Autologous Transplantation of Shock Wave-Treated Bone Marrow-Derived Mononuclear Cells Enhances Vascularization and Connexin43 Expression in Rat Dilated Cardiomyopathy

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Objective: This study tested the hypothesis that autologous transplantation of shock wave (SW)-treated bone marrow-derived mononuclear cells (BMDMNCs) is superior to BMDMNC therapy alone for improving left ventricular (LV) function in dilated cardiomyopathy (DCM).

Methods: Adult male Sprague-Dawley rats were divided into group 1 (saline-treated DCM), group 2 (BMDMNC implantation), group 3 (pre-implant SW applied to BMDMNCs), and group 4 (normal control). Rats were euthanized on day 90 following DCM induction.

Results: The number of small vessels in implanted area was highest in group 3 (all p < 0.005). Connexin43 (Cx43) protein expression was significantly enhanced in group 3 compared to groups 1 and 2 (all p < 0.0003). While mRNA expressions of endothelial nitric oxide synthase and interleukin-10 were higher in groups 2-4 than in group 1 (all p < 0.05), caspase-3, endothelin-1 and matrix metalloproteinase-9 mRNA expressions were lower in groups 2-4 compared with group 1 (all p < 0.05). LV ejection fraction was higher, while mitochondrial oxidative stress and fibrosis of LV myocardium were lower, in groups 2-4 compared to group 1 (all p < 0.003).

Conclusion: Pre-implantation SW treatment of BMDMNCs significantly enhanced myocardial vascularization and Cx43 expression which, however, offered no added improvement in LV function compared with BMDMNC treatment alone in rat dilated cardiomyopathy.

Key Words: Bone marrow stem cell therapy • Dilated cardiomyopathy • Shock wave therapy

INTRODUCTION

Dilated cardiomyopathy (DCM) is a primary myocardial disease of unknown cause characterized by ventricular chamber dilatation with normal or decreased wall thickness and impaired systolic function.1-3 This disease, which represents the final common expression of primary myocardial damage from interstitial and perivascular fibrosis,4-6 is also a common cause of congestive heart failure (CHF), which is an important cause of mortality worldwide.3,4,7 Owing to its significant prevalence1,8 and high rates of hospitalization, mortality
and morbidity. DCM is a global health concern. In spite of improvements in treating DCM-induced CHF in the past decade, clinical outcomes following symptom onset have not substantially changed.

Despite growing evidence showing that autologous bone marrow cell (BMC) transplantation improves ischemia-induced or infarct-related cardiac dysfunction, data on the impact of BMC therapy on improving cardiac function in the DCM setting are seldom reported. Furthermore, the molecular-cellular mechanisms whereby BMC therapy improves cardiac function have not been discussed.

Not only do changes in connexin43 (Cx43) expression patterns contribute to various cardiac pathologies, they are also known to participate in initiating cardiac arrhythmia. However, Cx43 protein expression and the integrity of Cx43 in DCM have not been thoroughly investigated. In addition, cardiac generation of reactive oxygen species causes left ventricular (LV) contractile dysfunction, cardiomyocyte death, and remodeling of extracellular matrix in the heart. Also, proinflammatory cytokines, which are indexes of inflammation, are known to rise in individuals with DCM. On the other hand, mesenchymal stem cells are known to have immunomodulation properties. Moreover, in vitro studies indicate that SW therapy enhances vascular endothelial growth factor (VEGF) mRNA expression in cultured human umbilical vein endothelial cells and rat bone marrow cells (BMCs). It also promotes BMC differentiation into endothelial phenotype cells. Accordingly, this study hypothesizes that autologous bone-marrow-derived mononuclear cell (BMDMNC) transplantation improves DCM heart function by attenuating inflammatory responses, oxidative stress, and myocardial fibrosis by up-regulating Cx43 expression and subcellular signal transduction. This investigation further tested the hypothesis that autologous transplantation of shock wave (SW) - treated culturing BMDMNCs is better than BMDMNC therapy alone for improving LV function in a rat model of DCM.

METHODS

Ethics

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

Animal model of DCM

Experimental procedures were performed on pathogen-free adult male Sprague-Dawley (SD) rats weighing 250-300 g (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan). Dilated cardiomyopathy was induced through immunogenic myocarditis as described previously. Briefly, 1 mg (0.1 mL) of porcine heart myosin (Sigma) mixed with equal volume of Freund complete adjuvant (Sigma) was injected into the footpad of each animal on days 1 and 7. Five weeks after immunization, these rats served as models for heart failure due to DCM, as confirmed by pathology.

Randomization

The 30 induced DCM rats and 8 normal rats were divided into group 1 (DCM treated with 750 µL saline, n = 10), group 2 (DCM treated with 1.2 × 10^6 BMDMNCs on day 35 after DCM induction, n = 10), group 3 (SW applied to culturing BMDMNCs prior to implantation, n = 10), and group 4 (normal control, n = 8). All rats were euthanized on day 90 following DCM induction.

Preparation of BMDMNCs with and without shock wave treatment

Rats in groups 2 and 3 were anesthetized with chloral hydrate (35 mg/kg i.p.) on day 21 following DCM induction. After carefully separating the ligament from the patella, a 0.2-mm diameter electric rotablator was used to drill directly into the femoral bone from the distal end. A sterile 22-gauge needle syringe was then used to aspirate the bone marrow from the created orifice.

BMCs from each rat were buffered in 10 mL RPMI1640 medium (Gibco), 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. The cells were washed twice with PBS and then centrifuged at 400 g for 5 min. The BMDMNCs were then cultured in a 60 mm-diameter dish with 10 mL...
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DMEM culture medium containing 10% 5637 cell-culture supernatant. Approximately 1.2-2.0 × 10⁶ BMDMNCs were obtained from each rat. Then 5-azacytidine (Sigma) 100 μM was added to the culture medium on day 3 following BMDMNC culture for 24-h stimulation.

This study utilized 280-SW as the optimal energy dose of SW therapy, based on the results of our recent report.²⁹ Hence, 280-SW (defined as 280 shots in total, given at 0.09-mJ/mm²) was applied once to BMDMNCs on day 0 before cell culture was performed.

BMDMNC labeling and implantation

On day 35 after DCM induction and 14 days of cell culture, 30 min before implanting BMDMNCs, CM-Dil (Vybrant™ Dil cell-labeling solution, Molecular Probes, Inc.) (50 μg/mL) was added to the culture medium to facilitate cell identification after implantation. Cell viability by trypan blue exclusion was > 95% in all experiments. After completion of BMDMNC labeling, all animals were anesthetized by chloral hydrate (35 mg/kg i.p.) and placed in a supine position on a warming pad at 37 °C. Endotracheal intubation was then performed with positive-pressure ventilation support with room air (180 mL/min) using a Small Animal Ventilator (SAR-830/A, CWE, Inc., USA). The heart was exposed via a left thoracotomy under sterile conditions. Approximately 1.0-1.2 × 10⁶ BMDMNCs in 750 μl culture medium IMDM without and with SW treatment were respectively implanted into the anterior and lateral LV myocardium in group 2 and 3 rats using a 30-gauge needle. Additionally, 750 μl normal saline was implanted into myocardium of the LV anterior and lateral walls in group 1 rats, while group 4 animals received only thoracotomy (without cardiac injection).

Functional assessment by echocardiography

Transthoracic echocardiography was performed prior to and on days 35 and 90 after DCM induction, with the anesthetized rats in a supine position 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). M-mode tracings of LV were obtained with the heart being imaged in 2-dimensional mode in the short-axis at the level of the papillary muscle. The procedure was described thoroughly in our recent study.³¹

Immunolabeling of connexin43 and quantitative Image data analysis

Six serial sections of LV myocardium (three longitudinal and three transverse) were prepared at 4 μm thickness by Cryostat (Leica CM3050S) for Cx43 immunolabeling. To co-localize troponin I and Cx43 in the same sample, tissue sections were first incubated with a mixture of polyclonal anti-Cx43 (1:200) plus anti-troponin I (1:200) for 24 h at 4 °C, then incubated with anti-mouse FITC (1:200) and anti-rabbit rhodamine (1:200) for 30 min at room temperature.

For each rat, six sections were chosen for immunolabeling of Cx43 gap junctions (GJs). For each section, the number of intact Cx43 GJs (defined as linear aggregation of Cx43-labeled spots between cardiomyocytes) was quantified under fluorescence microscope in three randomly chosen high-power fields (HPFs). The average number of Cx43-positively stained GJs for each animal per HPF was then obtained from the sum of the numbers of GJs divided by 18.

The integrated area (μm²) of Cx43 spots in the sections was calculated using Image Tool 3 (IT3) image analysis software (University of Texas, Health Science Center, San Antonio, UTHSCSA; Image Tool for Windows, Version 3.0, USA) as described previously.³¹ Three selected sections were quantified for each animal. Three randomly selected HPFs (400X) were analyzed in each section. The numbers of pixels obtained from the three HPFs were summed after determining the number of pixels in each Cx43 spot per HPF. The procedure was repeated in two other sections for each animal. The mean pixel number per HPF for each animal was then determined by summatin all pixel numbers and dividing by 9. The mean area of Cx43 per HPF was obtained using a conversion factor of 19.24 (i. e. 1 μm² represented 19.24 pixels).

Histological study of fibrosis area

Masson Trichrome staining was used to examine the extent of fibrosis in LV myocardium. The method of calculating the integrated area (μm²) of fibrosis in the tissue sections of LV myocardium was identical to that for quantifying Cx43 using Image Tool 3 (IT3) image analysis software.

Mitochondrial isolation

Samples of excised LV myocardium were washed
with buffer A (100 mM Tris-HCl, 70 mM sucrose, 10 mM EDTA and 210 mM mannitol, pH 7.4) and minced finely in cold buffer A, followed by incubation for 10 min. 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 min at 4 °C. The supernatant was centrifuged again at 8,500 g for 15 min, and the pellets were washed using buffer B (10 mM Tris-HCl, 70 mM sucrose, 1 mM EDTA, and 230 mM mannitol, pH 7.4). The mitochondria-rich pellets were collected and stored at -70 °C.

**Western blot analysis for Cx43 and PKC-ε 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 (PKC-ε) were electrophoresed as controls. Following electrophoresis, the separated proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific proteins were blocked by incubating the membrane in the blocking buffer (5% non-fat dry milk in T-TBS containing 0.05% Tween 20) overnight. The membranes were incubated with the indicated primary antibodies (Cx43, 1:1000, Chemicon; Cytochrome C, 1:1000, BD Biosciences; Actin, 1:10000, Chemicon) for 1 h at room temperature. Horseradish peroxidase-conjugated anti-mouse immunoglobulin IgG (1:2000, Amersham Biosciences) was applied as the secondary antibody for 1 h at room temperature. The washing procedure was repeated eight times within 1 h, 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.

**Real-time quantitative PCR analysis**

Real-time polymerase chain reaction (RT-PCR) was conducted for assessing changes in mRNA expressions of caspase 3, MMP-9, IL-10, ET-1, and endothelial nitric oxide synthase on day 90 following DCM induction using LightCycler TaqMan Master (Roche, Germany) in a single capillary tube according to the manufacturer’s guidelines for individual component concentrations. The forward and reverse primers were each designed based on individual exon of the target gene sequence in order to avoid amplifying genomic DNA, as described previously. During PCR, the probe was hybridized to its complementary single-strand DNA sequence within the PCR target. As amplification occurred, the probe was degraded due to the exonuclease activity of Taq DNA polymerase, thereby separating the quencher from reporter dye during extension. The light emission increased exponentially during the entire amplification cycle. A positive result was determined by identifying the threshold cycle value at which the reporter dye emission appeared above background.

**Oxidative stress reaction of LV myocardium**

An Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (S7150). The DNPH derivatization was carried out on 6 μg of protein for 15 min 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 h, then incubated with second antibody solution (1:300) for 1 h at room temperature. The washing procedure was run eight times within 40 min. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) which was then exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP). A standard control sample was loaded into each gel.

**Vessel density in LV myocardium**

Immunohistochemical staining of blood vessels was performed with α-SMA (1:400) as the primary antibody at room temperature for 1 h, followed by washing with PBS thrice. The anti-mouse-HRP-conjugated secondary antibody was then added and incubated for 10 min, followed by washing with PBS thrice. A 3,3’ diaminobenzidine (DAB) (0.7 gm/tablet) (Sigma) was added and then incubated for 1 min, followed by washing with PBS thrice. Finally, the sections were treated with hematoxylin for 1 min as a counterstain for nuclei, followed...
by washing twice. Three sections of LV myocardium were analyzed in each rat. Three randomly selected HPFs (100X) were analyzed in each section for quantification. The mean number of vessels per HPF for each animal was then determined from the sum of all numbers divided by 9.

Statistical Analysis
Data were expressed as mean values (mean ± SD). The significance of group differences was evaluated with t-test. Continuous variables among the four groups were compared by the Kruskal-Wallis test followed by multiple comparison procedure using Wilcoxon rank sum test and Bonferroni correction. Statistical analyses were performed using SAS statistical software for Windows version 8.2 (SAS Institute, Cary, NC). A probability value < 0.05 was considered statistically significant.

RESULTS

Group mortality rates
No mortality was noted in normal controls (group 4) within the study period. Whereas, two rats died in the DCM group (group 1), and one rat died each in the DCM group treated by BMDMNCs (group 2) and in SW-treated BMDMNCs (group 3) respectively, within the study period. Fischer exact test revealed no significant difference in mortality rates among the four groups (p = 0.891).

Histological findings, final body weight and heart weight and serial echocardiographic findings (Table 1)
The initial and final body weights were similar among the four groups, whereas the final heart weight was significantly higher in group 1 than in groups 2-4, and notably higher in groups 2 and 3 than in group 4. Additionally, no significant difference was observed in initial LV ejection fraction (EF), LV end-diastolic diameter (EDD) or LV end-systolic diameter (ESD) among the four groups. On day 35 following DCM induction, although LVEF, LVEDD and LVESD did not differ between groups 1-3, LVEF was significantly lower and LVEDD and LVESD were significantly increased in groups 1-3 than in group 4. By day 90 following DCM induction, LVEF were significantly lower, whereas LVEDD and LVESD were significantly higher, in group 1 than in groups 2-4. Moreover, LVEF and LVESD were significantly decreased in groups 2 and 3 compared with

Table 1. Summary of Baseline and Cardiac Parameters of the Studied Animals

<table>
<thead>
<tr>
<th>Variables†</th>
<th>Group 1*</th>
<th>Group 2*</th>
<th>Group 3*</th>
<th>Group 4*</th>
<th>p value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (gm)</td>
<td>364.7 ± 16.5</td>
<td>368.1 ± 15.9</td>
<td>365.0 ± 15.6</td>
<td>368.8 ± 15.5</td>
<td>0.938</td>
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<tr>
<td>Final body weight (gm)</td>
<td>504.3 ± 28.0</td>
<td>524.7 ± 25.3</td>
<td>523.9 ± 33.4</td>
<td>540.1 ± 37.8</td>
<td>0.198</td>
</tr>
<tr>
<td>Final heart weight (gm)</td>
<td>1.64 ± 0.12a</td>
<td>1.38 ± 0.03b</td>
<td>1.37 ± 0.05b</td>
<td>1.23 ± 0.04c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Initial LVEF (%)</td>
<td>92.1 ± 2.0</td>
<td>92.5 ± 1.9</td>
<td>92.7 ± 2.6</td>
<td>92.4 ± 2.3</td>
<td>0.889</td>
</tr>
<tr>
<td>Initial LVEDD (cm)</td>
<td>0.74 ± 0.04</td>
<td>0.73 ± 0.06</td>
<td>0.75 ± 0.05</td>
<td>0.74 ± 0.05</td>
<td>0.893</td>
</tr>
<tr>
<td>Initial LVESD (cm)</td>
<td>0.28 ± 0.04</td>
<td>0.30 ± 0.04</td>
<td>0.28 ± 0.04</td>
<td>0.30 ± 0.02</td>
<td>0.777</td>
</tr>
<tr>
<td>Day-35 LVEF (%)</td>
<td>84.7 ± 3.2a</td>
<td>84.3 ± 2.9a</td>
<td>84.7 ± 2.6a</td>
<td>92.3 ± 2.9b</td>
<td>0.001</td>
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<tr>
<td>Day-35 LVEDD (cm)</td>
<td>0.81 ± 0.04a</td>
<td>0.80 ± 0.03a</td>
<td>0.81 ± 0.04a</td>
<td>0.73 ± 0.04b</td>
<td>0.001</td>
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<tr>
<td>Day-35 LVESD (cm)</td>
<td>0.40 ± 0.04a</td>
<td>0.42 ± 0.03a</td>
<td>0.42 ± 0.03a</td>
<td>0.32 ± 0.03b</td>
<td>0.0002</td>
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<tr>
<td>Day-90 LVEF (%)</td>
<td>82.7 ± 1.4a</td>
<td>87.6 ± 2.9b</td>
<td>87.6 ± 1.9b</td>
<td>93.1 ± 2.3c</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Day-90 LVEDD (cm)</td>
<td>0.87 ± 0.02a</td>
<td>0.77 ± 0.06b</td>
<td>0.77 ± 0.04b</td>
<td>0.74 ± 0.05b</td>
<td>0.0002</td>
</tr>
<tr>
<td>Day-90 LVESD (cm)</td>
<td>0.48 ± 0.03a</td>
<td>0.38 ± 0.04b</td>
<td>0.37 ± 0.03b</td>
<td>0.31 ± 0.02c</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
LVEF = left ventricular ejection fraction; LVEDD = left ventricular end diastolic dimension; LVESD = left ventricular systolic dimension.
*group 1 (saline-treated), group 2 (bone marrow-derived mononuclear cell [BMDMNC]-treated), group 3 (pre-implanted shock-wave applied to culturing BMDMNCs) and group 4 (control).
†indicates n = 10 on day 0 in each group and n = 8 in each group on day 90 for statistical analysis.
‡by Kruskal-Wallis test. Letters (a, b, c) indicate significant difference (at 0.05 level) (by Wilcoxon rank sum test with Bonferroni’s correction).
group 4, whereas LVEDD was similar in groups 2-4.

**Identification of implanted BMDMNCs in LV myocardium (Figure 1)**

All rats were sacrificed for identifying differentiated BMDMNCs in LV myocardium by day 90 following DCM induction. Numerous CM-Dil-stained undifferentiated BMDMNCs were found to have engrafted (Figures 1A & C). However, only some CM-Dil-stained engrafted cells presenting as myogenic-like cells were stained positively for troponin I (Figures 1B & D). Additionally, the density of CD31-positively stained vessels was lower in DCM rats than in DCM rats treated by BMDMNCs or DCM rats treated by SW-treated BMDMNCs (Figures 1E-H).

**Small arteriolar density analysis (Figure 2)**

The number of small arteries (≤100 μm in diameter) in LV myocardium was notably lower in groups 1 and 4 than in groups 2 and 3 on day 90 following MCT induction. Additionally, the number of small arteries was significantly higher in group 3 than in group 2. These findings indicate that BMDMNC transplantation induced angiogenesis and vasculogenesis, and SW-treated culturing BMDMNCs before transplantation further enhanced angiogenesis and vasculogenesis.

**Fibrosis of LV Myocardium (Figure 3)**

The mean area of fibrotic tissue of LV myocardium was significantly higher in group 1 than in groups 2-4 on Masson Trichrome staining, and higher in groups 2 and 3 than in group 4 (i.e. negative staining) on day 90 following DCM induction. However, the mean area of fibrotic tissue of LV myocardium did not differ between groups 2 and 3. These findings reveal that SW-treated culturing BMDMNCs did not provide an additional benefit over BMDMNC therapy only for reducing fibrosis of LV myocardium.

**Connexin43 expression in LV (Figure 4)**

Figure 4A illustrates the number of intact Cx43 gap junctions in each group of LV myocardium on day 90 following DCM induction. The mean number of intact Cx43 gap junctions was similar among groups 2-4. However, the mean number of intact Cx43 gap junctions was significantly higher, and its distribution was more homogenous, in groups 2-4 than in group 1 (Figures 4C-F).

Figure 4B illustrates the quantification results of the integrated area (μm²) of clustered Cx43 spots in each group of LV myocardium. The summation area did not significantly differ among groups 2-4. However, the area was substantially lower in group 1 than in groups 2-4.

Western blotting for Cx43 in LV (Figure 4G) showed that Cx43 protein expression was substantially lower in group 1 than in groups 2-4, and in group 2 and 4 than in group 3, on day 90 following DCM induction. These findings reveal that SW-treated culturing BMDMNCs...
PKC-ε levels in mitochondria (Figure 5)

PKC-ε expression in mitochondria (Figure 5A) did not differ between group 2 (i.e. DCM+ BMDMNCs) and 3 (DCM+SW-treated BMDMNCs), or between group 1 (DCM) and 4 (i.e. normal controls), on day 90 following DCM induction. However, PKC-ε expression in mitochondria was significantly higher in groups 2 and 3 than in groups 1 and 4, whereas the expression in cytosol (lower panel) was significantly lower in group 2 than in groups 1, 3 and 4 on day 90 following DCM induction. These findings indicate that BMDMNC treatment markedly enhanced the translocation of PKC-ε from cytosol to the mitochondrial domains for cardioprotection following DCM induction. Furthermore, these findings imply that SW-treated culturing BMDMNCs did not offer an additional benefit over BMDMNC therapy alone for upregulating mitochondrial PKC-ε expression on day 90 following DCM induction.

Caspase 3, MMP-9, IL-10, ET-1, and eNOS mRNA expressions (Figure 6)

The mRNA expressions of caspase 3 (Figure 6A) and MMP-9 (Figure 6B) were significantly higher in group 1 than in groups 2-4. Additionally, ET-1 mRNA expression (Figure 6C) was significantly higher in group 1 than in groups 2 and 4, and also notably higher in groups 2 and 3 compared to group 4. However, these
mRNA expressions were similar between groups 2 and 3. Conversely, the mRNA expressions of IL-10 (Figure 6D) and eNOS (Figure 6E) were significantly lower in group 1 than in groups 2-4, and no difference was observed between group 2 and 3. These findings demonstrate that SW-treated culturing BMDMNCs before transplantation offered no extra benefits in terms of suppressing inflammatory response or cellular apoptosis, or enhancing anti-inflammatory response, as compared with BMDMNC therapy only.

**Oxidative stress reaction (Figure 7)**

Western blotting revealed that the oxidative stress index in mitochondria was significantly higher in group 1 than in groups 2-4, whereas there was no significant difference between group 2 and 3. This finding suggests that pre-implant SW treatment of BMDMNCs did not downregulate cardiac generation of reactive oxygen species compared to BMDMNC therapy alone.

**DISCUSSION**

**BMDMNC transplantation enhances angiogenesis/vasculogenesis and improves cardiac function through unknown mechanisms**

Nagaya et al. previously demonstrated that transplantation of mesenchymal stem cells improves cardiac function in a DCM rat model. More recently, our work also demonstrated that autologous BMDMNC therapy improves rat LV function and mitigates molecular and cellular perturbation following acute myocardial infarction. The principal finding of the present study is that BMDMNC transplantation attenuates LV remodeling and improves LV function on day 90 following DCM induction. Thus, the results of this study further support those of Nagaya et al. and the observation that bone marrow-derived stem cells are highly advantageous for cell therapy.
Consistent with the results of a previous study that demonstrated angiogenesis induction in ischemic myocardium through intramyocardial autologous BMDMNC implantation, the present study demonstrated that BMDMNC implantation enhanced angiogenesis/vascularization in a rat model of DCM. On the other hand, the results of this study, which showed significantly higher myocardial angiogenesis/vascularization after implantation of SW-pretreated BMDMNCs compared to BMDMNCs alone, are also in concert with those from our previous investigation that revealed promotion in VEGF production and differentiation of BMDMNCs into cells with endothelial phenotype. Functionally, however, the current study revealed no additional benefits from such an increase in angiogenesis/vascularization following BMDMNC implantation with prior SW-treatment compared to BMDMNC treatment alone on day 90 following DCM induction. The reason for such a morphological-functional discrepancy is still unclear. Although various mechanisms underlying improved cardiac function following BMDMNC therapy in ischemia- or infarct-related myocardium, including angiogenesis, myogenesis, cytokine effects, effects of paracrine mediators, or myocardial homing by stem cells to the myocardium for repair and angiogenesis have already been previously proposed, the principal mechanism is still controversial. These proposals and our findings together imply that other unidentified mechanisms underlie the improved cardiac function following BMDMNC therapy in DCM.

**BMDMNC transplantation limits inflammation, oxidative stress and fibrosis in LV myocardium—role of immunomodulation**

This study found that MMP-9 and ET-1, which are indexes of inflammatory chemokines and oxidative stress, respectively, were significantly higher in the saline-treated DCM group than in the control group. Our findings are, therefore, consistent with those of previous studies demonstrating that oxidative stress and inflammatory chemokines increase in DCM as in CHF, irrespective of the etiology. One important finding of the present study is that BMDMNC therapy markedly suppressed both oxidative and inflammatory responses in LV myocardium as reflected in the drastically reduced levels of oxidative index and anti-inflammatory mediators (i.e. eNOS and IL-10) in DCM treated with BMDMNCs compared with saline treatment only. Furthermore, caspase 3, an index of apoptosis, was remarkably lower in BMDMNC-treated groups than in the saline-treated DCM group. Interestingly, stem cell therapy has recently been shown to modulate immune reactivity by down-regulating innate and adaptive immunity.

Therefore, the findings of this study not only strengthen this hypothesis but also highlight the mechanisms underlying reduction in fibrosis and cellular apoptosis of LV myocardium in DCM following BMDMNC implantation, which may explain the significant reduction in LV remodeling and preservation in heart function demonstrated in this study.
BMDMNC implantation upregulates PKC-ε and Cx43 protein expressions—repressive molecular perturbations

This investigation also found that PKC-ε expression was significantly increased in mitochondrial compartment, but reduced in cytosol, after BMDMNC implantation in DCM rats. An increased PKC-ε expression in the membrane compartment following ischemic preconditioning, and in mitochondria after DCM induction, has been shown to occur frequently. This sub-cellular signal transduction has been documented as a crucial protective mechanism against subsequent cardiac injuries. Therefore, the current finding not only provides a reasonable explanation for the improved cardiac function after BMDMNC implantation in DCM rats, but also reinforces the findings from previous experimental studies.

Gap junctions, which are composed of connexin
subunits, play a fundamental role in electrical coupling between cardiomyocytes. Changes in these connexin expression patterns, which have been linked with various cardiac pathologies, contribute to development of cardiac arrhythmia. This study revealed a distinctive reduction in Cx43 expression, both morphologically and in terms of protein expression, in saline-treated DCM rats, highlighting the perturbation in cell-to-cell interconnections and hence electrical coupling as well as cellular signal transductions in DCM rats. Of importance in the present study is that BMDMNC therapy enhanced Cx43 expression in DCM rats. Therefore, we propose that the reduced LV remodeling and preserved heart function are, at least partly, due to the preservation in Cx43 expression following BMDMNC implantation.

CONCLUSIONS

In conclusion, pre-implantation SW treatment of BMDMNCs significantly enhanced myocardial vasculization and Cx43 protein expression in a rat DCM model. However, while autologous BMDMNC transplantation with or without SW pretreatment in DCM rats preserved LV function by inhibiting inflammation and oxidative stress as well as molecular-cellular perturbation of LV myocardium, SW application on culturing BMDMNCs provided no added benefit over BMDMNC therapy alone for improving LV function.

REFERENCES

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