paque (Amersham, Piscataway, NJ) density-gradient centrifugation. Finally, the interphase of BMDMNCs was collected. These cells were washed twice with phosphate buffered saline (PBS) and finally centrifuged at 400 *g* for 5 minutes. About 1.5×10^7 BMDMNCs were obtained from each rabbit (both femoral bones) using this method and then cultured in 60 mm diameter dish with 10 mL Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum) culture medium.

Application of SW to the Culturing BMDMNCs

On the basis of the results of our recent report,[21] this study used 280-SW as the optimal energy dose of SW therapy. Hence, 280-SW (defined as 280 shots in total, given at 0.09-mJ/mm^2) (Machine: EvoTron RFL0300; Probe: EvoTrode R05, High Medical Technologies AG) was applied once to BMDMNCs in group 4, 30 minutes before BMDMNCs transplantation into the infarct area (IA).

Autologous Implantation of BMDMNCs into the IA of Left Ventricle

By day 7 after cell cultures, AMI was induced through LCAL. The BMDMNCs were implanted 15 minutes after LCAL. Thirty minutes before BMDMNCs implantation, CM-Dil (Vybrant Dil cell-labeling solution, Molecular Probes, Inc., Carlsbad, CA) (50 μg/mL) was added to the culture medium for identifying cells in an implanted area. Approximately 1.0×10^7 BMDMNCs in 750-μL culture medium Dulbecco's modified Eagle's medium were implanted into the IA in groups 3 and 4 rabbits, and 750 μL saline was injected into the IA using a 30-gauge syringe in group 2 rabbits.

Functional Assessment by Echocardiography

Transthoracic echocardiography was performed preoperatively using a commercially available echocardiographic system (UF-750XT) equipped with a 8-MHz linear-array transducer for animals (FUKUDA Denshi Co. Hongo, Bunkyo-Ku, Tokyo, Japan)—with rabbits in a supine position—on day 30, 90, and 180 after BMDMSC implantation under anesthesia. LV internal dimensions—(end-systolic diameter and end-diastolic diameter)—were measured according to the American Society of Echocardiography leading-edge method using at least three consecutives cardiac cycles. The LV ejection fraction (LVEF) was calculated as follows: LVEF (%) = (LVEDD [left ventricular end-diastolic dimension]^3−LVEDS^3]/LVEDD^3 ×100.

All measurements were performed by an animal cardiologist blind to treatment and nontreatment groups.

Immunoflourescence Imaging Study for Troponin I

On day 42 after BMDMNC implantation, two rabbits in groups 3 and 4 were sacrificed for identification of differentiated BMDMNCs in IA.

Assessment of viable myocardium in IA was performed through immunolabeling of troponin-I positively stained cells. The primary antibody (anti-troponin I [Abcam, Cambridge, MA]) and secondary antibody (anti-mouse FITC [Molecular Probes, Carlsbad, CA]) were incubated for 30 minutes at room temperature. Irrelevant antibodies were used as controls.

Histological Study of Fibrotic Area

Masson trichrome staining was used for studying the extent of fibrosis in LV myocardium. The integrated area (μm^2) of fibrosis in sectioned LV myocardium was calculated using Image Tool 3 (IT3) image analysis software (University of Texas, Health Science Center, San Antonio, TX, UTHSCSA; Image Tool for Windows, Version 3.0), as was recently described.[22] Three selected sections were quantified for each animal. Three randomly selected high-power fields (HPFs) (400×) were analyzed in each section. After determining the number of pixels in each Cx43 spot per HPF, the numbers of pixels obtained from the three HPFs were summated. The procedure was repeated in two other sections for each animal. The mean pixel number per HPF for each animal was then determined by summating all pixel numbers and dividing by nine. The mean area of fibrosis per HPF was obtained using a conversion factor of 19.24 (1 μm^2 represented 19.24 pixels).

Isolation of Mitochondria

The LV myocardium was excised and washed with buffer A (Tris-HCl, 100 mM; sucrose, 70 mM; EDTA, 10 mM; and mannitol, 210 mM; pH 7.4). Samples were minced finely in cold buffer A and incubated for 10 minutes. All samples were homogenized in an additional 3 mL of buffer A using a motor-driven grinder. The homogenate was centrifuged twice at 700 *g* for 10 minutes at 4 °C. The supernatant was centrifuged again at 8,500 *g* for 15 minutes, and the pellets were washed with buffer B (Tris-HCl, 10 mM; sucrose, 70 mM; EDTA, 1 mM; and mannitol, 230 mM; pH 7.4). The mitochondria-rich pellets were collected and stored at −70°C.

Western Blot Analysis for Cx43 and Cytochrome-C in Mitochondria

Equal amounts (10-30 μg) of protein extracts from remote viable LV myocardium were loaded and separated by SDS-PAGE using 8-10% acrylamide gradients. Molecular weight standards and rat brain extracts rich in protein kinase C epsilon were electrophoresed as controls. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Amersham Biosciences). Nonspecific proteins were blocked by incubating the membrane in blocking buffer (5% nonfat dry milk in Tween-Tris buffered saline (T-TBS) containing 0.05% Tween 20) overnight. The membranes were incubated with the indicated primary antibodies (Cx43, 1:1,000, Chemicon; Cytochrome C, 1:1,000, BD Biosciences; Actin, 1:10,000, Chemicon) for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-mouse

immunoglobulin IgG (1:2,000, Amersham Biosciences) was applied as the second antibody for 1 hour at room temperature. The washing procedure was repeated eight times within 1 hour, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposure to Biomax L film (Kodak). For quantification, digitized ECL signals were analyzed using LabWork UVP software (Waltham, MA).

Oxidative Stress reaction of LV Myocardium

The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (S7150). The oxyblot procedure was performed according to our recent report.[22] The 2,4-dinitrophenylhydrazine (DNPH) derivatization was carried out on 6 μg of protein for 15 minutes, according to manufacturer's instructions. One-dimensional electrophoresis was carried out on 12% SDS/polyacrylamide gel after DNPH derivatization. Proteins were transferred to nitrocellulose membranes, which were then incubated in the primary antibody solution (anti-DNP 1:150) for 2 hours, followed by incubation with second antibody solution (1:300) for 1 hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by ECL (Amersham Biosciences, ST. Louis, MO), which was then exposed to Biomax L film (Kodak, Rochester, NY). For quantification, ECL signals were digitized using LabWork software (UVP, Waltham, MA). On each gel, a standard control sample was loaded.

Real-Time Quantitative PCR Analysis

Real-time polymerase chain reaction (RT-PCR) was conducted using LightCycler TaqMan Master (Roche, Germany) in a single capillary tube according to the manufacturer's guidelines for individual component concentrations. Forward and reverse primers were each designed on the basis of individual exons of the target gene sequence to avoid amplifying genomic DNA.

During PCR, the probe was hybridized to its complementary single-strand DNA sequence within the PCR target. As amplification occurred, the probe was degraded as a result of the exonuclease activity of Taq DNA polymerase, thereby separating the quencher from reporter dye during extension. During the entire amplification cycle, light emission increased exponentially. A positive result was determined by identifying the threshold cycle value at which reporter dye emission appeared above background.

Vessel Density in LV Myocardium

Immunohistochemical staining of blood vessels was performed with α-smooth muscle (α-SMA) (1:400) as primary antibody at room temperature for 1 hour, followed by washing thrice with PBS. The anti-mouse horseradish peroxidase-conjugated secondary antibody was then added for 10 minutes, followed by washing thrice with PBS. The 3,3′ diaminobenzidine(0.7 gm/tablet, Sigma) was added for 1 minute, followed by washing thrice with PBS. Finally, hematosylin was added for 1 minute as a counterstain for nuclei, followed by washing twice. Three sections of LV myocardium were analyzed in each rat. For quantification, three randomly selected HPFs (×100) were analyzed in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by nine.

Cardiac Catheterization and Definition

By 120 days after BMDMNC implantation, cardiac catheterization was performed using right femoral artery approach. A 3-French pigtail was used for measuring the arterial blood pressure in ascending aorta, LV systolic and end diastolic pressure, and left ventriculogram. Left ventriculogram was recorded for 30° right anterior oblique and 60° left anterior oblique views. The LVEF, LV contractility, and the presence or absence of mitral regurgitation were determined by left ventriculographic study. The MR was categorized into grades 1 (mild), 2 (moderate), 3 (moderate-severe), and 4 (severe), according to traditional method. All measurements were performed by two interventional cardiologists blind to treatment and nontreatment groups.

Statistical Analysis

Data are expressed as mean values (mean ± SD). The significance in differences in the data simply between 2 groups was determined by *t* test. The means among groups were compared by one-way analysis of variance followed by Tukey's multiple comparison procedure (on Table I). Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Table I. Summarized data on body weight, echocardiographic findings, angiographic results, and mortality rate in four groups of rabbits

Variables	Group 1* ($n = 8$)	Group 2* ($n = 8$)	Group 3* ($n = 8$)	Group 4* ($n = 8$)	p Value†
Initial BW (kg)	2.81 ± 0.23	2.77 ± 0.31	2.79 ± 0.28	2.68 ± 0.22	0.682
Final BW (kg)	4.36 ± 0.89	3.98 ± 1.01	4.12 ± 0.88	4.18 ± 1.12	0.486
Echocardiographic findings					
Initial LVEF (%)	75.9 ± 4.0	75.2 ± 4.2	75.1 ± 3.9	74.4 ± 3.7	0.910
Initial LVEDD (cm)	1.39 ± 0.14	1.36 ± 0.21	1.39 ± 0.15	1.39 ± 0.21	0.980
Initial LVESD (cm)	0.82 ± 0.14	0.81 ± 0.84	0.86 ± 0.14	0.85 ± 0.15	0.899
30-day LVEF (%)	76.1^{a} ± 4.0	52.4^{b} ± 5.3	54.9^{b} ± 5.0	58.2^{b} ± 4.3	<0.0001
30-day LVEDD (cm)	1.40 ± 0.14	1.57 ± 0.14	1.47 ± 0.08	1.50 ± 0.08	0.059
30-day LVESD (cm)	0.82^{a} ± 0.14	1.29^{b} ± 0.22	1.15^{b} ± 0.13	1.08^{b} ± 0.20	0.0002
120-day LVEF (%)	76.3^{a} ± 4.9	48.1^{b} ± 3.3	57.7^{c} ± 3.5	64.5^{d} ± 2.9	<0.0001
120-day LVEDD (cm)	1.46^{a} ± 0.10	1.61^{b} ± 0.11	1.48^{a} ± 0.07	1.46^{a} ± 0.09	0.006
120-day LVESD (cm)	0.81^{a} ± 0.08	1.37^{b} ± 0.17	1.11^{c} ± 0.14	0.92^{d} ± 0.11	<0.0001
120-day angiographic results					
AsAo BP (mm Hg)	98.4 ± 6.2	93.3 ± 4.2	95.3 ± 4.8	95.0 ± 5.4	0.301
LVEF (%)	69.4^{a} ± 2.4	44.0^{b} ± 5.5	56.4^{c} ± 2.7	61.5^{d} ± 2.8	<0.0001
Mitral regurgitation	0^{a} ± 0	1.88^{b} ± 1.46	0.63^{a} ± 0.52	0.50^{a} ± 0.93	0.003
120-day mortality	12.5% (1)	25% (2)	12.5% (1)	12.5% (1)	1.0

AsAo BP, ascending aortic blood pressure; BW, body weight; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension.

Different letters (a, b, c, d) being used for grouping, showing significant difference (i.e., $p < 0.05$) among different groups by Tukey's multiple comparison procedure.

*Group 1 = sham control; Group 2 = acute myocardial infarction (AMI); Group 3 = AMI plus BMDMNCs; Group 4 = AMI plus BMDMNCs and shock wave.

†One-way analysis of variance.

RESULTS

Body Weight, Echocardiographic, and Angiographic Findings and Mortality (Table I)

The initial and final body weight did not differ among the four groups. Additionally, echocardiographic study demonstrated no differences in terms of initial LVEF, left ventricular end-systolic dimension (LVESD), and LVEDD. By day 30 after AMI induction, the echocardiographic examination revealed that LVEDD was similar among the four groups. However, the echocardiographic findings showed that LVESD was significantly higher, whereas LVEF was significantly lower in groups 2-4 as compared with that in group 1 ($p < 0.005$). Additionally, by day 120 after AMI induction, both echocardiographic and angiographic studies (Fig. 1) revealed that LVEF was substantially lower in group 2 than in other groups, notably lower in group 3 than in groups 1 and 4, and also significantly lower in group 4 than in group 1 ($p < 0.001$). Furthermore, the echocardiographic finding identified that the LVESD and LVEDD were significantly higher in groups 2-4 than in group 1, and significantly higher in group 2 than in groups 3 and 4 ($p < 0.01$). Moreover, the LVESD was notably higher in group 3 than in group 4 on day 120 after AMI induction ($p < 0.0001$). These findings indicate that BMDMNC transplantation can improve LV function and reduce LV remodeling after AMI. In addition, these finding implicate that, as compared with BMDMNC therapy alone, preimplantation SW therapy of BMDMNCs can provide additional benefit in preserving LV function and attenuating LV remodeling.

LV angiogram showed that mitral regurgitation was notably higher in group 2 than in other groups ($p < 0.005$). The 120-day mortality did not differ among the four groups ($p = 1.0$).

Identification of Implanted BMDMNCs in LV Myocardium

By day 120 after AMI induction, the rabbits were sacrificed for identifying differentiated BMDMNCs in LV myocardium. Numerous CM-Dil-stained undifferentiated BMDMNCs were found to have engrafted (Fig. 2). However, only some CM-Dil-stained

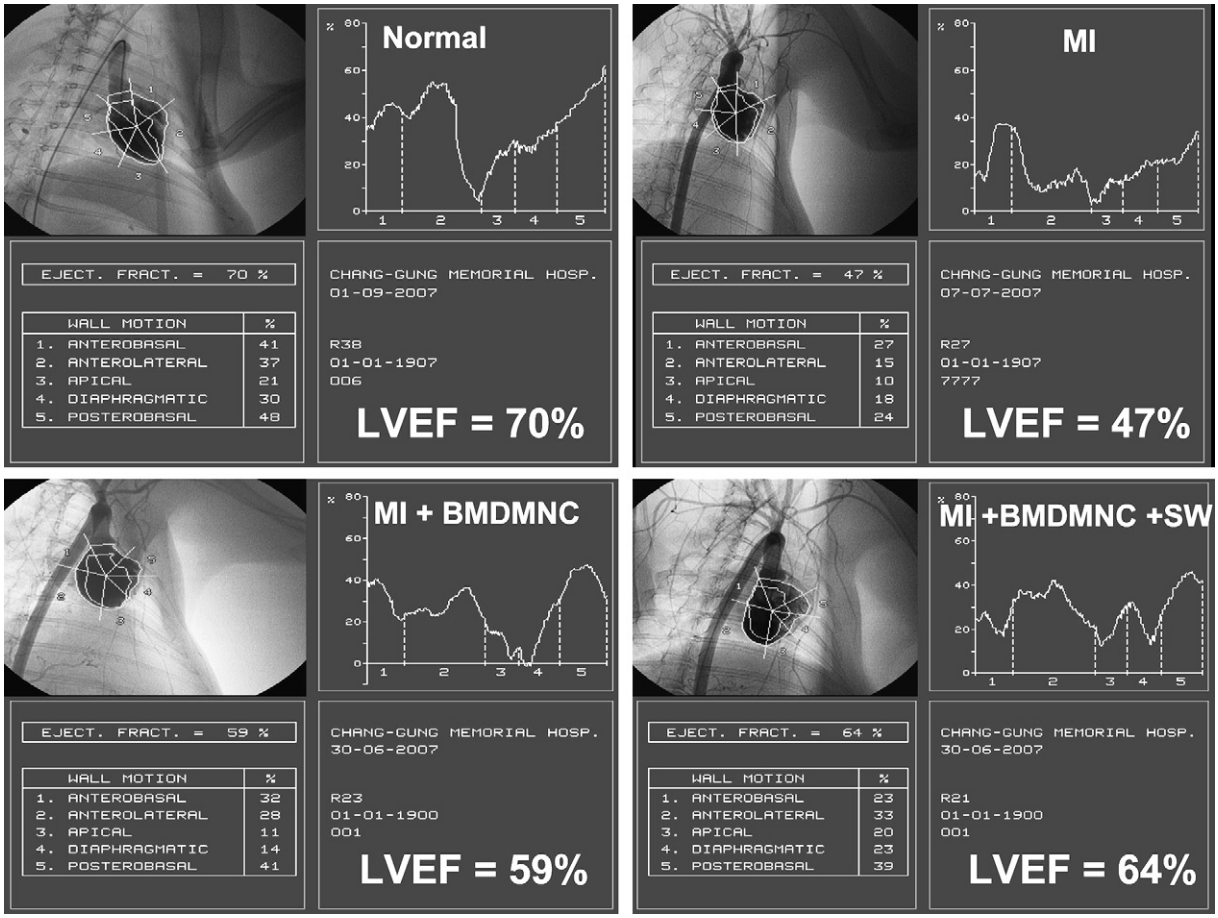

Fig. 1. A total of 120-day left angiogram of four groups. MI, myocardial infarction; BMDMNC, bone marrow-derived mononuclear cell; SW, shock wave.

engrafted cells presenting as myogenic-like cells were stained positively for troponin I.

Preimplantation SW Treatment Further Reduced IA on Day 120 after AMI

Immunofluorescence imaging was performed (Figs. 3A–D) to determine whether IA was reduced after BMDMNC implantation and further reduced by preimplantation SW treatment of BMDMNCs (Fig. 3). The results revealed that the summation area of troponin-I positively stained myocardium in IA was remarkably higher in groups 3 and 4 than in group 2, and significantly higher in group 4 than in group 3 (Fig. 3E) (all p values <0.007). Additionally, Masson trichrome staining (Figs. 3F-I) identified that the summation of fibrosis in IA was substantially higher in group 2 than in groups 3 and 4 and notably higher in group 3 than in group 4 (Fig. 3J) ($p < 0.04$). These findings indicate that SW application to preimplantation BMDMNCs further reduces myocardial death in IA.

mRNA Expressions of Matrix Metalloproteinase -9, IL-10, ET-1, and eNOS on Day 120 after AMI

To elucidate whether implantation of BMDMNC attenuated AMI-induced inflammatory response, mRNA expression of matrix metalloproteinase (MMP)-9, an index of inflammatory reactivity, was performed (Fig. 4). As expected, the MMP-9 mRNA expression was markedly increased in IA in group 2 compared with groups 3 and 4 (Fig. 4A) ($p < 0.05$). Moreover, to determine whether implantation of BMDMNC elicited an anti-inflammatory response, the mRNA expression of interleukin (IL)-10, an index of anti-inflammatory reactivity, was assessed. Interestingly, the results demonstrated that as compared with groups 3 and 4, IL-10 mRNA expression in IA was remarkably lower in group 2 (Fig. 4B) ($p < 0.05$). These findings support those of recent studies,[22,23] demonstrating an immunomodulatory action of stem cell therapy.

To determine the integrity of endothelial function in IA, mRNA expressions of endothelial

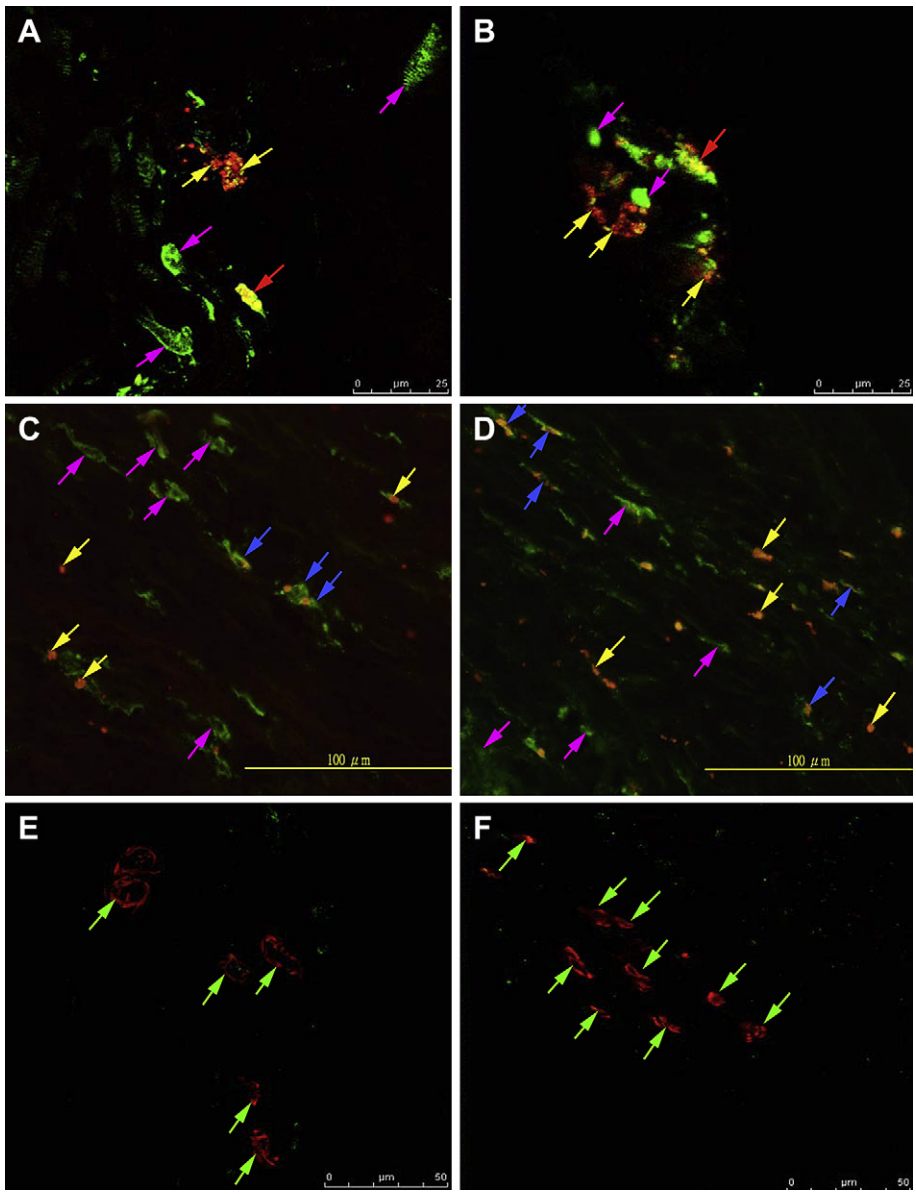

Fig. 2. A-D Confocal imaging study showing merged images from double staining (Dil and troponin-I) in groups 3 and 4 on days 42 (**A, B**) and 120 (**C, D**) after AMI induction, respectively. Note that only a few troponin I-positive myogenic-like cells (red *arrows*) in both groups, whereas the majority being undifferentiated BMDMNCs (yellow *arrows*) (implanted cells with red color from Dil staining). Troponin-I-positive native myocardium also present (pink *arrows*) with some BMDMNCs engrafted into native myocardium (blue *arrows*). **E, F** Confocal microfluorographs showing increased number of α-smooth muscle (SMA) actin-positive cells (green *arrows*) in (**F**) (group 4) than in (**E**) (group 3) on day 120 after AMI induction. Scale bars in right lower corner represent 25μm (**A, B, E, F**) and 100 μm (**C, D**), respectively.

(ET)-1 and endothelial nitric oxide synthase (eNOS) were evaluated. Results of RT-PCR showed that ET-1 mRNA expression, an index of endothelial dysfunction, was significantly increased in group 2 as compared with group 1 (Fig. 4C) ($p < 0.05$). However, this mRNA expression was significantly decreased in groups 3 and 4 than in group 2, and further decreased in group 4 than in group 3 ($p < 0.05$). Conversely, mRNA expression of eNOS was significantly decreased in group 2 as compared with group 1 (Fig. 4D) ($p < 0.05$). However, this mRNA expression was notably increased in group 3 than in group 4, and more remarkably increased in group 4 than in group 3 ($p < 0.05$). These findings support the fact that endothelial dysfunction is present after AMI that can be reversed by BMDMNC transplantation and be further improved by preimplantation SW treatment.

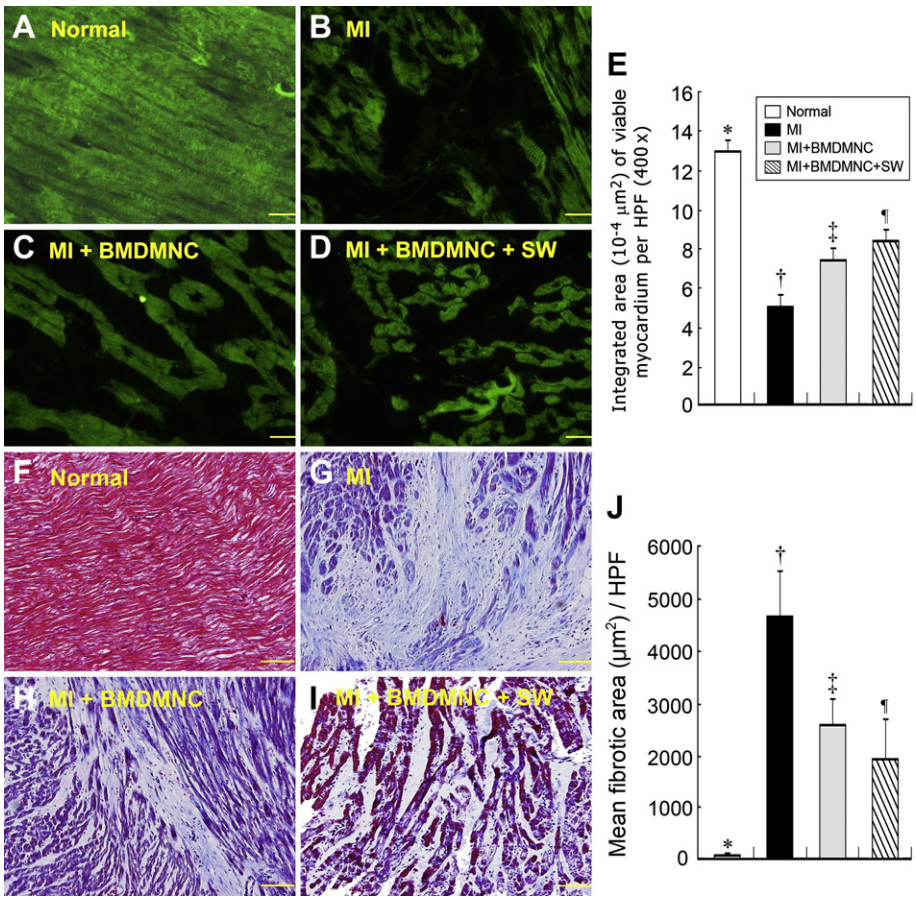

Fig. 3. **A-D** Immunofluorescence imaging study demonstrating significantly smaller integrated area (μm^2) of troponin-I-positive myocardium in infarct area (IA) in group 2 than in group 1 (**A**), group 3 (**C**) and group 4 (**D**) on day 120 after acute myocardial infarction (AMI) induction ($n = 8$). **E** Different symbols (*, †, ‡, ¶) indicate significant difference (all p values <0.007). Scale bars in right lower corner represent 20 μm. **F-I** Mean fibrotic area (μm^2)/high-power field (HPF) (100×) in each group ($n = 8$) of rabbits on day 90 after DCM induction. Masson trichrome staining showing markedly increased fibrosis area (whitish area) in group 2 (**G**) compared with group 1 (**F**), group 3 (**H**), and group 4 (**I**). **J** Different symbols (*, †, ‡, ¶) indicate significant difference (all p values <0.04). Scale bars in right lower corner represent 100 μm.

Western Blot Analyses of Cytochrome C and Cx43 Expressions in Mitochondria of IA on Day 120 after AMI

The total cytochrome C protein expression in mitochondria was markedly lower in group 2 than in groups 1, 3, and 4 (Fig. 5A) ($p < 0.05$). Moreover, this protein expression in mitochondria was significantly lower in groups 3 and 4 than in group 1, and notably lower in group 3 than in group 4 ($p < 0.05$). These findings indicated that the expression of cytochrome C, an index of energy supply and storage in mitochondria, was more reduced in group 2 than in other groups, suggesting significant mitochondrial damage in the myocardium of this group of animals.

The results of Western blotting showed that mitochondrial Cx43 protein expression in IA was substantially lower in group 2 than in groups 1, 3, and 4 (Fig. 5B) ($p < 0.05$). Besides, this protein expression in mitochondria was remarkably lower in groups 3 and 4 than in group 1, and notably lower in group 3 than in group 4 ($p < 0.05$). These findings reveal that preimplantation SW treatment of culturing BMDMNCs achieves better Cx43 protein expression in mitochondria than BMDMNC therapy only.

Western Blot Analysis of Oxidative Stress in Mitochondria of IA on Day 120 after AMI

Conversely, the results of Western blotting demonstrated that oxidative stress in mitochondria was markedly higher in group 2 than in groups 1, 3, and 4, and significantly higher in groups 3 and 4 than in group 1 (Fig. 6) ($p < 0.05$). These findings

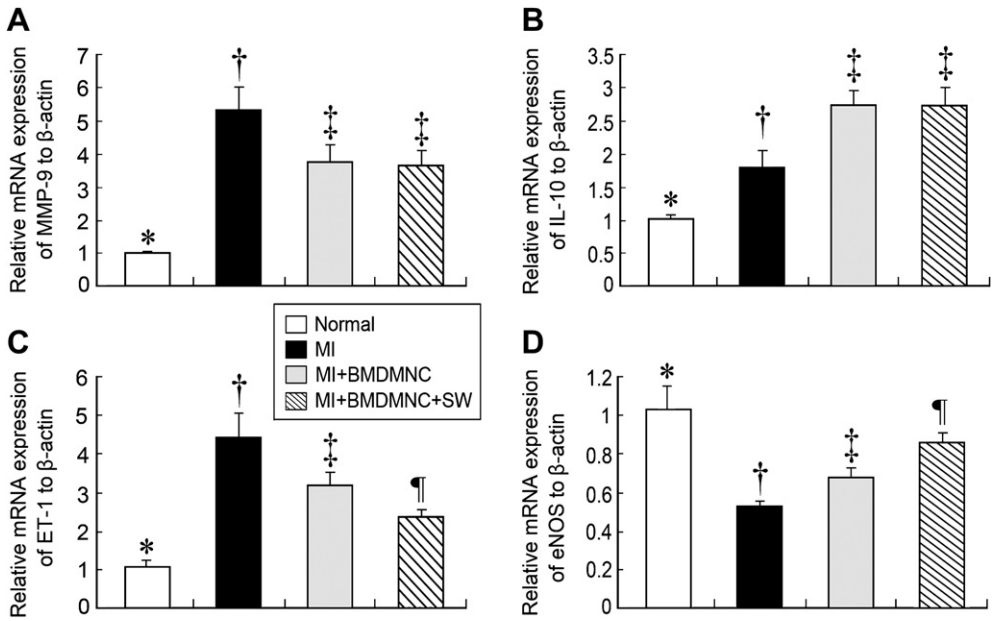

Fig. 4. A-D mRNA expressions of metalloproteinase (MMP)-9 (**A**), interleukin (IL)-10 (**B**), endothelial (ET)-1, and endothelial nitric oxide synthase (eNOS) in each group ($n = 8$) of rabbits on day 120 after AMI induction. Different symbols (*, †, ‡, ¶) indicate significant difference (all p values <0.05).

further support those from recent studies[22,23] showing modulation of immune reactivity by stem cell therapy through downregulating innate and adaptive immunity.

Small Arteriolar Density Analysis

The number of small arterioles (≤100 μm in diameter) in IA was notably lower in groups 1 and 2 than in groups 3 and 4 on day 90 after AMI induction ($p < 0.008$) (Fig. 7). Furthermore, the number of small arterioles was significantly higher in group 4 than in group 3 ($p < 0.05$). These findings indicate that BMDMNC transplantation induced angiogenesis and vasculogenesis, and SW-treated culturing BMDMNCs before transplantation further enhances the processes.

DISCUSSION

An Additional Benefit of Preimplantation SW Application to Culturing BMDMNCs for Improving LV Function and Attenuating LV Remodeling

Both experimental and clinical observational studies have established that bone marrow stem cell therapy improves ischemia- or infarct-related LV dysfunction.[14-19,24] The present study, using a rabbit AMI model, also revealed that BMDMNC implantation into IA improved 120-day LV function. Therefore, the results of the current study strengthen the findings of those previous studies.

In fact, although growing evidence indicates that various types of cellular therapy improve LV function in the setting of myocardial ischemia or AMI,[14-17,24,25] the recovery of LV function is usually shown to be partial and incomplete.[14-16,24,25] Therefore, in addition to cell therapy, various adjunctive strategies[26,27] have been attempted to further improve LV function. The most important finding in the present study is that preimplantation SW treatment for BMDMNCs offered an additional benefit in improving 120-day LV function and attenuating LV remodeling. Interestingly, recent in vivo experimental[19] and clinical observational[28] studies demonstrated that extracorporeal SW therapy could improve ischemia-induced myocardial dysfunction. Accordingly, the results of the current investigation, in addition to being consistent with those of the recent studies,[19,28]provide another potential therapeutic avenue to improving LV function in setting of AMI undergoing autologous BMDMNC transplantation by adopting SW cellular pretreatment.

Rationale and Mechanisms of Improving LV Function after BMDMNC Transplantation

Although the mechanisms for improving cardiac function after cellular therapy in ischemic or

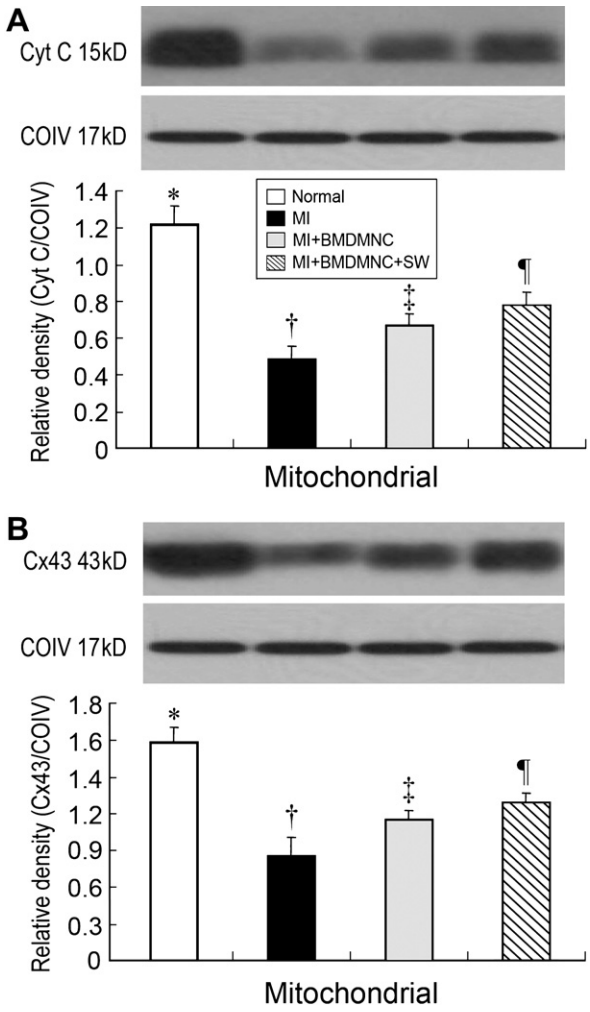

Fig. 5. A Western blot analysis ($n = 8$) demonstrating substantially lower mitochondrial cytochrome C (cyto C) protein expression in group 2 than in groups 1, 3, and 4 in IA on day 120 after AMI induction. Different symbols (*, †, ‡, ¶) indicate significant difference (all p values <0.05). **B** Western blot ($n = 8$) showing remarkably lower mitochondrial connexin43 (Cx43) protein expression in IA of group 2 as compared with groups 1, 3, and 4 on day 120 after AMI induction. Different symbols (*, †, ‡, ¶) indicate significant difference (all values of p <0.05).

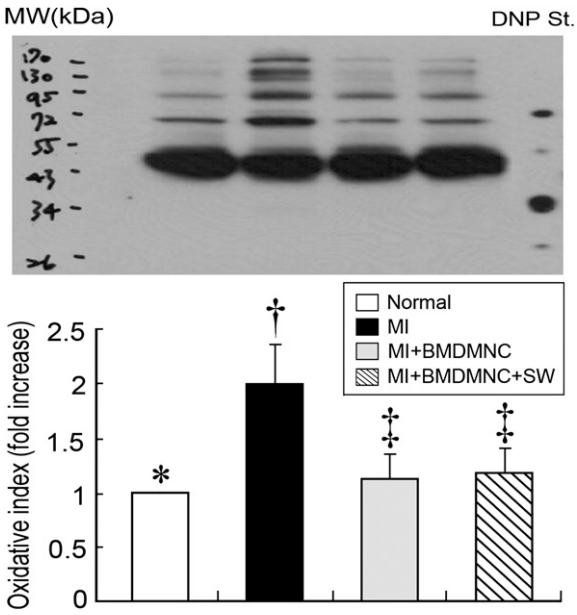

Fig. 6. Western blotting showing notable increase in the oxidative index, protein carbonyls, in IA of group 2 compared with groups 1, 3, and 4 on day 120 after AMI but no significant difference between groups 3 and 4 (*upper* panel), with quantification results of each group ($n = 8$) shown (*lower* panel). Note: *Right* lane and *left* lane shown on upper panel represent control oxidized molecular protein standard and protein molecular weight marker, respectively. Different symbols (*, †, ‡, ¶) indicate significant difference (all p values <0.05).

infarcted myocardium, including myogenesis,[14,29] cytokine production,[17]effects of paracrine mediators,[29-31]a myocardial homing by stem cells to the myocardium for repair and angiogenesis,[32,33]or angiogenesis,[14,16,22,27,29] has been extensively debated, the principal mechanism is still unclear. The principal finding in the present study was that immunofluorescence imaging study identified the differentiation of only a small population of BMDMNCs into troponin I-positive myogenic-like cells, whereas most of the implanted BMDMNCs were found to be undifferentiated in the implantation area. Therefore, the discrepancy between immunofluorescence imaging study for differentiation of implanted BMDMNCs into troponin I-positive myogenic-like cells and echocardiographic or angiographic findings of significantly improved LV function and attenuated LV remodeling by preimplantation SW therapy on day 120 after BMDMNC implantation suggests the existence of other unidentified confounders.

Studies have previously shown that innate immune mechanisms are activated after myocardial damage, which in turn, initiate the complement cascade, inflammatory reaction, and reactive oxygen species (ROS) generation,[34-36]giving rise to further damage of myocardium.[37] Interestingly, the current study identified that MMP-9 mRNA expression and oxidative stress in mitochondria, indexes of inflammatory responses, were markedly increased in IA after AMI. Therefore, our findings further support those from previous studies.[34-36] Conversely, mRNA expression of IL-10, an anti-inflammatory contributor, was significantly lower in IA after AMI. Notably, BMDMNC implantation into the IA not only notably downregulated

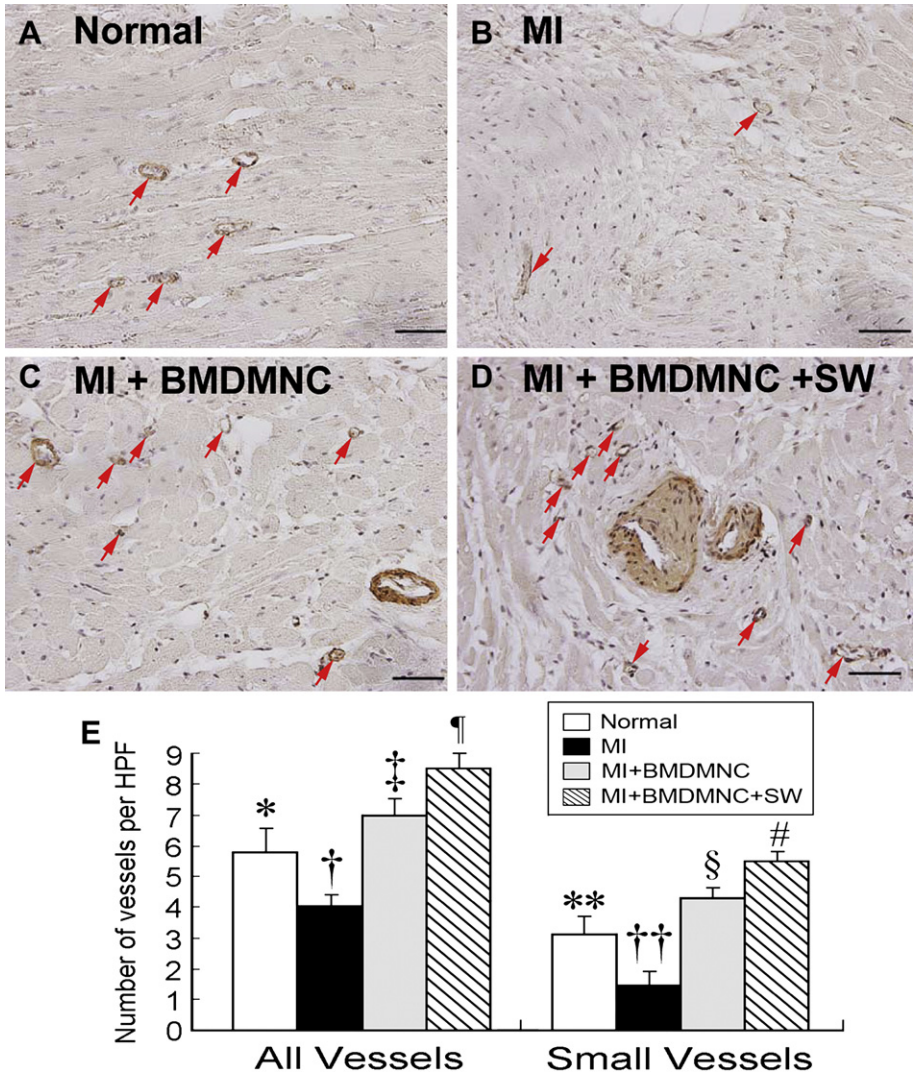

Fig. 7. Identification of blood vessel distribution (red *arrows*) in BMDMNC-implanted region using α-SMA immunohistochemical staining (*upper* panel) (200×). Small vessels (diameters ≤100 μm) quantification for each group ($n = 8$) on 120 day after AMI induction (*lower* panel) showing notably lower small vessel number in group 2 than in other groups. Scale bars in right lower corner represent 50 μm. Different symbols (*, †, ‡, ¶) and (§, #, **, ††) indicate significant difference (all p values <0.008) A-D indicate individual study group. E = indicates the statistically analytical results.

MMP-9 and oxidative stress levels but also remarkably upregulated the IL-10 level in IA. Our findings, in addition to reinforcing those of recent studies [22,23] demonstrating the immunomodulatory characteristics of stem cell therapy, could also explain the significant attenuation in fibrosis and death of myocardium in IA, the notable preservation of LV function and limitation in LV remodeling after BMDMNC therapy.

Another important finding in the present study was that mRNA expression of ET-1 was significantly increased, whereas the mRNA expression of eNOS was markedly suppressed in IA after AMI. However, these mRNA expressions were significantly reversed after BMDMNC implantation, suggesting that BMDMNC therapy significantly reversed endothelial dysfunction in IA. More importantly, ET-1 gene expression was further inhibited and eNOS gene expression and angiogenesis or vasculogenesis were further enhanced using BMDMNCs with preimplantation SW treatment than by BMDMNC therapy alone, suggesting SW pretreatment further improved 120-day LV function.

Previous study has revealed that changes in Cx43 expression patterns are related to various cardiac pathologies and have been shown to be associated with an initiation of cardiac arrhythmia.[38] Besides, enhancement of Cx43 expression in cardiomyocyte

mitochondria during ischemic preconditioning has been suggested to play an essential role in reducing infarct size.[39] In the present study, Western blot analysis identified that the protein expressions of Cx43 in mitochondria and the total amount of cytochrom C, an index of energy supply and storage in mitochondria, were notably increased after BMDMNC therapy than in AMI without treatment. Surprisingly, preimplantation SW treatment of culturing BMDMNCs was observed to further enhance the Cx43 and cytochrome C protein expressions in mitochondria. Therefore, the findings from our study provide a plausible explanation for the well-preserved LV function and attenuated LV remodeling in AMI rabbits with BMDMNC therapy than in those without. In addition, the results of the current study further highlight the use of combined therapy with SW and BMDMNCs as an effectual treatment strategy for reducing LV remodeling and preserving LV function after AMI.

This study has limitation. Both echocardiography and angiogram which were used in the current study for measurement of LVEF might not accurately refer the true LV function. Other methods, such as LV stain or tissue Doppler echo, may be the more reliable methods for assessment of the LV function.

In conclusion, the results of this study demonstrate that the adoption of preimplantation SW-treated BMDMNC is superior to BMDMNC alone in preventing LV remodeling and improving LV function in an experimental model of AMI.

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