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Five-year follow-up after transepicardial implantation of autologous bone marrow mononuclear cells to ungraftable coronary territories for patients with ischaemic cardiomyopathy☆

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Abstract

Objective: Cell therapy for patients with ischaemic cardiomyopathy (IC) is still an open issue. We aimed to assess the long-term safety and therapeutic potency of autologous bone marrow mononuclear cell (ABMMNC) implantation into ungraftable coronary artery (UCA) territories in patients with IC. **Methods:** Bone marrow was aspirated from the iliac crest, and transepicardial ABMMNC implantation ($n = 25$, 24 men, aged 57 ± 7 years) as an adjunct to coronary artery bypass grafting (CABG) was performed into an area of reversible ischaemia within the territory of UCA ($1.29 \pm 0.09 \times 10^9$ ABMMNCs). Control group ($n = 25$, 23 men, aged 59 ± 7 years) underwent incomplete CABG due to poor target vessel graftability. The study protocol consisted of coronary angiography, stress echocardiography, nuclear imaging and Holter monitoring at baseline and follow-up. The mean follow-up time was 988 ± 423 days. **Results:** There was no difference between the groups regarding postoperative complications and outcome. Overall 5-year survival for the ABMMNC group was $79 \pm 10\%$, and $71 \pm 12\%$ for the controls ($p = 0.48$). Left ventricular ejection fraction (LVEF) at baseline was 24.8 ± 3.7 versus 25.9 ± 3.1 in the ABMMNC group and the controls, respectively. After 6 months, mean global LVEF increased to 36.3 ± 7.4 ($p < 0.001$) versus 31.4 ± 4.1 ($p = 0.001$), respectively. A significant difference was noted in delta LVEF between the groups ($p < 0.001$, 95% confidence interval (CI): 3.4—8.9) at 6 months, and ($p = 0.001$, 95% CI: 2.0—7.4) at 1 year. Accordingly, perfusion scores in UCA segments detected by single-photon emission computed tomography (SPECT) improved with ABMMNC therapy to 18.0 ± 24.4 from 7.1 ± 25.7 ($p = 0.001$ vs control UCA segments). **Conclusion:** Cellular therapy for IC within UCA could augment myocardial perfusion and contractility but does not improve overall survival. No adverse events were detected after cell therapy at mid-term follow-up.

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Keywords: Heart failure; Stem cells; Ischaemia; Angiogenesis; Left ventricular function; Survival

Emerging cell-based therapies in the injured heart may influence the direction of heart failure (HF) treatment paradigms [1]. Experimental research has shown that bone marrow-derived stem/progenitor cell implantation improves cardiac function by paracrine signalling, reduced apoptosis, modification of matrix remodelling, neovascularisation, regeneration or stimulation of endogenous stem cell niches [2—4]. Despite sceptical reports about cardiomyocyte differentiation [5], skeletal myoblasts [6,7], various bone marrow-derived cells [8—15], endothelial progenitor cells [14,16—18] and stem cell mobilisation [19] have undergone testing in clinical trials. However, the mechanisms involved and the ideal cell source for cardiac cytotherapy remains to be defined. Furthermore, there are no uniform standards relating to the method of cell processing, delivery, quantity of transplanted cells and end points of the clinical studies. By and large, these studies indicate relative safety and modest degrees of clinical improvement after cellular therapy [1,6—19].

It remains unclear whether cellular therapies provide sufficient myocardial recovery for better event-free survival over the long term. We, therefore, evaluated the long-term safety and efficacy of autologous bone marrow mononuclear

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cell (ABMMNC) implantation in patients with ischaemic cardiomyopathy (IC) and who have at least one ischaemic region that cannot be re-vascularised by conventional means.

1. Materials and methods

1.1. Patients and protocol

The Research Ethics Committee of the Ankara University School of Medicine approved the study protocol on 24 June 2002 (20-2002/371) and all subjects gave written informed consent to participate in the study. The study was carried out in accordance with the Declaration of Helsinki (1964, revised 1989) and the current revisions of the Good Clinical Practice Guidelines of the European Commission; the safety data were reported to the Ethics Committee every 6 months. The study population consisted of patients with IC who were already scheduled for first-time isolated coronary artery bypass grafting (CABG) but had at least one ungraftable coronary artery (UCA) territory, which was not suitable for conventional forms of re-vascularisation. The decision for re-vascularisation was based on symptoms, presence of ischaemia, single-photon emission computed tomography (SPECT) and angiographic findings. Twenty-five patients (24 men, 57.1 ± 7.0 years of age, range: 43–68 years) underwent CABG and ABMMNC implantation at the Heart Centre, Ankara University School of Medicine (Table 1). Twenty-five patients (23 men, 59.6 ± 7.4 years of age, range: 43–71 years) who underwent incomplete re-vascularisation due to poor distal vessel quality within the same period, served as the control group. All patients had documented AMI. This is a prospective, case-controlled, safety study of patients with severe IC and at least one UCA territory.

1.2. Definitions

Heart failure of ischaemic aetiology was defined as the presence of any history of AMI or coronary re-vascularisation; or left main coronary artery with >50% stenosis; or two or more epicardial vessels with >70% stenosis. Inclusion criteria included: (1) end-stage IC with New York Heart Association (NYHA) classes 3–4; (2) left ventricular ejection fraction (LVEF) below 30%; (3) left ventricular (LV) end-systolic volume index above 50 ml m^{-2}; (4) mean pulmonary artery pressure above 20 mmHg; (5) an LV end-diastolic pressure or pulmonary capillary wedge pressure above 14 mmHg; (6) chronic coronary artery disease with reversible perfusion defect detectable by SPECT; (7) ineligibility for percutaneous or surgical re-vascularisation in at least one infarct-related ischaemic myocardial territory, as assessed by both a cardiovascular surgeon and an interventional cardiologist on the basis of its angiographic appearance and (8) signed informed consent. Patients were not enrolled in the study if any one of the following exclusion criteria were met: (1) severe fixed nuclear perfusion defect in the UCA territory; (2) significant ischaemic mitral regurgitation as assessed by quantitative Doppler and proximal isovelocity surface area (effective regurgitant orifice >20 mm^2 and regurgitant volume >30 ml per beat); (3) previous or current history of neoplasia or other co-morbidity that could impact the patient's survival; (4) previous sternotomy; (5) history of significant ventricular dysrhythmias; (6) atrial fibrillation or flutter; (7) second- or third-degree heart block; (8) primary haematologic disease, which may interfere with the yield of bone marrow harvest; (9) hepatic cirrhosis, chronic hepatitis; (10) significant multiorgan dysfunction or cardiac cachexia, (11) ethanol abuse and (12) previous psychiatric illness including depression and schizophrenia. Adverse events were defined as death, non-fatal AMI, stroke, pathological angiogenesis, ventricular arrhythmias and malignant disease. Perioperative myocardial infarction (MI) was defined as either new Q waves or ischaemic ST segment changes with concomitant elevations of creatine kinase isoenzyme (CK-MB) up to >5 times the upper limit of the reference range or a CKMB to total creatine kinase ratio at >10% occurring within 48 h after surgery or troponin I >1 ng ml^{-1}.

1.3. Baseline evaluation

On admission, complete laboratory tests and serologic profile including acute phase reactants (erythrocyte sedimentation rate and C-reactive protein), fibrinogen, complement measurements, thyroid function, hypercoagulability screen (plasma levels of protein C, protein S and antithrombin III, factor V Leiden and prothrombin 20210A gene mutation analysis) have been obtained for all patients. Baseline evaluation also included coronary angiography, left ventriculography, dobutamine stress echocardiography (DSE); Thalium201 SPECT and Tc99m MIBI gated SPECT perfusion scanning, Holter monitoring, ophthalmological exam and haematological evaluation. Aspirin and/or clopidogrel were discontinued 5–7 days before surgery.

1.4. Resting echocardiography

Two-dimensional echocardiography was performed with the patient lying in the left lateral decubitus position using Sonos-5500 device (Hewlett-Packard, Andover, MA, USA). Regional function was assessed according to the recently recommended 17-segment LV model [20], and was graded as 1 = normal, 2 = hypokinetic, 3 = akinetic or severely hypokinetic, 4 = dyskinetic and 5 = aneurysmal (diastolically deformed). Wall motion score index was determined by dividing the sum of the wall motion scores by the number of visualised segments. The LV volumes (end-diastolic and end-systolic) and LVEF were calculated from the apical two- and four-chamber views using the biplane Simpson method. The end-diastolic and end-systolic volumes were then indexed by the body surface area. Measurements of the LV internal diameter, interventricular septal and posterior wall thickness obtained at the papillary muscle level were used for the calculation of LV mass by the Devereux formula and normalised for body surface area to determine the LV mass index (g m^{-2}). The LV sphericity index was derived by the ratio of LV short- to long-axis dimension in the end-systolic apical four-chamber view. The average values of at least three measurements were calculated and used for final analysis. Intra- and inter-observer variability was less than 5%.

1.5. Dobutamine stress echocardiography

After the baseline echocardiograms were obtained, dobutamine was infused at doses of 5, 10, 20 and

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Table 1
Baseline clinical characteristics of each treatment group.

Variables	ABMMNC group (n = 25)	Control group (n = 25)	p value
Age (years), mean ± SD, median (min–max)	57.1 ± 7.0, 58 (43–68)	59.6 ± 7.4, 61 (43–71)	0.21
Male gender, *n* (%)	24 (96)	23 (92)	1.00
Body-mass index (kg m^{-2})	24.9 ± 3.1	25.6 ± 2.7	0.41
Hypertension, *n* (%)	15 (60)	19 (76)	0.23
Diabetes, *n* (%)	10 (40)	11 (44)	0.77
Insulin-dependent, *n* (%)	4 (16)	2 (8)	0.67
Hypercholesterolemia, *n* (%)	19 (76)	21 (84)	0.48
Current tobacco use, *n* (%)	22 (88)	20 (80)	0.70
Family history of CAD, *n* (%)	20 (80)	17 (68)	0.33
Time from last MI to procedure (days) mean ± SD, median (min–max)	397 ± 467, 152 (4–1697)	311 ± 272, 197 (9–970)	0.44
History of PCI, *n* (%)	14 (56)	11 (44)	0.40
History of cardiac arrest or IABP, *n* (%)	3 (12)	2 (8)	1.00
Previous hospital admission for HF	4 (16)	5 (20)	0.71
Previous stroke, *n* (%)	1 (4)	2 (8)	1.00
Peripheral vascular disease, *n* (%)	9 (36)	7 (28)	0.54
COPD, *n* (%)	8 (32)	9 (36)	0.77
Serum creatinine (mg dl^{-1})	1.05 ± 0.23	1.06 ± 0.25	1.00
Estimated GFR, ml min^{-1} per 1.73 m^2	81.5 ± 21.2	80.3 ± 18.7	0.82
Mean arterial pressure (mmHg)	76.4 ± 13.5	79.8 ± 15.4	0.41
Systolic PAP (mmHg)	45.4 ± 8.2	45.8 ± 7.9	0.86
LVEF (%)	24.8 ± 3.7	25.9 ± 3.1	0.20
LVEDP (mm Hg)	19.0 ± 3.6	18.5 ± 3.5	0.61
Heart rate (beats min^{-1})	81 ± 13	80 ± 13	0.82
Infarct territory, *n* (%)			
Anterior/septal	16 (64)	15 (60)	0.77
Lateral	7 (28)	10 (40)	0.37
Inferior	19 (76)	15 (60)	0.23
Left main stem stenosis, *n* (%)	3 (12)	2 (8)	1.00
Ungraftable coronary artery, *n* (%)			
Left anterior descending	1 (4)	0 (0)	1.00
Left circumflex	7 (28)	8 (32)	0.76
Right coronary	22 (88)	19 (76)	0.46
CCS class, mean ± SD, (median)	3.04 ± 0.54 (3)	2.88 ± 0.53 (3)	0.29
NYHA class, mean ± SD, (median)	3.16 ± 0.37 (3)	3.24 ± 0.43 (3)	0.49
Medication on admission, *n* (%)			
Aspirin	24 (96)	23 (92)	1.00
Clopidogrel	2 (8)	4 (16)	0.67
ACE inhibitors	18 (72)	15 (60)	0.55
Angiotensin receptor antagonists	8 (24)	12 (48)	0.39
Diuretics (loop diuretics or thiazides)	17 (68)	16 (64)	1.00
Aldosterone receptor antagonists	12 (48)	10 (40)	0.78
Beta-adrenoceptor antagonists	19 (76)	18 (72)	1.00
Nitrates	13 (52)	10 (40)	0.57
Statins	16 (64)	19 (76)	0.54
Digitalis	2 (8)	1 (4)	0.55
*Euro*SCORE[a] mean ± SD,	8.2 ± 1.9	7.5 ± 2.3	0.22
Median (min–max)	9.0 (4–13)	8.0 (3–11)	0.16
Estimated operative risk	11.8 ± 5.8	9.6 ± 4.8	

Values are mean ± SD or percentage of patients. ABMMNC, autologous bone marrow mononuclear cells; CAD, coronary artery disease; MI, myocardial infarction; PCI, percutaneous coronary intervention; IABP, intra-aortic balloon pump; COPD, chronic obstructive pulmonary disease; GFR, glomerular filtration rate; PAP, pulmonary artery pressure; LVEF, left ventricular ejection fraction; LVEDP, left ventricular end-diastolic pressure; LAD, left anterior descending artery; CX, circumflex artery; RCA, right coronary artery; CCS, Canadian Cardiovascular Society; NYHA, New York Heart Association.

[a] Based on *Euro*SCORE, European system for cardiac operative risk evaluation. A score of 6 plus indicates a high risk.

40 μg kg^{-1} min^{-1} at 3-min intervals in order to evaluate ischaemia and viability. Absence of improvement in motion at the akinetic regions during dobutamine infusion was considered as non-viable myocardium. Patients were considered to have viable myocardium if wall motion score improved by at least one full grade in two adjacent akinetic segments from the same vascular territory during low-dose dobutamine administration.

1.6. Coronary angiography

Selective coronary arteriography and contrast left ventriculography were performed from the femoral approach using 6F-Judkins diagnostic catheters. Multiple views of the right and left coronary arteries were recorded (Philips Integris H3000, Eindhoven, the Netherlands). Minimal luminal diameter was measured by quantitative analysis,

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and severity of the lesion was defined as percent stenosis calculated by using the minimal luminal diameter and the diameter of the reference segment (a normal segment proximal to the lesion).

1.7. Radionuclide imaging

Myocardial perfusion scintigraphy was performed using pharmacological stress in all patients. About 2–3 mCi Tl-201 was injected intravenously 45 min after per oral administration of 375 mg dipyridamole; stress images were acquired within 5 min of injection. Redistribution images were acquired 3–4 h after stress images. Two independent nuclear physicians (Nuriye Ozlem Kucuk and Gulseren Aras) blinded to patient identity and the territory of ABMMNC implantation interpreted the images. Inter-observer concordance was 96% before consensus reading; discrepancies were resolved by consensus. For the quantitative evaluation, we used 4D-M SPECT software. Semi-quantitative visual interpretation was performed for resting and stress perfusion images with short-axis and vertical long-axis myocardial tomograms using a 20-segment model of the LV. Each segment was scored with a five-point scoring system (0 = normal, 1 = mild reduction of radiotracer uptake, 2 = moderate reduction, 3 = severe reduction, and 4 = absent uptake). Scores $\geq$2 were taken to be perfusion defects; reversibility was defined as an improvement of at least one grade on subsequent images. Global cardiac perfusion score revealed the sum of the perfusion scores for each segment; regional perfusion score was calculated by the sum of the perfusion scores for segments on which ABMMNC implantation was performed. The Tc99m-sestamibi gated SPECT images were acquired using dipyridamole stress and a rest study. Siemens 4D-M software 2.01 program was applied to gated SPECT images for the estimation of LV volumes, wall thickness, wall motion and LVEF.

1.8. Bone marrow harvest and isolation of mononuclear cells

A bone marrow aspiration technique from the iliac crest and processing methods has been previously described [21,22]. The collected bone marrow (658 ± 66 ml) was immediately transferred to the Haemapheresis Unit for red blood cell depletion and volume reduction. Using this schedule, we targeted a total of 1×10^9 ABMMNCs while avoiding unnecessary red blood cell and platelet contamination and higher harvest volumes. The harvest material was processed on the COBE Spectra (Gambro BCT, Lakewood, CO, USA) using bone marrow processing program and software version 5.1. Using a continuous flow cell separator, we achieved 91 ± 2% red blood cell depletion. The final bone marrow cell preparation (57.5 ± 8.9 ml) contained a mean of $2.52 \pm 0.16 \times 10^9$ (range: 0.88–4.16×10^9) nucleated cells, $1.29 \pm 0.09 \times 10^9$ (range: 0.24–2.4×10^9) mononucleated cells and $31.9 \pm 4.4 \times 10^6$ (range 0.05–80.4×10^6) $CD34^+$ cells. Cytofluorimetric analysis of implanted stem cells was 99% viable and included $CD45^+$ cells in 97.5 ± 2.2%. Approximately 5 ml bone marrow cells were tested for viability and bacterial/fungal contamination.

1.9. Operation and transepicardial implantation of ABMMNCs

After bone marrow harvest, we performed a complete intraoperative transoesophageal echocardiography (TEE) examination in the supine position, including two-dimensional, colour flow and Doppler studies with the use of Sonos-5500 device (Hewlett-Packard, Andover, MA, USA). All operations were performed by the same surgical team through a median sternotomy followed by either standard non-pulsatile CPB with moderate hypothermia or the off-pump coronary artery bypass (OPCAB) technique. Grafting to coronary arteries of small calibre, diffuse disease or severe calcification requiring endarterectomy was avoided. The target region for ABMMNC implantation was selected by matching the area identified previously by SPECT imaging as ischaemic (viable infarct border zone) and by coronary angiography as ungraftable (Table 2). At the end of distal anastomoses, ABMMNC implantation was performed into the peri-infarct area (12–18 injections per patient), which surrounded the ungraftable vessel. ABMMNCs were delivered while the implanted needle was progressively removed from the myocardium. Septal injections (6–12 injections patient^{-1}) were performed via the anterior surface of the right ventricle in all patients. The needle tip was placed across the right ventricle in a perpendicular position and into the septal wall subendocardial target area under TEE guidance. Approximately 2 ml of ABMMNCs were implanted into each injection site, with a 27 G $\times$ 90 mm, K-3 point type spinal needle (Dr Japan Co., Ltd, Shinjuku, Japan). The number of bone marrow cells per injection is estimated as 0.46×10^8.

1.10. Follow-up

Serial blood samples for measuring the concentrations of the mass of isoenzyme MB of creatine kinase (CK-MB; Beckman Coulter, USA) and cardiac troponin I (cTnI; Access AccuTnI™, Beckman Coulter, USA) were obtained via a central venous line shortly before the induction of anaesthesia and at 1, 6, 12, 24, 48 h after the construction of the final anastomosis and, additionally whenever necessary. The upper limit of normal as defined by our laboratory was 0.04 ng ml^{-1} for cTnI and 6.3 ng ml^{-1} for CK-MB. Unless contraindications existed, all patients were treated with aspirin within 8 h postoperatively, an angiotensin-converting enzyme inhibitor or angiotensin-receptor blocker, beta-blocker and diuretics. A statin was commenced if preoperative low-density lipoprotein–cholesterol concentrations were above 80 mg dl^{-1}. Furthermore, the patients underwent follow-up evaluations at 6 and 18 months after the operation, which consisted of a repeat baseline clinical and laboratory evaluation, Thalium201 SPECT and Tc99m MIBI gated SPECT perfusion scanning, transthoracic echocardiogram and DSE. All patients underwent follow-up angiography 6–8 months after surgery to assess graft/vessel patency and angiographic coronary disease progression. Bypass grafts were analysed visually in an independent blinded fashion by two cardiologists (Mustafa Kilickap and Cetin Erol) and classified as having <50% stenosis, 50–99% stenosis or total occlusion. Safety measures included adverse events, resting

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Table 2
Operative and postoperative morbidity comparison data.

Variables	ABMMNC group (n = 25, %)	Control group (n = 25, %)	p value
Number of coronary grafts	2.6 ± 1.0	2.8 ± 0.6	0.43
Number of arterial distal anastomoses	1.1 ± 0.3	1.2 ± 0.4	0.39
Number of venous distal anastomoses	1.6 ± 1.0	1.7 ± 0.5	0.53
LITA grafts, n (%)	25 (100)	25 (100)	1.00
Radial artery grafts, n (%)	3 (12)	4 (16)	1.00
OPCAB, n (%)	7 (28)	5 (20)	0.74
Ischaemic time (min) (n; 18 vs 20)	50 ± 13	45 ± 9	0.26
CPB time (min) (n; 18 vs 20)	71 ± 18	61 ± 11	0.05
BM implant time, mean ± SD, (min–max)	12.9 ± 2.9 (8–18)	–	
Intra- or postoperative IABP, n (%)	4 (16)	5 (20)	1.00
Peak CK-MB, ng ml^{-1} (<6.3)[a]	25.2 ± 10.8	27.9 ± 8.9	0.34
Peak troponin I, ng ml^{-1} (<0.04)[a]	0.75 ± 0.48	0.85 ± 0.57	0.51
Troponin I at 48 h (ng ml^{-1}) (mean ± SD), median (min–max)	0.53 ± 0.33, 0.55 (0.02–1.20)	0.67 ± 0.47, 0.63 (0.01–2.25)	0.24
Postoperative blood loss at 24 h (ml)	775 ± 236	856 ± 150	0.15
Re-exploration due to bleeding (n)	0	0	1.00
Postoperative atrial fibrillation, n (%)	4 (16)	5 (20)	1.00
Mechanical ventilation (h)	15.5 ± 8.5	14.4 ± 6.7	0.60
Stay in intensive care unit (h)	51.7 ± 20.5	63.5 ± 24.9	0.07
Postoperative peak serum creatinine (mg dl^{-1})	1.15 ± 0.23	1.24 ± 0.25	0.18
Minimum estimated GFR (ml min^{-1} per 1.73 m^2)	72 ± 15	65 ± 13	0.09
Hospital stay (days)	9.6 ± 3.4	11.3 ± 3.6	0.10
Hgb, g dl^{-1} at discharge (13–18)[a]	9.6 ± 1.1	10.6 ± 1.3	0.004
Operative mortality, n (%)	0	1 (4)	1.00
Medication at 6 weeks follow-up, n (%)			
Aspirin	25 (100)	23 (96)	0.49
Clopidogrel	2 (8)	4 (17)	0.42
ACE inhibitors	22 (88)	17 (71)	0.17
Angiotensin receptor antagonists	10 (40)	13 (54)	0.40
Diuretics (loop diuretics or thiazides)	20 (80)	17 (71)	0.52
Aldosterone receptor antagonists	17 (68)	15 (63)	0.77
Beta-adrenoceptor antagonists	20 (80)	21 (88)	0.70
Nitrates	0	1 (4)	1.00
Statins	19 (76)	20 (83)	0.73
Digitalis	3 (12)	2 (8)	0.67
Overall mortality, n (%)	4 (16)	6 (24)	0.48

ABMMNC, autologous bone marrow mononuclear cells; LITA, left internal thoracic artery; OPCABG, off-pump coronary artery bypass grafting; CPB, cardiopulmonary bypass; BM, bone marrow; CK, creatine kinase; GFR, glomerular filtration rate; Hgb, haemoglobin. Values of CK-MB and troponin I are expressed as median and range.
[a] Reference values provided by our laboratory.

electrocardiograms, 24-h Holter monitoring, chest radiography, chemistry/haematology analyses, and ophthalmological examinations. We used 24 h Holter monitoring (Oxford Medilog 4500) to assess potential proarrhythmic effects of ABMMNC implantation, which were obtained before hospital discharge, and at 6-weeks' follow-up.

1.11. Statistics

The differences in proportions between the two groups were compared by using the Chi-square test or the Fisher's exact test, where appropriate. Normally distributed continuous variables were compared using the unpaired t-test. Ordinal and non-normally distributed continuous variables were evaluated by the Mann–Whitney U test. Differences between preoperative and postoperative for CCS, NYHA, LVEF, LV volume indices were assessed by the paired t test or the Wilcoxon signed ranks test, where applicable. Time–activity curves for CK-MB and cTnI were constructed for each patient, the peak levels were noted and the analysis of the difference in the peak enzyme release between the two groups was performed using a Mann–Whitney U test. Survival was estimated by the Kaplan–Meier method. Data were analysed using SPSS 16.0 (SPSS Inc, Chicago, IL, USA) for Windows (Microsoft Corp., Redmond, WA, USA). The significance level for difference for all tests was p value less than 0.05. Predicted risk scores for in-hospital mortality were calculated using previously validated models of the *Euro*SCORE. Intra- and inter-observer variabilities were assessed as the percentage of the difference between two readings to the mean.

2. Results

Between 4 June 2003 and 14 January 2007, 868 patients with triple vessel coronary artery disease were admitted to our unit for isolated CABG (Fig. 1). A total of 50 patients were included in the study, after consideration of inclusion and exclusion criteria. The final cohort included 25 controls and 25 patients in the ABMMNC group. The clinical characteristics of the study group are reported in Table 1. There were no clinically or statistically significant differences between the two groups with regard to age, gender, cardiac risk factors, symptoms, *Euro*SCORE, the number of viable and transmural segments, LVEF or LV dimensions. All patients presented with LV dysfunction and moderate to severe LV dilatation. LV end-diastolic volume index and LV end-systolic volume index were on average 107 ± 12 and 72 ± 5 ml m^{-2}, respectively.

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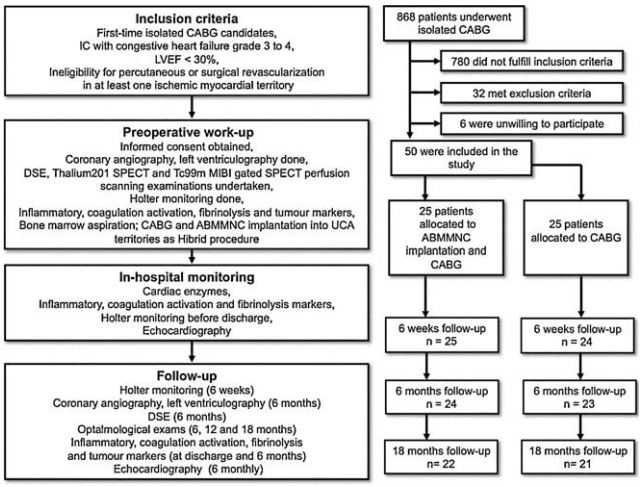


Fig. 1. Study design. CABG, coronary artery bypass grafting; IC, ischaemic cardiomyopathy; LVEF, left ventricular ejection fraction; DSE, dobutamine stress echocardiography; SPECT, single-photon emission computed tomography, ABMMNC; autologous bone marrow mononuclear cells; UCA, ungraftable infarct-related coronary artery.

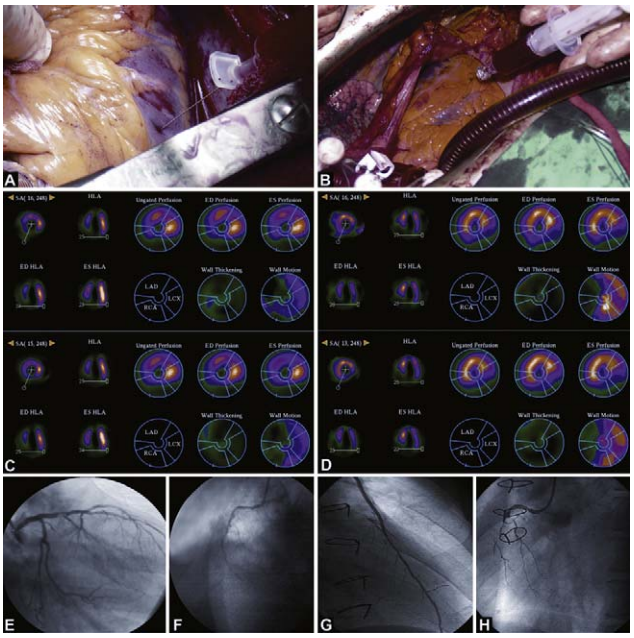


Fig. 2. ABMMNC implantation technique. Two millilitres of ABMMNCs into each injection site, with a 7 cm grid and 22-G spinal needle (A) implantation of ABMMNC's into acute margin of the heart at the diaphragmatic surface in a patient with ungraftable RCA territory; (B) septal implantation of ABMMNCs via right ventricular injections under transoesophageal echocardiography guidance; preoperative (C) and postoperative (D) Tc99m MIBI gated SPECT perfusion scan images showing myocardial perfusion with colour scale for subject 3, a 60-year-old man. Regional myocardial perfusion at the ungraftable RCA territory has increased from baseline; (E) preoperative angiogram of the same patient with proximal LAD stenosis; (F) and ungraftable RCA territory, (G) postoperative control angiogram demonstrating patent LITA-LAD anastomosis; (H) and preserved collaterals beyond proximally occluded RCA.

2.1. Procedural data

Multi-vessel disease was present in all patients. There were no differences between the two groups with regard to the surgical technique, number of bypass grafts performed per patient, ischaemic time, postoperative intra-aortic balloon pump use and medication at discharge (Table 2). The mean total bone marrow harvesting time was 27 ± 5 min and the mean total procedural time for ABMMNC implantation was 13 ± 3 min. The mean time between the harvest of bone marrow cells and injection was 152 ± 17 min. At each injection, we delivered approximately 2 million cells in a volume of 2 ml (Fig. 2). Overall high-dose inotropic support (intravenous dopamine at $>5\ \mu g\ kg^{-1}\ min^{-1}$, dobutamine, epinephrine, phosphodiesterase inhibitors or levosimendan) was used in 28 patients (56%) but no significant difference was detected between the two groups.

2.2. Safety data

There were no hospital deaths in the ABMMNC group; however, one death was encountered in the control group as a result of the low cardiac output syndrome. None of the patients had a stroke. No patient experienced periprocedural complications. There was no significant difference in myocardial injury markers (cTnI and CK-MB) postoperatively between the two groups (Table 2). The peak cTnI levels were $>1\ ng\ ml^{-1}$ in four patients of both groups. Postoperative blood loss and blood product transfusion were also comparable, but haemoglobin values measured before discharge were higher in the control group ($p = 0.004$). Furthermore, four patients (16%) in the ABMMNC group, who were diagnosed with iron deficiency-induced hypochromic microcytic anaemia, were treated by oral iron replacement therapy for 3 months. Transient atrial fibrillation detected in both groups was converted to sinus rhythm with amiodarone hydrochloride or beta-blockers; no patients required electrocardioversion. Respiratory insufficiency necessitating prolonged (>24 h) respiratory support occurred in three patients (12%) versus five patients (20%) in controls ($p = 0.70$). No patient required renal replacement therapy in neither of the groups in the postoperative period.

2.3. Follow-up evaluations

Follow-up was complete on 14 September 2008. Mean follow-up time for both the groups, including hospital deaths, was 988 ± 423 days (range: 36–1860 days; 1011 ± 384 for the ABMMNC group vs 965 ± 466 for the controls, $p = 0.70$), and 18 months follow-up was complete in all survivors.

NYHA functional class at baseline was comparable between the two groups. In the group ABMMNC, NYHA class improved significantly from 3.16 ± 0.37 at baseline to 1.24 ± 0.52 at 6 months ($p < 0.001$), and this improvement was maintained to a value of 1.30 ± 0.47 at 18 months ($p < 0.001$ vs baseline). In control group, NYHA class decreased from 3.24 ± 0.43 to 1.68 ± 0.57 at 6 months ($p < 0.001$) and 1.76 ± 0.54 at 18 months ($p < 0.001$ vs baseline). However, significant difference was also noted in delta NYHA between the two groups at 6 months ($p = 0.004$) and at 18 months ($p = 0.012$). At 6-month follow-up, there was decrease in at least two angina classes noted in 96% of the patients treated with ABMMNC transplantation and CABG, whereas a reduction in two angina classes in the control group occurred in 86% of patients ($p = 0.33$).

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Table 3
Results of 24-h Holter ambulatory ECG monitoring, qualitative assessment of 6-month angiograms and inflammatory, coagulation activation and fibrinolysis markers.

Variables	ABMMNC group (n = 25, %)	Control group (n = 25, %)	p values
Ventricular activity at 6 weeks			
Total PVC (n)	614 ± 549	933 ± 1907	0.43
Single PVC (n)	545 ± 472	430 ± 462	0.39
Sustained VT (n)	2.88 ± 3.56	2.96 ± 4.07	0.94
Couplet (n)	7.04 ± 8.88	6.08 ± 8.51	0.70
Bigeminy/trigeminy (n)	0.52 ± 0.87	0.36 ± 0.64	0.46
Angiographic assessment at 6–8 months			
Totally occluded arterial grafts, n (%)	1 (4)	2 (7)	1.00
Totally occluded ITA grafts, n (%)	0	0	1.00
Totally occluded SV grafts, n (%)	5 (13)	7 (16)	0.69
Inflammatory, coagulation activation and fibrinolysis markers			
White blood cell count, $\times 10^9\ l^{-1}$ (4–11)[a]			
At baseline	6.6 ± 1.1	6.5 ± 1.1	0.58
At discharge	11.1 ± 2.3	10.5 ± 2.1	0.33
At 6 months	6.8 ± 1.1	6.7 ± 1.5	0.86
hC-reactive protein, mg dl^{-1} (<0.320)[a]			
At baseline	0.25 ± 0.07	0.21 ± 0.09	0.07
At discharge	2.86 ± 1.45	3.06 ± 1.87	0.68
At 6 months	0.31 ± 0.27	0.58 ± 0.82	0.14
Fibrinogen, mg dl^{-1} (180–380)[a]			
At baseline	231 ± 80	212 ± 78	0.39
At discharge	586 ± 225	612 ± 241	0.69
At 6 months	246 ± 65	213 ± 81	0.16
D-dimer, $\mu g\ l^{-1}$ (50–250)[a]			
At baseline	144 ± 56	145 ± 66	0.96
At discharge	636 ± 91	657 ± 92	0.42
At 6 months	173 ± 55	181 ± 68	0.69

ABMMNC, autologous bone marrow mononuclear cells; PVC, premature ventricular complex; VT, ventricular tachycardia; ITA, internal thoracic artery; SV, saphenous vein.

[a] Reference values provided by our laboratory.

Data obtained from resting echocardiography, DSE and SPECT scans showed statistically significant improvement in cardiac function after surgery. Mean global LVEF improved significantly from 24.8 ± 3.7% to 36.3 ± 7.4% in the ABMMNC group ($p < 0.001$), whereas LVEF improved modestly from 25.9 ± 3.1% to 31.4 ± 4.1% in the control group ($p < 0.001$). However, a significant difference was also noted about the mean improvement in LVEF between the groups ($p < 0.001$, 95% CI: 3.4–8.9) at 6 months, and ($p = 0.001$, 95% CI: 2.0–7.4) at 18 months. At baseline, parameters of LV dimensions were similar in both the ABMMNC-treated and the control groups (Table 4). On average, both LV end-diastolic and end-systolic volume indices decreased significantly at 6 months ($p = 0.001$, $p < 0.001$, respectively) in the ABMMNC group. Delta LV end-diastolic volume index at 6 months did not differ between the two groups ($p = 0.32$) whereas significant difference was noted in delta LV end-systolic volume index at 6 months ($p < 0.001$) suggesting better systolic ventricular function recovery with cell therapy.

A total of 24 left internal thoracic artery (LITA) grafts, 3 radial artery grafts and 39 saphenous vein grafts were evaluated at follow-up angiography in the ABMMNC group whereas 25, 4 and 43 grafts were evaluated in the control group, respectively. No evidence of accelerated coronary artery disease or graft occlusion was detected in either group during follow-up (Table 3). All LITA grafts were patent in both the groups. Complete arterial graft occlusion occurred in 3.7% versus 6.9% in the ABMMNC group and controls, respectively (1 of 27 vs 2 of 29, $p = 1.00$). Of 39 saphenous vein grafts, 5 (13%) were occluded in the ABMMNC group as opposed to 7 (16%) out of 43 in the control group ($p = 0.69$). There were no differences in inflammatory markers at baseline, discharge and 6-month follow-up between the two groups.

A total of 500 LV myocardial segments evaluated by SPECT for each group were available for comparative analysis; 360 segments were re-vascularised with CABG alone, and 140 received ABMMNC implantation in the study group whereas 366 were re-vascularised and 134 did not receive any form of treatment (in controls). On average, graftable segments had higher perfusion scores than UCA segments in both groups at baseline and follow-up (Table 4). However, perfusion scores in UCA segments improved with ABMMNC therapy to 18.0 ± 24.4 from 7.1 ± 25.7 at 6 months ($p = 0.001$ vs control UCA segments) and this difference between the groups was maintained at 1 year. Four late deaths occurred in the ABMMNC group on the 240th, 356th, 645th and 1177th postoperative days compared to five late deaths in the control group. One patient in the ABMMNC group, with preoperative LVEF of 18%, developed an episode of respiratory insufficiency as a result of pulmonary embolism on the 220th postoperative day and died on the 240th day; in one, multi-organ failure developed; in other, cardiac failure was the cause of death and yet another presented with acute

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Table 4
Baseline and changes across the study in left ventricular volumes and function at 6 and 18 months as measured by echocardiography and scintigraphy.

	Baseline			6 months			18 months		
	ABMMNC group ($n = 25$)	Control group ($n = 25$)	p value	ABMMNC group ($n = 24$)	Control group ($n = 23$)	p value	ABMMNC group ($n = 22$)	Control group ($n = 19$)	p value
Echocardiographic									
Global LVEF (%)	24.8 ± 3.7	25.9 ± 3.1	0.20	36.3 ± 7.4	31.4 ± 4.1	0.007	34.3 ± 6.1	30.3 ± 4.6	0.02
Delta LVEF				11.6 ± 6.1	5.4 ± 2.7	<0.001	9.4 ± 4.7	4.7 ± 3.9	0.001
LVSI	0.47 ± 0.09	0.49 ± 0.09	0.43	0.54 ± 0.09	0.51 ± 0.08	0.41	0.52 ± 0.09	0.51 ± 0.09	0.61
WMSI	2.70 ± 0.25	2.69 ± 0.31	0.94	2.47 ± 0.28	2.66 ± 0.29	0.04	2.50 ± 0.28	2.66 ± 0.17	0.04
LVMI (g m^{-2})	110.6 ± 10.2	109.9 ± 10.9	0.81	100.4 ± 9.5	100.5 ± 10.5	0.95	103.9 ± 9.8	103.8 ± 10.8	0.96
Gated SPECT data									
LVEDVI (ml m^{-2})	107.4 ± 11.8	106.8 ± 9.9	0.86	99.3 ± 15.2	101.4 ± 8.9	0.56	98.0 ± 10.9	102.4 ± 10.6	0.20
LVESVI (ml m^{-2})	71.7 ± 4.9	71.8 ± 3.1	0.92	60.0 ± 4.1	66.4 ± 4.3	<0.001	61.2 ± 5.4	66.5 ± 4.8	0.002
Perfusion score in graftable territories	13.9 ± 19.2	10.4 ± 15.4	0.48	68.7 ± 27.9	57.4 ± 37.3	0.24	62.8 ± 28.9	51.4 ± 36.9	0.26
Delta perfusion score in graftable territories				54.5 ± 30.1	47.9 ± 28.1	0.44	48.9 ± 30.7	42.7 ± 25.6	0.47
Perfusion score in UCA territories	7.1 ± 25.7	9.7 ± 24.9	0.72	18.0 ± 24.4	10.0 ± 22.9	0.25	13.6 ± 21.5	11.5 ± 25.6	0.77
Delta perfusion score in UCA territories				10.9 ± 11.4	0.93 ± 6.7	<0.001	11.0 ± 10.0	1.3 ± 7.8	<0.001

ABMMNC, autologous bone marrow mononuclear cells; LVEF, left ventricular ejection fraction; LVSI, left ventricular sphericity index; WMSI, wall motion score index; LVMI, left ventricular mass index; SPECT single-photon emission computed tomography; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; UCA; ungraftable infarct-related coronary artery. Delta values indicate versus baseline.

renal failure, which progressed despite an improvement in cardiac function. Cardiac autopsy findings of the patient, who died of pulmonary embolism, are demonstrated in detail in Fig. 3. The causes of late deaths in the control group were sudden death, ventricular tachyarrhythmia, lung cancer and HF in two patients. Overall, Kaplan–Meier survival estimation at 5 years, including in-hospital mortality, was 78.9 ± 10%, in the ABMMNC group versus 71.1 ± 11% for controls ($p = 0.48$) (Fig. 4A). Event-free survival (cardiac events) including mortality was also similar between the two groups (29.6 ± 13.1% vs 17.1 ± 14.4%; $p = 0.99$) (Fig. 4B). Patients neither received an automatic implantable cardiac defibrillator nor developed significant ventricular arrhythmias during the follow-up. Compared with the control group, the incidence of premature ventricular complexes was unchanged at 6 weeks after ABMMNC implantation as was the incidence of sustained ventricular arrhythmias (Table 3). All cases remained free of retinopathy observed in funduscopic examinations and teratoma formation detectable by chest radiography during follow-up.

3. Discussion

The results of our study suggest that implantation of ABMMNCs to UCA territories, as an adjunct to CABG, is safe and feasible, augments perfusion and LVEF contractile recovery but does not improve overall survival. Ungraftable ischaemic myocardial territories pose a number of problems for clinicians in their operative decisions because, at present, patients with poor distal run-off of the coronary arteries are untreatable by conventional treatments. Furthermore, failure to re-vascularise even a single ischaemic myocardial territory due to poor native vessel anatomy is associated with a suboptimal outcome despite a successful left internal thoracic artery bypass to the left anterior descending artery.

Based on the results from preclinical studies, cellular therapy is a novel option to improve cardiac regeneration [2] or vascularisation [3]. However, clinical studies addressing the effects of ABMMNC implantation showed modest therapeutic benefit after AMI or chronic myocardial ischaemia [8–11]. Knowledge, as to the mechanisms of improvement in clinical trials, is also inadequate. In particular, potential links between cardiac regeneration and differentiation, cell fusion, cytokine-mediated paracrine signalling, prevention of regional infarct expansion and stimulation of endogenous stem cell niches in the clinical setting remain poorly defined. Furthermore, there is relatively little previous work using cellular treatment strategies as an adjunct to CABG for patients with IC and UCA territories. The inclusion criteria in our study were designed to select high-risk patients because of ethical concerns. In chronic myocardial ischaemia, human bone marrow may be the best-established source for adult stem cells, which consists of several subpopulations of pluripotent cells. Fresh methodology is based on implementation of ABMMNCs aspirated from bone marrow directly to the ischaemic myocardium after removal of erythrocytes; no time is required to expand the cells in culture with this method while ABMMNCs ($1.29 \pm 0.09 \times 10^9$ ml^{-1} total mononuclear cells) are directly delivered to the target border zone of the infarct with multiple injections within approximately 3 h. Further advantages of these autologous cells are the avoidance of immunologic rejection, and their ready availability. Angiogenic proteins released from ABMMNC's (mostly $CD34^+$ haematopoietic stem cells) improve angiogenesis and/or vasculogenesis at the ischaemic myocardium; this is in keeping with the findings of previously reported series [11,18]. However, the use of unfractionated ABMMNCs still raises many unanswered questions regarding the mechanisms involved and the dose–response effect, which are beyond the scope of this study. Furthermore, IC was recently shown to be associated with

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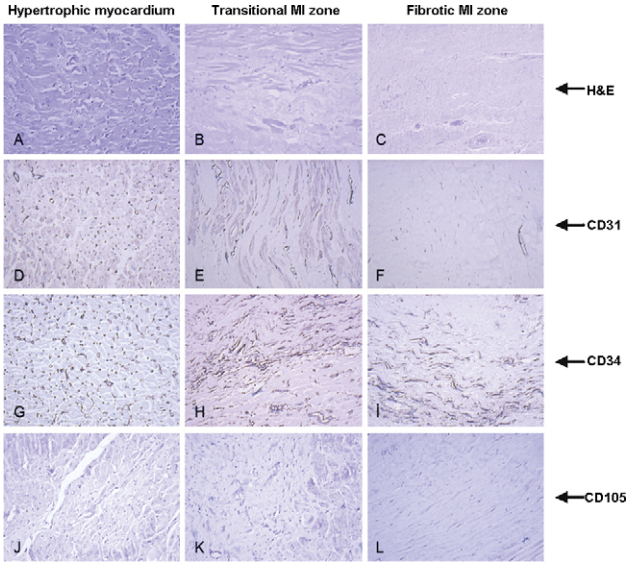


Fig. 3. Summary of the post-mortem findings of a patient from ABMMNC group. Autopsy findings of a patient, who died of a pulmonary embolism originating from iliac veins on the 240th postoperative day after CABG and ABMMNC implantation. The hypertrophic myocardial areas (A, D, G, J) were consistent with several capillary vessels shown by CD31 (D) and CD34 (G) whereas CD105 positive active proliferating endothelial cells were lining nearly half of the CD31 positive vessels. The transitional myocardial infarct zone represents the fibrotic areas and hypertrophied myocardium (B, E, H, K). (E) CD31 staining demonstrated decreased amount of capillary vessels in fibrotic areas but increased amount of CD34 positive cells (H) was present. When compared to CD31 and CD105 (K) it is clear that many of the CD34 positive spindle cells do not constitute the endothelial lining vessels. The fibrotic scar areas (C, F, I, L) were poorly vascularised when examined with CD31 (F). However, CD34 positive cells (I) were also increased in these areas which may be related to the myofibroblastic proliferation. Positive CD105 (L) in this area revealed that the amount of active proliferating vessels was decreased when compared to the transitional zone of the myocardial infarction. Since CD105 can be either positive on active proliferating endothelial cells or bone marrow mesenchymal cells, it is hard to be sure of the origin of CD105 positive endothelial cells whether they originate from the injected bone marrow mesenchymal cells or resident proliferating endothelial cells. Magnification: ×20 (H&E, CD31, CD34, CD105).

functional impairment of progenitor cell function within bone marrow niche and in the peripheral blood [23].

Different cellular implantation techniques have been performed including transepicardial [17], selective intracoronary infusion [8,9,12,14], percutaneous transfemoral endoventricular [10,11] or coronary venous [6] routes. Unexpected high rates of coronary in-stent restenosis after primary angioplasty and intra-coronary stem cell delivery mobilised by granulocyte-colony-stimulating factor (G-CSF) (corroborate) indicate caution in this regard [16]. It has been recently shown that ABMMNCs isolated from patients with IC have a significantly reduced migratory and colony-forming activity *in vitro* and a reduced neovascularisation capacity *in vivo* despite similar content of haematopoietic stem cells [24]. Thus, ischaemic heart disease and/or the presence of cardiovascular risk factors may contribute significantly to the functional impairment of ABMMNCs in patients with IC. Choosing the transepicardial implantation rather than intracoronary administration might overcome the problems of functionally impaired stem cells to extravasate against a chemo-attractant gradient to invade and home in on the ischaemic tissue. Recently, Hou et al. [25] have evaluated the actual fate of delivered cells (intra-myocardial vs intracoronary vs retrograde coronary venous) in an ischaemic swine model. They demonstrated that the majority of the delivered cells were not retained in the heart, but distributed to the visceral organs, mostly to the lungs, after each delivery modality. They also found that intramyocardial injection was more efficient ($11 \pm 3\%$) compared with intracoronary ($2.6 \pm 0.3\%$) or coronary venous ($3.2 \pm 1.0\%$) delivery techniques in terms of retaining cells in the heart. Our study is the first human study to surgically implant ABMMNCs to not only ungraftable territories but also ventricular septum via right ventricle under TEE guidance. Overall, we found no difference between the two groups regarding CK-MB/troponin rise postoperatively. The lack of bleeding through multiple injections or any evidence of cell embolisation such as systemic organ dysfunction in this series was gratifying and suggests the safety of the procedure.

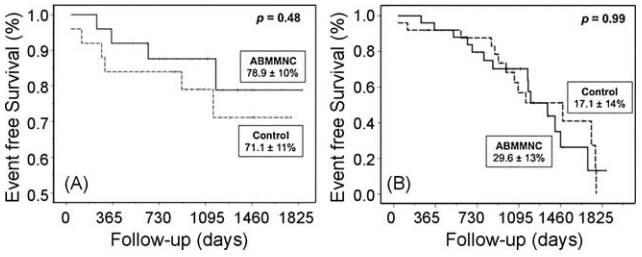


Fig. 4. Kaplan–Meier estimates for overall survival and event-free survival for ABMMNC group versus controls. (A) Kaplan–Meier survival plot categorized by ABMMNC (solid line) and control groups (dashed line) at 5 years (log rank analysis, $78.9 \pm 10\%$ vs $71.1 \pm 11\%$, $p = 0.48$). Ordinate graphs the proportion survived. Abscissa graphs person days of follow-up. (B) Event-free survival (cardiac events) including mortality in both groups ($29.6 \pm 13\%$ vs $17.1 \pm 14\%$; $p < 0.99$). ABMMNC group indicates combined autologous bone marrow mononuclear cell transplantation and coronary bypass surgery; control, incomplete coronary bypass surgery alone due to ungraftable territories.

Inappropriate electrophysiological coupling of bone marrow derived stem cells or skeletal myoblasts and cardiomyocytes were questioned previously [1,6]. Prior to discharge, the incidence of atrial fibrillation and ventricular arrhythmias was similar between the two groups in our study. Data from the present study showed no evidence of proarrhythmic potential on any Holter monitoring studies after ABMMNC implantation at 6 weeks. This observation is supported by a previous study in which no malignant arrhythmias and no change in the number of premature ventricular complexes after ABMMNC injection were detected [11].

Arteriosclerosis is being increasingly viewed as an inflammatory disease. Studies of inflammatory markers have consistently shown a strong correlation between the levels of these markers and the risk of cardiovascular events. Since the pro-inflammatory potential of ABMMNC implantation via growth factors and cytokines exists, we closely monitored serum inflammatory markers in our patient population. However, our data on C-reactive protein, white blood cell count, fibrinogen at baseline and 6-month follow-up did not show any difference between the two groups. Furthermore, accelerated arteriosclerosis via pro-inflammatory growth factors is one of the possible deleterious effects of cell therapy. However, repeat coronary angiography at 6 –8 months

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confirmed acceptable graft patency rates in both arterial and venous grafts as well as native coronary circulation. Our follow-up data for ABMMNC transplantation at 12 months confirm observations of earlier studies [10,11]. Furthermore, to date, there have been no analyses of 5-year follow-up data after cellular therapy in patients with IC. We did not detect any growth of a previously undetected cancer or calcification at follow-up. Moreover, no cell aggregates or nodules were detected at the autopsy of a patient, who died as a result of pulmonary embolism; however, CD34 and CD105 positive cells were prominent within ABMMNC-injected viable infarct zones but not in the fibrous scar areas.

The major limitations of this study are the non-randomised study design and the sample size, which limits significant conclusions about efficacy. The control group was not assigned concurrently with treated patients and did not receive placebo injections because of Ethics Committee concerns. It is possible that this inadvertent selection bias influenced the study results. The study is not randomised but it is reassuring that both groups were matched in terms of demographics, baseline laboratory values, functional status classification, coronary anatomy and LV function. Furthermore, the treatment and control groups had similar follow-up of at least 18 months. Although such a comparison does not reach the power of a randomly allocated, blinded control group, our study provides certain differences from the published data in terms of patient selection, and long-term adverse event monitoring. Lack of positron emission tomography investigation for the measurement of perfusion, which is considered the gold standard for assessment of myocardial viability in patients with IC, should be mentioned as another limitation for this study. In this initial prospective, non-randomised study in patients with UCA and IC, symptomatic benefit, improvement in exercise capacity and myocardial perfusion was noted at 6 months and maintained at 18 months after transepicardial ABMMNC therapy in conjunction with CABG to graftable territories. More importantly, there was marked increase in perfusion scores among ungraftable territories where ABMMNC were implanted. Furthermore, there was no clinical evidence of significant side effects from the procedure itself. However, this study could not provide scientific evidence for the mechanism of action such as myocardial regeneration or angiogenesis because of the lack of *in vivo* labeling and tracking methods but still provides a rationale for a prospective, randomised trial in patients with viable ungraftable coronary artery territories in order to assess the best strategy. This is because the selection of optimal cell type and content should be based on clinical evidence supported with experimental data.

4. Conclusion

In summary, cellular therapeutic strategies as an adjunct to the restoration of adequate flow to viable myocardium may preclude LV distortion and ongoing LV remodelling; but may not prevent progressive HF and death. Further investigation in prospective randomised trials with long-term follow-up is warranted.

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