Six-Month Angiographic Study of Immediate Autologous Bone Marrow Mononuclear Cell Implantation on Acute Anterior Wall Myocardial Infarction Using a Mini-Pig Model

Jiunn-Jye SHEU,[1] MD, Chun-Man YUEN,[2] MD, Cheuk-Kwan SUN,[3] MD, Li-Teh CHANG,[4] PhD, Chia-Hung YEN,[5] PhD, Chiang-Hua CHIANG,[6] PhD, Sheung-Fat KO,[7] MD, Sung-Nan PEI,[8] MD, Sarah CHUA,[9] MD, Anuj BHASIN,[9] MD, Chiung-Jen WU,[9] MD, *and* Hon-Kan YIP,[9] MD

SUMMARY

This study investigated six-month angiographic results of autologous bone marrow mononuclear cell (BMMNC) transplantation immediately following acute myocardial infarction (AMI) in a mini-pig model.

AMI was induced by left anterior descending artery ligation. Twenty-four mini-pigs were equally divided into group 1 [AMI plus saline injection in infarcted area (IA)], group 2 (AMI plus BMMNC transplantation into non-IA), group 3 (AMI plus BMMNC implantation into IA), and group 4 (sham control). One-week cultured BMMNCs (3.0×10^7) were immediately transplanted following AMI induction. Angiographic studies over 6 months demonstrated that mitral regurgitation (MR) was lower in groups 3 and 4 than in groups 1 and 2 (all $P < 0.01$). Wall motion scores and left ventricular ejection fraction (LVEF) were higher in groups 3 and 4 than in groups 1 and 2 (all $P < 0.05$). Collateral circulation was higher in group 3 than in groups 1 and 2 ($P < 0.01$). The wall thickness of the IA was higher, whereas the heart weight was lower in group 3 than in groups 1 and 2 (all $P < 0.01$).

Immediate autologous BMMNC transplantation into IA is superior to saline-treated only or BMMNC transplantation into non-IA following AMI for reducing MR and improving LVEF. (Int Heart J 2009; 50: 221-234)

Key words: Acute myocardial infarction, Bone-marrow cell transplantation, Mini-pig, Angiographic studies

From the [1] Department of Cardiovascular Surgery, [2] Department of Neurosurgery, [3] Department of General Surgery, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, [4] Basic Science, Nursing Department, Meiho Institute of Technology, [5] Department of Life Science, National Pingtung University of Science and Technology, [6] Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, [7] Department of Radiology, [8] Department of Hematology, and [9] Department of Cardiology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, R.O.C.

Address for correspondence: Hon-Kan Yip, MD, Division of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, 123, Ta Pei Road, Niao Sung Hsiang, Kaohsiung Hsien, 83301, Taiwan.

This study was supported by a program grant from the National Science Council, Taiwan, R.O.C (grant no. NSC-96-2314-B-182A-135).

Received for publication August 29, 2008.

Revised and accepted October 23, 2008.

ACUTE myocardial infarction (AMI) is the leading cause of death of patients hospitalized for cardiovascular disease,[1-3] mainly due to the fact that dead cardiomyocytes following AMI cannot be regenerated. Growing data, including those from animal models and limited clinical trials, have shown that autologous transplantation of bone marrow mononuclear cells (BMMNCs) improves left ventricular (LV) function in the settings of ischemic cardiomyopathy and acute myocardial infarction (AMI).[4-8]

Although the majority of the investigators prefer utilizing small animals, eg, either rats or mice, for their experimental models,[4,6,7,9] anesthesia can significantly suppress the heart rate, blood pressure, and especially LV contractility, resulting in inaccurate measurements using pressure transducers, electrocardiogram (ECG), and transthoracic echocardiography.[10,11] In addition, the anatomical distribution of coronary arteries in small animals is different from that in humans, which consists of the left main trunk, left anterior descending artery, and left circumflex artery. Furthermore, the LV end diastolic pressure, an index of left ventricular compliance, is difficult to measure accurately in small animals due to their rapid heart rate. Hence, the findings from the use of small animal AMI models may not truly reflect the clinical picture of AMI. Moreover, the size of small animals precludes the application of coronary angiographic studies in assessing the impact of BMMNC therapy on LV performance. Similarly, the effectiveness of BMMNC implantation into the noninfarcted area (non-IA) in improving LV function cannot be easily assessed in small animals. To investigate the impact of BMMNCs implantation in distinctive anatomical locations of the heart (ie, IA versus non-IA) and to study the subsequent changes in cardiac functions, a large animal model of AMI comparable to a clinical setting is needed. Mini-pigs have many baseline characteristics that are similar to those of humans, including heart rate, anatomical distribution of coronary arteries, ratio of heart to body weight, arterial blood pressure, LV pressure, and LV ejection fraction. Furthermore, coronary angiographic studies and LV angiograms can be easily evaluated in a mini-pig model. Therefore, this study utilized a mini-pig AMI model prepared by ligation of the middle left anterior descending artery (LAD) to investigate the impact of immediate autologous bone BMMNC implantation following AMI on cardiac function using angiography and echocardiography over a six-month period.

METHODS

Ethics: All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at our hospital and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication

No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

Animals, protocol, and procedure: The mini-pigs (Taitung Animal Propagation Station, Livestock Research Institute, Taiwan) were anesthetized by intramuscular injections of ketamine (15 mg/kg) and maintained with an inhalation of 1.5% isoflurane during the procedure. After being shaved on the chest, the mini-pigs were placed in the supine position on a warming pad at 37°C and then endotracheally intubated with positive-pressure ventilatory support (180 mL/minute) with room air using a ventilator (Sn: Q422ZO, SIMS PneuPAC, Ltd.) during the procedure. An ECG monitor and defibrillator were connected to the chest wall of each mini-pig. One ampule of amiodarone (150 mg) was administered intravenously to each min-pig before the procedure.

Prior to opening the chest wall, local anesthesia with 8 mL of 2% xylocaine was injected into the third, fourth, and fifth intercostal spaces. Under sterile conditions, the heart was exposed through a mid-thoracotomy. The pericardium was gently opened and the mid-LAD was ligated just after the first diagonal branch at two separated sites with 5-0 prolene suture (Figure 1). Regional myocardial ischemia was confirmed by ECG tracings (Figure 1) and rapid color change from reddish to whitish-dark red and then to a reddish-black color of the anterior surface of the LV, and the rapid development of akinesia as well as dilatation in the area at risk (Figure 1). Acute anterior wall myocardial infarction was confirmed from the ECG findings following the procedure.

LAD ligation was performed in 23 mini-pigs. Five mini-pigs died due to ventricular tachycardia/ventricular fibrillation ($n = 3$), injury to the trachea during intubation ($n = 1$), and perforation of a lung ($n = 1$) during the procedure. The remaining 18 mini-pigs were divided equally into group 1 [AMI plus saline (1000 μL) injection in IA, $n = 6$], group 2 [AMI plus BMMNC transplantation into non-IA (ie, the remote viable myocardium of left ventricle)], group 3 (AMI plus BMMNC implantation into IA), and group 4 (sham control: thoracotomy only without coronary artery ligation). One-week cultured BMMNCs (3.0×10^7) in 1000 μL culture medium DMEM were immediately implanted into non-IA of group 2 and IA of group 3 following AMI induction (Figure 1). Muscle and skin were closed in layers. The mini-pigs remained on the warm pad and were allowed to recover under care.

Preparation of BMMNCs for autologous transplantation: Under general anesthesia, BMMNCs were aspirated from the iliac crest of group 2 and group 3 animals 1 week before AMI induction (Figure 1). The detailed procedures of separating BMMNCs after aspiration and cell culturing were performed according to our recently described method.[9] Briefly, the BMMNCs were cultured in DMEM high glucose medium (supplemented with 10% bovine serum and antibiotics). Nonadherent hematopoietic cells were removed and the medium was replaced.

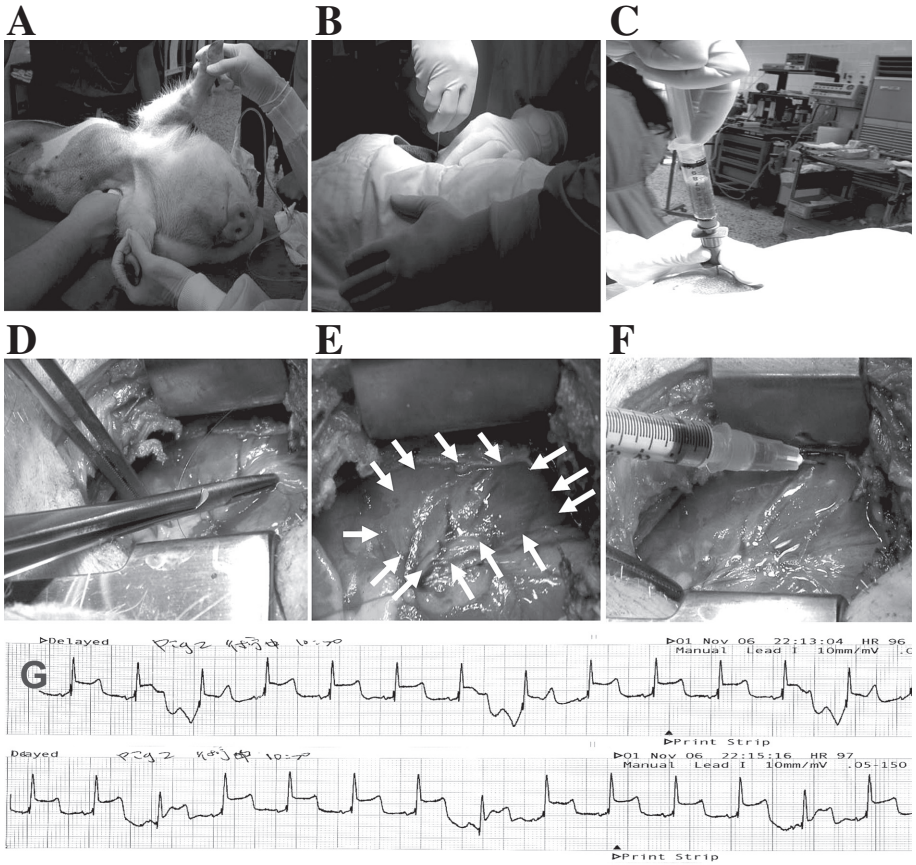

Figure 1. **A:** Echocardiographic examination. **B:** Aspiration of bone marrow mononuclear cells (BMMNCs) from iliac crest of a mini-pig. **C:** BMMNC aspiration. **D:** Ligation of anterior descending artery (LAD). **E:** Reddish-black discoloration of left ventricular (LV) anterior wall (yellow arrows) just after successful LAD ligation. **F:** BMMNC implantation into infarct area one week after culture. **G:** Electrocardiogram (ECG) monitor showed acute ST-segment elevation after LAD ligation.

An adherent BMMNC population of about 3.0×10^7 cells was obtained one week after they were first plated.

For 24 hour cellular stimulation, 5-azacytidine (Sigma) [300 μL in 3 mL DMEM-high glucose (10% FBS)] was added to the culture medium on day 3 following BMMNC culture. On day 7 and 30 minutes before implanting BMMNCs, CM-Dil (Vybrant^{TM} Dil cell-labeling solution, Molecular Probes, Inc.) [3 μL in 3 mL DMEM high glucose (serum free)] was added to the culture medium. This highly lipophilic carbocyanine dye, which has properties of low cytotoxicity and high resistance to intercellular transfer, can be added directly to normal culture media to visualize uniformly label suspended or attached culture cells in an implanted area due to the distinctive fluorescent colors.

Functional assessment by echocardiography (Figure 2): With the animals in a supine position, transthoracic echocardiography was performed preoperatively and on day 90 after AMI induction under general anesthesia as previously described[9,12] 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). Left ventricular internal dimensions, including end-systolic diameter (ESD) and end-diastolic diameter (EDD), were measured according to the American Society of Echocardiography leading-edge method using at least 3 consecutives cardiac cycles.[13] The LV ejection fraction (LVEF) was calculated as follows:

$$LVEF\ (\%) = [(LVEDD^3\text{-}LVEDS^3)/LVEDD^3] \times 100$$

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

Cardiac catheterization and definition (Figure 3): By 6 months after BMMNC implantation, cardiac catheterization was performed using a right common ca-

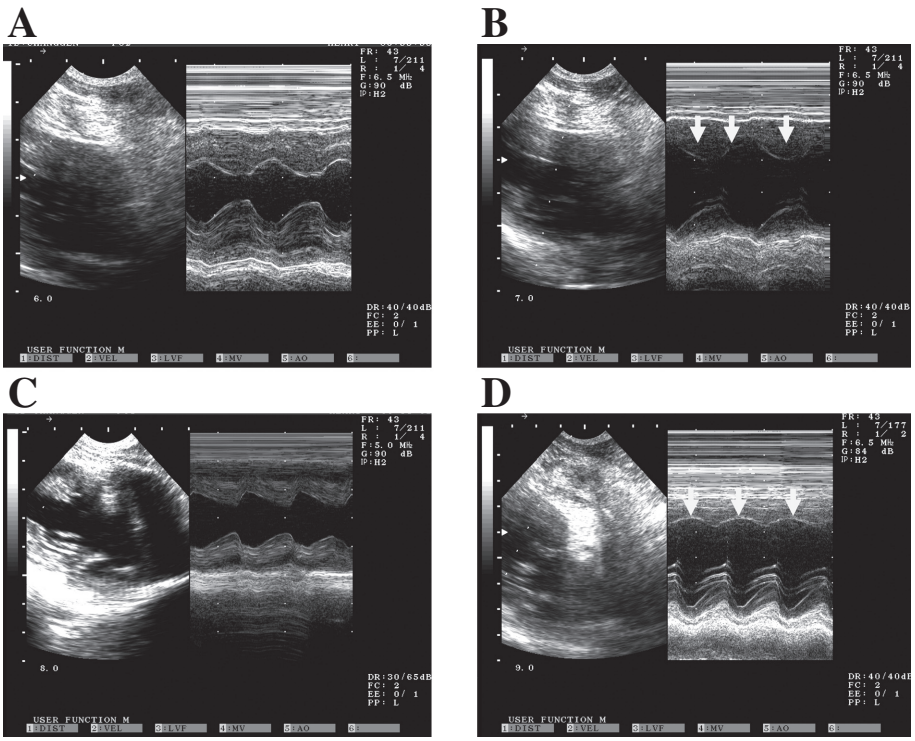

Figure 2. Echocardiographic examinations before and on day 90 after AMI induction. Good LV performance was observed before acute myocardial infarction (AMI) induction (**A** and **C**). Improved left ventricular ejection fraction (LVEF) in AMI treated by BMMNC implantation (**B**) compared to by saline (**D**) (56% versus 45%, respectively). Better septal motion (yellow arrows) with less dilated LV chamber in BMMNC therapy group than in saline-treated group.

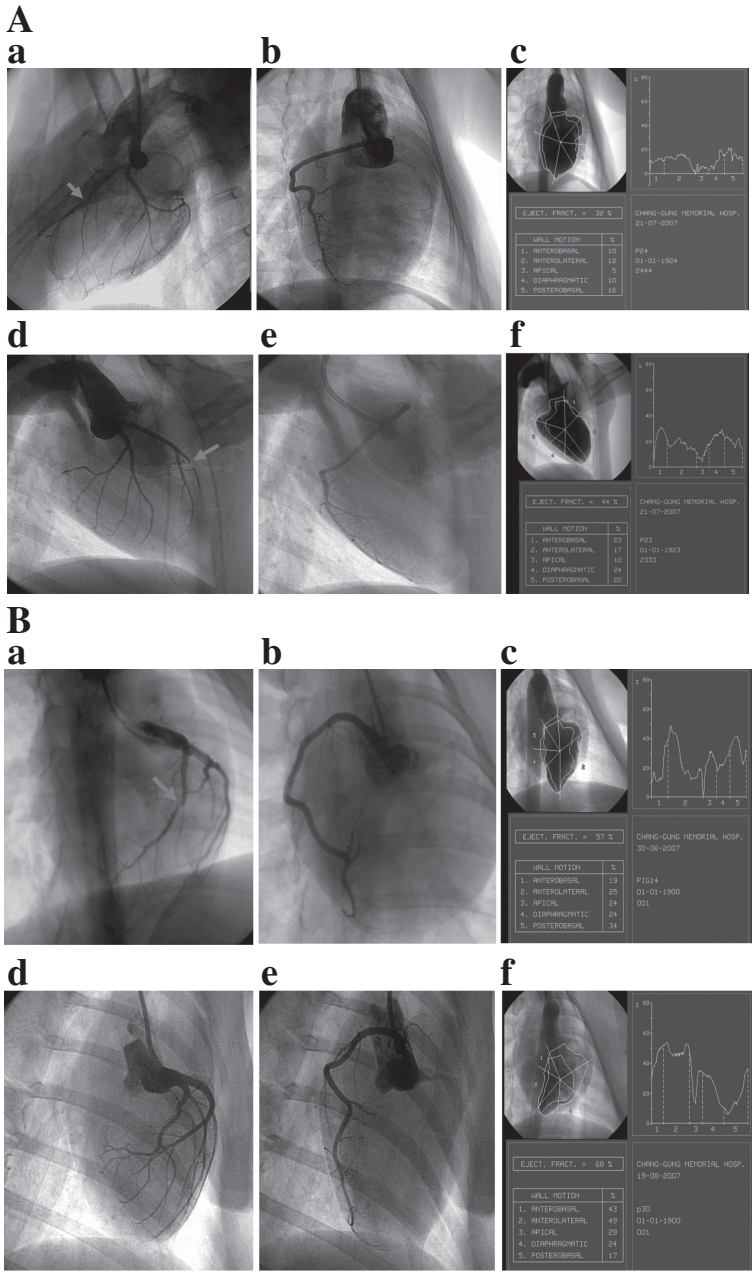

Figure 3A. Upper panel and lower panel indicate AMI treated with saline only and AMI treated with BMMNCs implanted into remote viable LV myocardium [defined as noninfarct area (non-IA)], respectively. **a and b:** Mid-LAD ligation just after first diagonal branch (yellow arrows) with poor antegrade blood flow. **b and e:** No collateral circulation from right coronary artery (RCA) to LAD. **c and f:** Marked LV dysfunction (34% and 44%, respectively) in these two groups of mini-pigs.

Figure 3B. Upper panel and lower panel indicate AMI treated with BMMNCs implanted into remote IA and sham control, respectively. **a:** Mid-LAD ligation just after first diagonal branch (yellow arrows) with normal antegrade blood flow. **b:** Therefore, no collateral circulation from RCA to LAD. **c:** LV function was notably preserved following BMMNC transplantation into IA. **d, e and f:** Normal LAD, RCA and LVEF were observed in a sham-control mini-pig.

rotid artery approach. A 6-French pigtail was used for measuring the arterial blood pressure in the ascending aorta, LV systolic and end diastolic pressure, as well as performing a left ventriculogram. Coronary angiographic study was performed using a 6-French Kimny guiding catheter (Boston Scientific, Scimed, Inc., Maple Grove, MN).

A left ventriculogram, which was immediately performed after the arterial sheath was inserted into the right common carotid artery, 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 (MR) were determined by left ventriculographic study. The severity of the MR was categorized into grades 1 (mild), 2 (moderate), 3 (moderate-severe), and 4 (severe) in accordance with a traditional method. Coronary collateral flow was determined according to a method previously published.[14)]

The mini-pigs were sacrificed by intracoronary injection of potassium chloride after the procedure. The heart was carefully removed and weighed with the infarct area excised for measuring wall thickness at the papillary muscle level and for immunohistochemical study.

Immunohistochemical study: Engraftment of troponin I-positive and CD31-positive BMMNCs was assessed by examining the previously implanted areas after immunohistochemical labeling with the respective primary antibodies, including anti-troponin I (Abcam) and anti-CD31 (Serotec) as well as secondary anti-mouse conjugate FITC antibody (Molecular Probe), followed by incubation for 30 minutes at room temperature. Irrelevant antibodies were used as negative controls.

Measurement of infarct wall thickness at papillary muscle level: To determine the wall thickness of the IA, cross sections at the papillary muscle level of the left ventricle were observed with the three thickest regions chosen and the thickness measured for each mini-pig. The variables were further summated and divided by 3 for statistical analysis for each animal. All measurements were performed by a technician blinded to the treatment and nontreatment groups.

Statistical analysis: Data are expressed as the mean ± SD or (%) of mini-pigs. The significance of differences between groups was evaluated with one-way analysis of variance. Continuous variables among the 4 groups were compared using Duncan's multiple comparison procedure. The data on MR which were not normally distributive were analyzed using the Kruskal-Wallis test, followed by Duncan's multiple comparison procedure with the Wilcoxon rank sum test and Bonferroni's correction. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS Institute, Cary, NC). A probability value < 0.05 was considered to be statistically significant.

Results

Group morbidity and mortality: No malignant ventricular tachyarrhythmia or mortality was noted in the AMI group with saline injection (group 1), AMI with BMMNC implanted into non-IA (group 2), or the control group (group 4) within the study period. However, by two months after AMI induction, right forelimb infection was observed in 1 mini-pig of AMI with BMMNC implantation into IA (group 3). To avoid transmission of infection to other mini-pigs, this animal was prematurely sacrificed. A replacement mini-pig was utilized following the same procedure and treatment as in group 2.

Initial and final body weight, heart weight and serial echocardiographic findings (Table): The initial body weight did not differ among the 4 groups. Additionally, the final body weight did not differ between groups 1 and group 2 or between groups 3 and group 4. However, the final body weight was significantly lower in groups 1 and 2 than in groups 3 and 4. The final heart weight was not different between groups 3 and group 4. However, the final heart weight was significantly higher in group 1 than in groups 2, 3, and 4, and significantly higher in group 2 than in groups 3 and 4. Furthermore, the ratio of heart to body weight was significantly higher in groups 1 and 2 than in groups 3 and 4. We, therefore, speculated that the above findings, including body weight and heart weight, suggest that congestive heart failure should develop in groups 1 and 2.

There was no significant difference in initial LVEF, LVEDD, or LVESD among the 4 groups. On day 90 following AMI induction, the LVEF and LVESD did not differ between groups 1 and 2. Additionally, the LVEDD was also similar among groups 1, 2, and 3. However, the LVEF was remarkably higher, whereas the LVESD was notably lower in groups 3 and 4 than in groups 1 and 2. Furthermore, the LVEF was significantly lower, whereas the LVESD was significantly higher in group 3 than in group 4.

Six-month angiographic follow-up results (Table and Figure 3): By 6 months after AMI induction, cardiac catheterization was performed via the right common carotid artery for each group of mini-pigs. There were no significant differences in terms of heart rate, systolic and diastolic blood pressure in the ascending aorta, and LV systolic blood pressure among the 4 groups. The LV end diastolic blood pressure tended to be higher in groups 1 and 2 than in groups 3 and 4, although there was no statistical significance. These findings indicate that the compliance of left ventricle was poorer in groups 1 and 2 than in groups 3 and 4 following AMI induction.

Left ventriculograms demonstrated that LVEF was significantly higher in group 2 than in group 1. Additionally, LVEF was remarkably higher in groups 3 and 4 than in groups 1 and 2, and significantly higher in group 4 than in group 3.

These findings highlight the positive impact of BMMNC transplantation on LV function after AMI and further indicate that the effect of BMMNC implantation in IA is better than BMMNC implantation in non-IA for improving heart function.

The wall motion of the individual segments, including anterobasal, anterolateral, apical diaphragm, and posterobasal, was also evaluated by left ventriculographic study. The results demonstrated that the wall motion of the anteroba-

Table. Summarized Data on Body Weight, Final Heart Weight, LV Dimension, LV Function and 6-Month Angiographic Results in the 4 Study Groups of Mini-Pigs

Variable	Group 1* (n = 6)	Group 2* (n = 6)	Group 3* (n = 6)	Group 4* (n = 6)	P
Initial BW (kgm)	17.1 ± 0.9	17.1 ± 0.7	16.9 ± 1.0	16.8 ±1.0	0.910
Final BW (kgm)†	20.0 ± 1.7[a]	20.8 ± 1.3[a]	25.1 ± 1.2[b]	26.6 ± 1.5[b]	< 0.0001
Final HW (gm)†	133.8 ± 4.5[a]	127.6 ± 6.5[b]	105.8 ± 4.3[c]	102.0 ± 3.6[c]	< 0.0001
Ratio of HW to BW ($\times 10^{-3}$)†	6.6 4 ± 0.86[a]	6.01 ± 0.61[a]	4.23 ± 0.29[b]	3.84 ± 0.28[b]	< 0.0001
Initial LVEF (%)	70.5 ± 7.3	68.7 ± 5.8	71.3 ± 5.6	70.3 ± 5.9	0.904
Initial LVEDD (mm)	3.06 ± 0.43	3.32 ± 0.27	2.99 ± 0.23	3.07 ± 0.28	0.300
Initial LVESD (mm)	1.87 ± 0.36	2.05 ± 0.11	1.93 ± 0.33	1.86 ± 0.19	0.610
90-day LVEF (%)†	46.8 ± 4.0[a]	50.0 ± 3.7[a]	58.1 ± 3.6[b]	71.4 ± 5.1[c]	< 0.0001
90-day LVEDD (mm)†	3.68 ± 0.23[a]	3.78 ± 0.19[a]	3.44 ± 0.29[a]	2.93 ± 0.38[b]	0.0002
90-day LVESD (mm)†	2.94 ± 0.23[a]	2.95 ± 0.15[a]	2.43 ± 0.27[b]	1.74 ± 0.24[c]	< 0.0001
6-month angiographic results					
Heart rate (beats/minute)	99.8 ± 13.9	98.0 ± 22.7	98.2 ± 14.5	103.0 ± 8.2	0.941
AsAo SBP (mmHg)	127.3 ± 25.2	120.8 ± 34.4	130.5 ± 34.4	117.2 ± 12.8	0.841
AsAo DBP (mmHg)	74.3 ± 31.5	77.3 ± 35.2	94.8 ± 34.7	76.3 ± 11.8	0.619
LV-SBP (mmHg)	141.2 ± 44.9	151.7 ± 29.5	142.8 ± 29.6	126.0 ± 19.6	0.588
LV-EDBP (mmHg)	19.2 ± 5.9	19.8 ± 9.6	13.7 ± 4.8	10.7 ± 5.6	0.083
LVEF (%) by ventriculogram†	33.3 ± 9.5[a]	43.8 ± 10.0[b]	58.3 ± 11.0[c]	64.0 ± 6.4[c]	< 0.0001
Wall motion (%)					
anterobasal†	14.3[a] ± 5.1	16.0[a] ± 13.3	27.0[b] ± 5.7	33.0[b] ± 9.9	0.005
anterolateral†	12.0[a] ± 8.4	19.5[b] ± 13.0	30.2[c] ± 9.3	33.7[c] ± 10.2	0.006
apical	12.8 ± 9.1	11.5 ± 1.8	15.0 ± 8.3	18.8 ± 7.4	0.343
diaphragmatic	19.0 ± 6.3	16.5 ± 7.8	28.7 ± 12.1	29.5 ± 11.2	0.064
posterobasal†	18.0[a] ± 8.4	17.8[a] ± 1.7	27.8[b] ± 6.5	23.8[a, b] ± 6.6	0.034
Ischemia-related MR‡	2.17[a] ± 0.98¶	2.17[a] ± 1.33¶	0.33[b] ± 0.82¶	0[b] ± 0¶	0.002
Collateral circulations‡	0.50 ± 0.55¶	0.83 ± 0.41¶	2.83 ± 0.41¶	0 ± 0¶	0.0008
Wall thickness of IA (mm)	0.69 ± 0.10[a]	0.80 ± 0.10[a]	1.14 ± 0.13[b]	1.40 ± 0.05[c]	< 0.0001

Data are expressed as the mean value ± SD or (%) of mini-pigs.

AsAo SBP indicates ascending aorta systolic blood pressure; BW, body weight; DBP, diastolic blood pressure; EDBP, end diastolic blood pressure; HW, heart weight; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular systolic dimension; and MR, mitral regurgitation.

* Group 1 = acute myocardial infarction (AMI) by saline treated in infarcted area (IA); Group 2 = AMI plus bone marrow mononuclear cell (BMMNC) implantation into non-IA; Group 3 = AMI plus BMMNC implantation into the IA; Group 4 (sham control).

† Letters ([a, b, c]) indicate significant difference (at 0.05 level) by one-way analysis of variance followed by Duncan's multiple comparison procedure.

‡ Letters ([a, b]) indicate a significant difference (at 0.05 level) by the Kruskal-Wallis test followed by multiple comparison procedure with the Wilcoxon rank sum test and Bonferroni's correction.

¶ Grading of collaterals and MR are expressed as mean values.

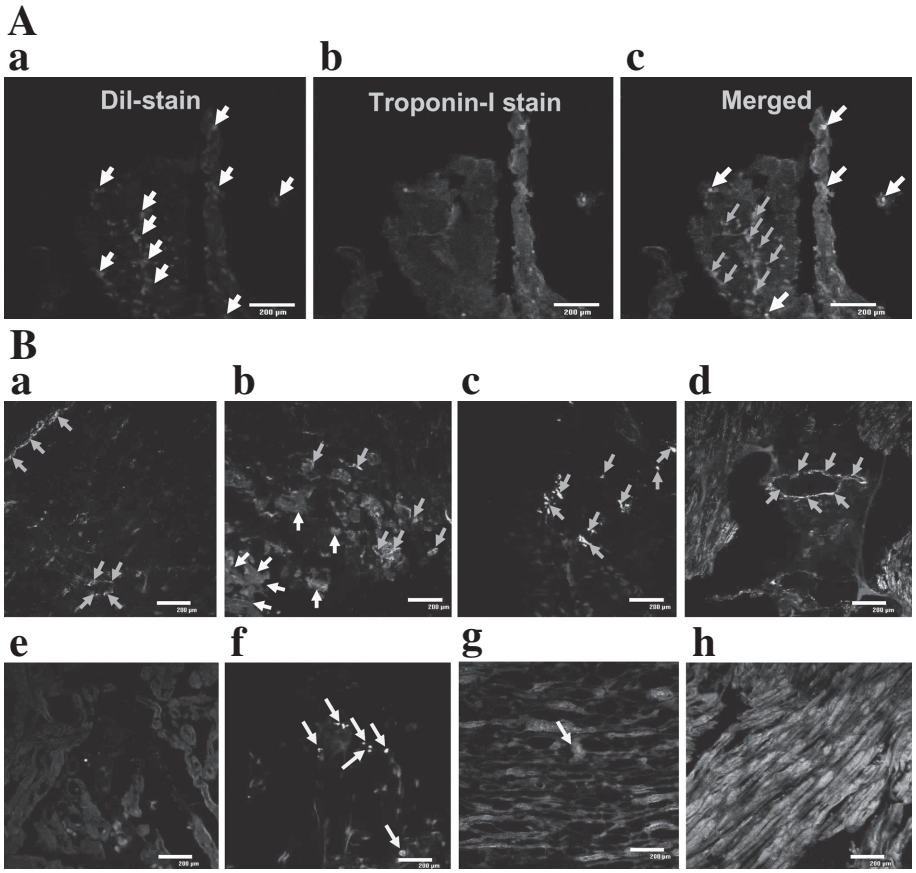

Figure 4A. Confocal imaging studies on day 90 after AMI induction in a mini-pig. **a:** Implanted BMMNCs (yellow arrows) identified in IA on day 90 after implantation with CM-Dil staining. **b:** Positive troponin I staining in IA, indicating viable myocardium in IA. **c:** Merged image after double staining with CM-Dil and Troponin I (**a**) and (**b**). Double staining with CM-Dil and troponin I indicating differentiation of only a minority of implanted BMMNCs into myogenic-like phenotype (yellow arrows) with the majority of IA-engrafted BMMNCs staying undifferentiated (red arrows). Nuclei counter-stained by 4',6-diamidino-2-phenylindole (DAPI) (blue color). Scale bars in right lower corner represent 200 μm.

Figure 4B. Confocal imaging studies 6 months after AMI induction.

CD31 (surface marker of endothelial cell) staining (Upper panel). **a:** AMI treated by saline only. CD31-positively stained cells (red arrows) observed in both longitudinal and cross sections, indicating some viable small vessels in IA. **b:** AMI treated by BMMNC implantation into IA, showing numerous Dil-positive and undifferentiated BMMNCs (yellow arrows). Doubly stained (CM-Dil and CD31) cells also frequently observed in IA (red arrows), indicating differentiation of implanted BMMNCs into endothelial phenotype. **c:** AMI treated by BMMNC implantation into non-IA, showing CD31positive staining (red arrows). **d:** Sham control. CD31-positively stained cells identified in an intact vessel (cross section), implicating development of angiogenesis following BMMNC implantation [(**c**) and (**d**)].

Troponin I staining (lower panel). **e:** AMI treated by saline only. **f:** AMI treated by BMMNC implantation into IA. Identification of only a minority of BMMNCs having differentiated into myogenic-like cells (double stains of CM-Dil and Troponin I) in IA (greenish arrows). **g:** AMI treated by BMMNC implantation into non-IA, showing CM-Dil positively stained BMMNCs. **h:** Positive troponin I staining in sham control indicating intact myocardium.

Nuclei counter-stained with 4',6-diamidino-2-phenylindole (DAPI) (blue color). Scale bars in right lower corner represent 200 μm.

sal, anterolateral, and posterobasal segments was significantly higher in groups 3 and 4 than in groups 1 and 2. These findings implicate an improvement in contractility of the left ventricle after BMMNC implantation into the IA following AMI.

The mean grade of coronary collateral circulation was significantly higher in group 3 than in groups 1 and 2. Interestingly, more than 80% of collaterals were intracoronary rather than intercoronary in the mini-pigs. These findings suggest that the collateral circulation was elicited by BMMNC implantation rather than by spontaneous development.

To investigate whether ischemic-related MR is present following AMI induction and whether BMMNC implantation can ameliorate this complication, the degree of MR was evaluated by a biplane left ventriculogram. As expected, MR was significantly higher in groups 1 and 2 than in groups 3 and 4. This finding indicates that BMMNC implantation in IA markedly inhibits the development of ischemic-related MR after AMI.

Identification of implanted BMMNCs in infarct and noninfarct areas (Figure 4): By day 90, numerous Dil-stained undifferentiated BMMNCs were identified in IA (Figure 4A). Additionally, some Dil-stained engrafted cells present as troponin I-positive myogenic-like cells were also identified in IA (Figure 4A). By 6 months following transplantation, numerous Dil-stained undifferentiated BMMNCs were engrafted into the IA and non-IA (Figure 4B). On the other hand, only some Dil-stained engrafted cells were present as troponin I-positive myogenic-like cells in the IA and non-IA (Figure 4B). Moreover, some Dil-stained engrafted cells positive for CD31 (an endothelial cell surface marker) were also clearly identified in IA and non-IA (Figure 4B).

Measurement of wall thickness in infarct area (Table): The wall thickness in IA at the level of papillary muscle was significantly thinner in groups 1 and 2 than in groups 3 and 4, and significantly thinner in group 3 than in group 4. This finding indicates that BMMNC implantation into the IA may be able to repair the infarct myocardium.

Discussion

Evidence for angiogenesis/vasculogenesis and long-term survival of BMMNCs at the implantation sites: Although there is evidence showing the survival of BMMNCs in ischemic LV myocardium for up to 3 months after implantation,[4,6,7,9] the long-term survival of implanted BMMNCs in an ischemic-related organ is currently unclear. One of the distinctive findings in the present study was that engrafted autologous BMMNCs in IA and non-IA of the LV myocardium can survive for more than 6 months. Therefore, our findings further support and ex-

tend the findings of recent studies.[4,6,7,9)]

Angiogenesis and vasculogenesis in infarcted LV myocardium were identified in the IA 3 months after autologous BMMNC implantation in a rat AMI model in our recent study.[9)] Interestingly, in the present study, we also found that implanted BMMNCs participated in angiogenesis/vasculogenesis (indirectly proved by the presence of CD31-positive cells) in IA. Therefore, the findings of the present study corroborate those from our recent study.[9)] Also of importance in the current study was that angiographic investigation after 6 months demonstrated that the majority of the collateral circulation was intracoronary rather than intercoronary. Furthermore, the incidence of collateral circulation was significantly higher in group 3 than in groups 1 and 2. Taken together, our basic and angiographic findings support that BMMNC therapy elicits angiogenesis/vasculogenesis which in turn enhances the intracoronary collateral circulation in the infarcted myocardium.

Prevention of LV remodeling following autologous BMMNC therapy: LV remodeling following AMI, as a consequence of LV dilatation and pump failure, largely accounts for the poor clinical outcomes.[15-17)] The severity of pump failure typically depends on infarct size and ischemic area.[15-18)] In the present study, the principal finding was the significantly elevated LVEDD and LVESD in groups 1 and 2 compared to group 4 (ie, control group) in a 90-day transthoracic echocardiographic study. Conversely, LVEF was significantly lower in groups 1 and 2 than in group 4. However, LVEDD and LVESD were lower in group 3 than in groups 1 and 2, whereas LVEF was significantly higher in group 3 than in groups 1 and 2. Additionally, the wall thickness of the IA was significantly increased in group 3 compared to in groups 1 and 2. These findings suggest that immediate autologous BMMNC transplantation into IA, in addition to improving LV function, also effectively prevented LV remodeling early after AMI.

Implications of 6-month angiographic findings: The exact mechanisms underlying LV functional improvement after BMMNC therapy remain uncertain.[5,7,9,19)] In fact, proposed mechanisms, including angiogenesis/vasculogenesis,[4,5,9)] myogenesis,[4,20)] chemokine effects,[5,9)] effect of paracrine mediators,[7,20,21)] or a myocardial homing by bone marrow-derived mesenchymal stem cells to the myocardium for angiogenesis and repair,[22,23)] have been extensively debated. The most important finding in the current study was that LV wall motion was notably improved and LVEF was significantly preserved following BMMNC implantation into the IA of LV myocardium. Surprisingly, immunohistochemical investigation demonstrated that only a small proportion of implanted BMMNCs differentiated into myogenic-like cells in the IA. Conclusively, our findings, based on both bench work and angiographic studies, suggest that the improvement in heart function following BMMNC implantation to the infarcted myocardium

could be due to a broad-spectrum of mechanisms rather than a single one.

Ischemic-related MR following AMI has been well recognized in many clinical observational studies.[24-26] An association between ischemic-related MR and poor prognostic outcome has been extensively studied in patients following AMI with or without undergoing primary coronary angioplasty.[24-27] Another distinctive finding in the present study was the high incidence of MR observed in groups 1 and 2 of mini-pigs following AMI induction. Interestingly, the incidence of MR was substantially reduced in group 3. Our findings, in addition to strengthening the findings of previous clinical observational studies,[24-27] further suggest that immediate implantation of BMMNCs to the IA following AMI can abrogate the development of ischemic-related MR. The result may raise a need for prospective clinical trials for further elucidation of the safety and effectiveness of BMMNC therapy for ischemic-related MR in human subjects.

Study limitations: This study has several limitations. Firstly, the sample size was relatively small. Secondly, 6-month follow-up may be still not long enough to extrapolate the favorable results to the conclusion of improved long-term outcomes. Finally, the design protocol of this study did not provide a satisfactory parameter for evaluating the incidence and severity of AMI-induced congestive heart failure in the animals studied.

Conclusion: The present study demonstrated that autologous BMMNC transplantation into the IA in acute phase of AMI provides persistent benefits of improving heart function and abrogating ischemia-induced MR. These findings encourage us to prospectively investigate the application of BMMNC therapy in the clinical setting of AMI.

References

1. The Multicenter Postinfarction Research Group. Risk stratification and survival after myocardial infarction. N Engl J Med 1983; 309: 331-6.
2. Murray GJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet 1997; 349: 1436-42.
3. Murray GJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet 1997; 349: 1269-76.
4. Tomita S, Li RK, Weisel RD, *et al*. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999; 100: II247-56.
5. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet 2003; 361: 47-9.
6. Nagaya N, Kangawa K, Itoh T, *et al*. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005; 112: 1128-35.
7. Dai W, Hale SL, Martin BJ, *et al*. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. Circulation 2005; 112: 214-23.
8. Schächinger V, Erbs S, Elsässer A, *et al*. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006; 355: 1210-21.
9. Yip HK, Chang LT, Wu CJ, *et al*. Autologous bone marrow-derived mononuclear cell therapy prevents

the damage of viable myocardium and improves rat heart function following acute anterior myocardial infarction. Circ J 2008; 72: 1336-45.
10. Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. Am J Physiol 1999; 277: H1967-74.
11. Roth DM, Swaney JS, Dalton ND, Gilpin EA, Ross J Jr. Impact of anesthesia on cardiac function during echocardiography in mice. Am J Physiol Heart Circ Physiol 2002; 282: H2134-40.
12. Litwin SE, Katz SE, Morgan JP, Douglas PS. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. Circulation 1994; 89: 345-54.
13. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978; 58: 1072-83.
14. Rentrop KP, Thornton LC, Feit F, Van Buskirk M. Determinants and protective potential of coronary arterial collaterals as assessed by an angioplasty model. Am J Cardiol 1988; 61: 677-84.
15. Erlebacher JA, Weiss JL, Weisfeldt ML, Bulkley BH. Early dilation of the infarcted segment in acute transmural myocardial infarction: role of infarct expansion in acute left ventricular enlargement. J Am Coll Cardiol 1984; 4: 201-8.
16. Pfeffer MA, Pfeffer JM. Ventricular enlargement and reduced survival after myocardial infarction. Circulation 1987; 75: IV93-7. (Review)
17. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling-- concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J Am Coll Cardiol 2000; 35: 569-82. (Review)
18. Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation 1999; 100: 999-1008.
19. Makino S, Fukuda K, Miyoshi S, *et al*. Cardiomyocytes can be generated from marrow stromal cell in vitro. J Clin Invest 1999; 103: 697-705.
20. Mangi AA, Noiseux N, Kong D, *et al*. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003; 9: 1195-201.
21. Davani S, Marandin A, Merisin N, *et al*. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 2003; 108: II253-8.
22. Jackson KA, Majka SM, Wang H, *et al*. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107: 1395-402.
23. Quaini F, Urbanek K, Beltrami AP, *et al*. Chimerism of the transplanted heart. N Engl J Med 2002; 346: 5-15.
24. Tcheng JE, Jackman JD Jr, Nelson CL, *et al*. Outcome of patients sustaining acute ischemic mitral regurgitation during myocardial infarction. Ann Intern Med 1992; 117: 18-24.
25. Nesković AN, Marinković J, Bojić M, Popović AD. Early predictors of mitral regurgitation after acute myocardial infarction. Am J Cardiol 1999; 84: 329-32.
26. Grigioni F, Enriquez-Sarano M, Zehr KJ, Bailey KR, Tajik AJ. Ischemic mitral regurgitation: long-term outcome and prognostic implications with quantitative Doppler assessment. Circulation 2001; 103: 1759-64.
27. Chung SY, Lin FC, Chua S, *et al*. Clinical profile and outcome of first acute myocardial infarction with ischemic mitral regurgitation. Chang Gung Med J 2008; 31: 268-75.