Autologous Bone Marrow-Derived Mononuclear Cell Therapy Prevents the Damage of Viable Myocardium and Improves Rat Heart Function Following Acute Anterior Myocardial Infarction

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Background We examined the effects of bone marrow-derived mononuclear cells (BMDMNCs) on preventing viable myocardium damage from myocardial infarction (MI) in a rat MI model.

Methods and Results Saline (group 1) or BMDMNCs (group 2) were implanted into the infarct area (IA) of 1-week-old anterior wall MI Sprague–Dawley (SD) rats. Twenty SD rats without MI served as the controls (group 3). The results demonstrated that in remote viable myocardium, the integrated area (μm²) of connexin43 spots was lower, whereas the number of apoptotic nuclei were higher in group 1 than in groups 2 and 3 on day 90 following BMDMNC implantation (all p<0.001). Additionally, the number of vessels and survival myocardium in the IA was lower in group 1 than in groups 2 and 3 (all p<0.005). Furthermore, the mRNA expressions of nitric oxide synthase, interleukin-8/Gro-α, interleukin-10 and matrix metalloproteinase-9 were higher in group 2 than in groups 1 and 3 in peri-IA (all p<0.05). On days 42 and 90, the left ventricular (LV) function was lower in group 1 than in groups 2 and 3 (p<0.001).

Conclusions Autologous BMDMNC therapy improves LV function, and mitigates molecular and cellular perturbation following MI. (Circ J 2008; 72: 1336–1345)

Key Words: Bone marrow-derived mononuclear cell therapy; Left ventricular function; Molecular and cellular perturbation; Myocardium infarction

C}linal observational studies have demonstrated that both angiotensin-converting enzyme inhibitors and angiotensin type I receptor blockers can attenuate left ventricular (LV) enlargement in association with reduced mortality after myocardial infarction (MI).1–3 However, despite the application of pharmacotherapeutics1–3 or mechanical intervention,4 dead cardiomyocytes following MI cannot be regenerated.

Growing experimental evidence indicates that bone marrow-derived mesenchymal stem cells (BMDMSCs) appear to be highly advantageous for cell therapy.5–7 Experimental and clinical studies have recently demonstrated that autologous BMDMSC transplantation improved ischemic-induced or infarct-related cardiac dysfunction.5–7 Thus, autologous BMDMSC transplantation has been proposed as a promising novel therapeutic strategy for ameliorating ischemia-induced myocardial dysfunction and restoring heart function following acute MI (AMI).5–11 Although short-term and intermediate-term results5–8,12 are attractive and promising, the mechanisms underlying improvement of cardiac function following BMDMSC therapy remain poorly defined.7,10,13

Experimental studies have demonstrated that damage to injured and remote viable myocardium from AMI is frequently progressive.14,15 Additionally, living cells are challenged by myriad acute and chronic stresses,16–18 and programmed cell death, or “apoptosis,” has been shown to increase following various cardiac insults, such as ischemia-reperfusion injury and cardiac failure.19 Moreover, gap junctions, which are composed of connexin (Cx) subunits, play a key role in electrical coupling between cardiomyocytes.20,21 Changes in Cx expression patterns, which are associated with a variety of cardiac pathologies, contribute to the development of cardiac arrhythmia.22–24 Nevertheless, the impact of AMI on remote (non-infarct area) viable myocardium, which undoubtedly plays an essential role in maintaining heart function following infarction, remains uncertain. This study tested the hypothesis that AMI induced apoptotic body formation and suppressed Cx43 gap junction expression in viable myocardium, and examined the effects of autologous transplantation of bone marrow-derived mononuclear cells (BMDMNCs) on preserving LV function and preventing molecular and cellular perturbations in a rat MI model. Additionally, this study also measured the impact of BMDMNC therapy on mRNA
expressions of endothelial nitric oxide synthase (eNOS), interleukin (IL)-8/Gro-1, CXC chemokine family, IL-10, and matrix metalloproteinase (MMP)-9, which were identified to regulate the myocardial homing and neovascularization from bone marrow to damaged myocardium.25

Methods

Animals, Protocol and Procedure

Pathogen-free, adult male Sprague–Dawley (SD) rats, weighing 250–300 g (Charles River Technology, BioLASCO Taiwan Co, Ltd, Taipei, Taiwan) were anesthetized by chloral hydrate (35 mg/kg, ip) and placed in a supine position on a warming pad at 37°C, followed by endotracheal intubation with positive-pressure ventilation (180 ml/min) with room air using a Small Animal Ventilator (SAR-830/A, CWE, Inc, Ardmore, PA, USA). Under sterile conditions, the heart was exposed via a left thoracotomy.

AMI was induced in 40 SD rats by left coronary artery ligation (LCAL) just below the left atrium in the atrioventricular groove with 7-0 prolene sutures. These rats were assigned to a saline-treated group (group 1, n=20) and a BMDMNC-treated group (group 2, n=20). Rats (n=20) receiving thoracotomy without LCAL served as over sham control (group 3).

Preparation of BMDMNCs for Autologous Transplantation

The group 2 rats were anesthetized by chloral hydrate (35 mg/kg, ip) on day 3 following LCAL and placed on a warming pad at 37°C. After carefully separating the ligament from the patella, an electric rotablator with a diameter of 0.2 mm was used to screw straight into the femoral bone. A sterile 22-gauge needle syringe was then used to aspirate the bone marrow.

Bone marrow cells from each rat were buffered in 10 ml RPMI1640 medium (Gibco) and then digested for 40 min with 0.01% collagenase B and DNase1, and filtered through a 30 μm nylon mesh. The BMDMNCs were then isolated by Ficoll-paque (Amersham) density-gradient centrifugation. Finally, the interphase of BMDMNCs was collected. These cells were washed twice with PBS and finally centrifuged at 400 g for 5 min. Approximately 1.2–2.0×10⁶ BMDMNCs were obtained from each rat (both femoral bones) via this method and then cultured in a 60 mm diameter dish using 10 ml DMEM culture medium containing 10% FBS, 1% penicillin–streptomycin (HPF)-conjugated anti-mouse immunoglobulin IgG (Kodak). Protein bands were quantified using a Labwork software (UVP) densitometer.

Immunocytochemical Staining

BMDMNCs were also obtained from 4 other rats. These BMDMNCs (1.2×10⁶ cells/rat) were then cultured in 10 ml DMEM culture medium containing 10% FBS cell culture supernatant for 21 days to examine the presence of eNOS, CD31, CD105, and D1-SMA-positive stained cells. The 5-azacytidin was added into the culture medium on day 3 following culturing for 24 h stimulation.

Western Blot Analysis for Cx43

To investigate the impact of BMDMNC therapy on preventing down-regulation of Cx43, protein aliquots (30 μg) of LV myocardium were Western blotted for Cx43 according to manufacturer instructions. The membranes were incubated with the indicated primary antibody (Cx43: 1:1,000, Chemicon) for 1 h at room temperature. Horseradish peroxidase (HPF)-conjugated anti-mouse immunoglobulin IgG (1:2,000, Amersham Biosciences) was used as a secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to Biomax L film (Kodak). Protein bands were quantified using a Labwork software (UVP) densitometer.

Real-Time (RT) Quantitative Polymerase Chain Reaction (PCR) Analysis for IL-8/Gro-1, IL-10, MMP-9 and eNOS

A RT-PCR was conducted using a LightCycler TaqMan Master (Roche, Mannheim, Germany) in a single capillary tube according to the manufacturer’s guidelines for individual component concentrations.
Vessel Density in Infarct Area

Immunohistochemical stain of blood vessels was performed with І-СМА (1:400) as a primary antibody at room temperature for 1 h, followed by washing with PBS thrice. The anti-mouse-HRP conjugated secondary antibody was then added for 10 min, followed by washing with PBS thrice. The 3,3’ dianinobenzidine (0.7 gm/tablet) (Sigma) was added for 1 min, followed by washing with PBS thrice. Finally, hematosylin was added for 1 min as a counter stain for nuclei, followed by washing twice. For quantification, 3 sections of the infarct area were chosen for each rat and 3 randomly selected HPFs (x100) were analyzed in each slide. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9.

TUNEL Assay for Apoptotic Nuclei

For each rat, 6 sections (3 longitudinal and 3 transverse sections of LV myocardium) were analyzed by an in situ Cell Death Detection Kit, AP (Roche), according to the manufacture’s guidelines. The TUNEL-positive cells were examined in 3 randomly chosen HPFs (x400) and normalized to the total number of cells divided by 18.

Statistical Analysis

Data are expressed as mean values (mean±SD). The significance of differences between groups was evaluated with a t-test. Continuous variables among 4 groups were compared using the Kruskal-Wallis test, followed by a multiple comparison procedure with the Wilcoxon’s rank sum test and Bonferroni’s correction. SAS statistical software for
Bone Marrow Cells Therapy on MI

Fig 2. (A) Cells positively-stained for CD31 (red arrows, 400×) (FITC stain). (B) Cells positively stained for troponin I (red arrow, 400×) (FITC stain). (C) Cells positively stained for α-smooth muscle actin (α-SMA) (400×) (rhodamine stain) on day 21 following cell culture. (D) Spindle-shaped mesenchymal stem cells over the culture plate was observed on day 30 culturing (200×). The scale bars in the right lower corner represent 50 μm (A–C). Flow cytometry showed cells positively stained for troponin I (E) and CD31 (F) on day 21 following culturing. BMDMNCs, bone marrow-derived mononuclear cells.

Table 1  Body Weight and Echocardiographic Results in 3 Group Rats

<table>
<thead>
<tr>
<th></th>
<th>Group 1**</th>
<th>Group 2**</th>
<th>Group 3**</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (gm) (n=20)</td>
<td>297.3±24.8</td>
<td>301.8±25.9</td>
<td>300.6±26.0</td>
<td>0.776</td>
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<tr>
<td>Final body weight (gm) (n=12)</td>
<td>414.6±53.8</td>
<td>436.3±48.1</td>
<td>442.2±46.2</td>
<td>0.652</td>
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<tr>
<td>Pre-procedural LVEF (%)</td>
<td>90.1±2.6</td>
<td>90.3±2.3</td>
<td>90.7±2.4</td>
<td>0.874</td>
</tr>
<tr>
<td>Pre-procedure FS (%)</td>
<td>56.8±5.6</td>
<td>57.3±5.5</td>
<td>56.6±4.8</td>
<td>0.935</td>
</tr>
<tr>
<td>Pre-procedural EDD (mm)</td>
<td>0.69±0.06</td>
<td>0.71±0.05</td>
<td>0.70±0.04</td>
<td>0.890</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>0.32±0.04</td>
<td>0.31±0.07</td>
<td>0.31±0.06</td>
<td>0.392</td>
</tr>
<tr>
<td>Post-implanted (day 21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>75.9±4.8*</td>
<td>78.7±3.9*</td>
<td>87.3±4.4†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FS (%)</td>
<td>39.9±6.3*</td>
<td>43.7±6.0*</td>
<td>57.3±5.1†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EDD (mm)</td>
<td>0.81±0.05*</td>
<td>0.80±0.04*</td>
<td>0.69±0.05†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>0.51±0.08*</td>
<td>0.47±0.05*</td>
<td>0.30±0.04†</td>
<td>&lt;0.0001</td>
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<tr>
<td>Post-implanted (day 42)</td>
<td></td>
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<td></td>
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<tr>
<td>LVEF (%)</td>
<td>77.0±5.3*</td>
<td>82.5±3.6*</td>
<td>89.9±3.9*</td>
<td>&lt;0.0001</td>
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<tr>
<td>FS (%)</td>
<td>41.7±5.8*</td>
<td>49.2±5.8*</td>
<td>56.9±5.0*</td>
<td>&lt;0.0001</td>
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<td>EDD (mm)</td>
<td>0.83±0.08b</td>
<td>0.77±0.06b</td>
<td>0.69±0.07b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>0.40±0.06b</td>
<td>0.32±0.03b</td>
<td>0.32±0.03b</td>
<td>&lt;0.0001</td>
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<tr>
<td>Final (day 90)</td>
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<tr>
<td>LVEF (%)</td>
<td>76.1±5.5*</td>
<td>85.5±3.6*</td>
<td>90.5±3.0*</td>
<td>&lt;0.0001</td>
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<td>FS (%)</td>
<td>42.4±5.7*</td>
<td>52.5±5.9*</td>
<td>56.7±4.6*</td>
<td>&lt;0.0001</td>
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<tr>
<td>EDD (mm)</td>
<td>0.84±0.07f</td>
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<td>0.70±0.04f</td>
<td>&lt;0.0001</td>
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<tr>
<td>ESD (mm)</td>
<td>0.50±0.06f</td>
<td>0.36±0.02f</td>
<td>0.31±0.03f</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

LVEF, left ventricular ejection fraction; FS, fractional shortening; EDD, end-diastolic diameter; ESD, end-systolic diameter.

**Group 1, myocardial infarction (MI) only; Group 2, MI plus bone marrow-derived mononuclear cells; Group 3, sham control. Symbols (*, †, ¤) or letters (a, b, c) indicate significant difference (at 0.05 level) by Wilcoxon’s rank sum test with Bonferroni’s correction.
Windows version 8.2 (SAS institute, Cary, NC, USA) was used. A probability value <0.05 was considered statistically significant.

Results

Mortality in Each Group

No mortality was observed within group 3 in the study period. However, 5 rats in group 1 and 6 rats in group 2 were dead within 7 days after the procedure. Therefore, in total, 25 rats in group 1 and 26 rats in group 2, respectively, were originally used in the present study.

Histological Findings Indicating LV Anterior Wall Infarction (Fig 1: Upper Panel)

Extensive LV MI was identified grossly (Fig 1A) and further confirmed histopathologically using H.E. (Fig 1B) and Masson Trichrome (Fig 1C) stains.

Immunofluorescence and Flow Cytometry Findings of Cultured BMDMNCs (Fig 2)

By immunofluorescence staining, the cells positive for CD31, a marker of an endothelial phenotype, and cells positive for troponin I, a cardiomyocyte specific marker, and those positive for ß-SMA were observed on day 21 following culturing (Figs 2A–C). Plentiful spindle-shaped BMDMCS were also noted on day 30 of culture (Fig 2D). Furthermore, flow cytometric measurement also identified the presence of troponin-I (Fig 2E) and CD31 positively stained cells (Fig 2F) following 21-day culture.
Initial and Final Body Weight and Serial Echocardiographic Findings (Table 1)

Initial and final body weight did not differ between the 3 groups. There were no significant differences in initial LV ejection fraction (LVEF), fractional shortening (FS), LVEDD, and LVESD between the 3 groups. The LVEF, FS, LVEDD, and LVESD did not differ between groups 1 and 2 on day 21 following BMDMNC implantation. However, LVEF and FS on day 21 were significantly lower, whereas LVEDD and LVESD were markedly higher in groups 1 and 2 than in group 3. Both LVEF and FS on days 42 and 90 (Figs 1D–G) following BMDMNC implantation were significantly lower in group 1 than in groups 2 and 3. Additionally, LVEDD and LVESD were significantly higher in group 1 than in groups 2 and 3. The LVEF and FS were significantly lower, whereas LVED and LVESD were significantly higher in group 2 than in group 3 on day 42 following BMDMNC implantation. Moreover, LVEF was significantly lower in group 2 than in group 3 on day 90 following BMDMNC implantation. However, FS, LVEDD, and LVESD did not differ between groups 2 and 3 on day 90 following BMDMNC implantation.
By days 21 and 42 following transplantation, numerous DAPI-stained undifferentiated BMDMNCs were engrafted into the infarct area (Figs 3A, B). By days 42 and 90, some DAPI-stained engrafted cells presenting as muscle-like cells were stained positively for troponin I in the infarct area (Figs 3C, D). Additionally, DAPI-stained engrafted cells, which were stained positively for α-SMA, were also clearly identified (Lower panel of Fig 3H). Moreover, the number of vessels stained positively for α-SMA was notably higher in group 2 than in group 1 rats [group 2 (5.67±1.07) vs group 3 (2.0±0.89) vs group 1 (0.83±0.75), p=0.0012] (Lower panel of Figs 3E–G).

**Cx43 Expression in the LV Remote Area**

Fig 4A shows the results of quantification of an integrated area (μm²) of clustered Cx43 spots in each group of LV myocardium on day 90 following BMDMNC therapy. The summation area of Cx43 did not differ between group 2 and group 3. However, the number of vessels stained positively for α-SMA was notably higher in group 2 than in group 1 rats [group 2 (5.67±1.07) vs group 3 (2.0±0.89) vs group 1 (0.83±0.75), p=0.0012] (Lower panel of Figs 3E–G).

**Western Blot Analysis of Cx43 in an LV Remote Area** (Fig 5)

The Cx43 protein expression of an LV remote area was substantially lower in group 1 than in groups 2 and 3.

**RT-PCR Analysis**

Changes in the peri-infarct area of mRNA expressions of eNOS, IL-8/Gro-α, IL-10, and MMP-9 were measured by RT-PCR. On day 90 following BMDMNC treatment, mRNA expressions of IL-8/Gro-α (Fig 6A), IL-10 (Fig 6B), and MMP-9 (Fig 6C) in the peri-infarct area of LV were...
significantly higher in groups 1 and 2 than in group 3, and significantly higher in group 2 than in group 1. Conversely, eNOS mRNA expression (Fig 6D) in the peri-infarct area was significantly lower in group 1 than in groups 2 and 3 and significantly higher in group 2 than in group 3.

**Apoptotic Nuclei Formation in Remote Viable Myocardium**

Fig 6E shows the results of quantitative TUNEL detection of apoptotic nuclei. Experimental results showed that the number of apoptotic nuclei was significantly increased in group 1 than in groups 2 and 3, and in group 2 than in group 3.
Vascular Density and Quantitative Survival Myocardium in an Infarct Area

The number of large-sized vessels in an infarct area did not differ between groups 1 and 2. However, the number of small-sized and medium-sized vessels in the infarct area was significantly higher in group 2 than in group 1 on day 90 after BMDMNC implantation (Figs 7A–C). Additionally, the integrated area of survival myocardium was significantly higher in group 2 than in group 1 on day 90 after implantation (Figs 7D–F).

Discussion

Experimental evidence has suggested that BMDMSCs are highly advantageous for cell therapy. Increasing data has also shown that BMDMSCs can be successfully implanted into ischemic or infarct myocardium by different methods. Additionally, these engrafted BMDMSCs can differentiate into myogenic cells and cells with CD31 endothelial phenotype in the infarct area. In the present study, we observed that BMDMNCs were successfully implanted into an infarct myocardium and survived in the infarct area as long as 3 months. Additionally, differentiation of these engrafted BMDMNCs into myogenic cells and β-SMA-positive cells were clearly identified in the infarct area. Thus, our results are consistent with those obtained from recent experimental studies and further support findings acquired by in vitro study.

In this study, although the engrafted BMDMNCs differentiated into troponin I-positive cells in the infarct myocardium, a rather large number of these engrafted BMDMNCs were found to present either as undifferentiated or β-SMA-positive phenotype. The mechanism by which mesenchymal stem cells develop into cardiac myocyte-like cells remains uncertain. Furthermore, poor cell viability associated with transplantation and difficulty obtaining clinically meaningful numbers of transplanted BMDMSCs that have differentiated into myocyte-like cells have been suggested as limitations in the reparative capacity for replacing lost myocardium through these cells. A recent study also demonstrated that implanted allogenic mesenchymal stem cells in post-infarcted rat myocardium did not fully evolve into adult cardiomyocytes. Accordingly, our findings further support the observations that the number of myogenic-like cells in the infarct area having differentiated from BMDMSCs is typically insufficient for restoring MI.

Interestingly, serial echocardiographic measurements showed that LV function was significantly improved and LV chamber size was notably reduced in the BMDMNC therapy group compared with those in the saline-treated MI groups on post-transplant days 42 and 90 in this study. The findings of an inadequate number of BMDMNCs differentiating into myogenic-like cells and the distinctive improvement in LV remodeling and post-transplantation LV function in this study suggest that other unidentified confounders exist. In fact, proposed mechanisms, including angiogenesis, myogenesis, cytokine effects, or effect of paracrine mediators, or a myocardial homing by BMDMSCs to the myocardium for reparation and angiogenesis underling improved cardiac function following BMDMNC therapy in ischemic or infarcted myocardium have been extensively debated. However, the exact mechanism remains poorly defined. Collectively, we suggest that the significance of any single mechanism acting alone is unlikely. Furthermore, because it appeared that the site of BMDMSC injection is irrelevant, the BMDMSC-derived paracrine mediators might be the key elements in improving LV function and remodeling.

The effects of cytokines on myocardial homing of bone marrow angioblasts and neovascularization have been demonstrated in a recent experimental study. Importantly, RT-PCR identified that mRNA expressions of IL-8/Gro-1, IL-10, and MMP-9 in a peri-infarct area were significantly higher in the BMDMNC therapy group than that in the saline-treated MI group in the present study. Moreover, eNOS gene expression in the peri-infarct area and the number of vessels in the infarct area were both markedly higher in the BMDMNC therapy group than in the saline-treated MI group. Therefore, our findings, together with those from other studies, might explain the substantially higher summation area of survival myocardium in the infarct zone after BMDMNC treatment compared to the saline-treated MI group in this study. More importantly, our findings suggest that the new vessel formation is the result of angiogenesis and vasculogenesis and the effects of cytokines following BMDMNC transplantation. Interestingly, in vitro studies have recently demonstrated that either cytokine or shock wave therapy applied to BMDMNC can enhance angiogenesis, expression of vascular endothelial growth factor, and positive CD31 cellular staining. Other recent investigations have also shown that autologous bone marrow cell transplantation elicits angiogenesis, which in turn improves ischemia-induced organ dysfunction. Accordingly, the current findings could also, at least in part, explain the improved cardiac function after BMDMNC transplantation.

 DAMAGE to injured and remote viable myocardium from AMI is often progressive as in post-infarcted LV remodeling. Most previous experimental studies focused on examining the differentiation of implanted mesenchymal stem cells into myogenic-like cells or endothelial cells in an infarcted area rather than the additional beneficial effects of these implanted cells on remote viable myocardium. The principal finding in this study was that the total area of Cx43 and the number of intact Cx43 gap junctions were significantly higher, whereas cellular apoptosis was significantly lower in the remote myocardium in the BMDMNC therapy group compared with the saline-treated group. Studies have previously demonstrated that Cx43 gap junctions are clusters of cell membrane aqueous channels linked to cytoplasmic compartments of adjacent cells, thereby providing pathways with minimal resistance for electrical coupling, direct intercellular ion exchanges, secondary messengers, and small signaling molecules. Programmed cell death, or “apoptosis”, which has been shown to increase following a variety of cardiac insults, such as ischemia-reperfusion injury and cardiac failure, would be initiated following a loss of cell-to-cell communication mediated by gap junctions. The decreased number of apoptotic nuclei in the remote area of LV in this study, therefore, might explain the reduction in LVEFS and LVEDD on post-transplantation days 42 and 90 in the BMDMNC therapy compared with the saline-treated group.

In conclusion, autologous BMDMNC implantation into the infarct myocardium can elicit more than 1 harmonically biological effect—reducing LV remodeling, improving LV function, and reversing the adverse effects of AMI on molecular and cellular perturbation in the remote viable myocardium.
Acknowledgement

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References


