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CELL THERAPY

Red Blood Cell Contamination of the Final Cell Product Impairs the Efficacy of Autologous Bone Marrow Mononuclear Cell Therapy

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Objectives The aim of this study was to identify an association between the quality and functional activity of bone marrow-derived progenitor cells (BMCs) used for cardiovascular regenerative therapies and contractile recovery in patients with acute myocardial infarction included in the placebo-controlled REPAIR-AMI (Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial.

Background Isolation procedures of autologous BMCs might affect cell functionality and therapeutic efficacy.

Methods Quality of cell isolation was assessed by measuring the total number of isolated BMCs, CD34+ and CD133+ cells, their colony-forming unit (CFU) and invasion capacity, cell viability, and contamination of the final BMC preparation with thrombocytes and red blood cells (RBCs).

Results The number of RBCs contaminating the final cell product significantly correlated with reduced recovery of left ventricular ejection fraction 4 months after BMC therapy ($p = 0.007$). Higher numbers of RBCs in the BMC preparation were associated with reduced BMC viability ($r = -0.23$, $p = 0.001$), CFU capacity ($r = -0.16$, $p = 0.03$), and invasion capacity ($r = -0.27$, $p < 0.001$). To assess a causal role for RBC contamination, we coincubated isolated BMCs with RBCs for 24 h in vitro. The addition of RBCs dose-dependently abrogated migratory capacity ($p = 0.003$) and reduced CFU capacity ($p < 0.05$) of isolated BMCs. Neovascularization capacity was significantly impaired after infusion of BMCs contaminated with RBCs, compared with BMCs alone ($p < 0.05$). Mechanistically, the addition of RBCs was associated with a profound reduction in mitochondrial membrane potential of BMCs.

Conclusions Contaminating RBCs affects the functionality of isolated BMCs and determines the extent of left ventricular ejection fraction recovery after intracoronary BMC infusion in patients with acute myocardial infarction. These results suggest a bioactivity response relationship very much like a dose–response relationship in drug trials. (Reinfusion of Enriched Progenitor cells and Infarct Remodeling in Acute Myocardial Infarction [REPAIR-AMI]; NCT00279175) (J Am Coll Cardiol 2010;55:1385–94) © 2010 by the American College of Cardiology Foundation

Although reperfusion therapy has significantly improved prognosis of patients with acute myocardial infarction (AMI), the development of post-infarction heart failure remains a major challenge, particularly in patients with large AMIs (1,2). Numerous experimental and several clinical studies suggest that cell therapy might provide an attractive novel option to beneficially interfere with left ventricular (LV) remodeling processes, and thereby attenuate the development of post-infarction heart failure. In experimental studies, infusion of various types of cells—including circulating endothelial progenitor cells, bone marrow mononuclear progenitor cells (BMCs), CD34+ cells, mesenchymal stem cells, adipose-tissue derived cells, and cardiac stem cells—leads to improvement of neovascularization and cardiac function (3–6). So far, most of the clinical trials used autologous BMCs for cell therapy of AMI (7). In contrast to an allogeneic therapy with an "off the shelf" cell product, autologous

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Abbreviations and Acronyms

AMI = acute myocardial infarction
BMC = bone marrow mononuclear progenitor cell
CFU = colony-forming unit
FACS = fluorescent-activated cell sorting
LV = left ventricle/ventricular
LVEF = left ventricular ejection fraction
NO = nitric oxide
RBC = red blood cell (erythrocyte)
SDF = stromal cell-derived factor

cell therapy requires isolation of cells from each individual patient, and thus the applied cell composition and function might vary individually. Indeed, risk factors for atherosclerosis such as diabetes and age were shown to impair cell functionality (8–11). Previous experimental studies and clinical pilot trials showed that a reduction of functional activity of the infused cells defined as impaired migratory capacity was associated with reduced therapeutic effects (12,13). However, the impact of the composition of the cell product and the potential effect of contaminating cells are unclear.

Therefore, we determined whether total cell number, the number of selected subpopulations of progenitor cells as measured by fluorescent-activated cell sorting (FACS, Becton Dickinson, Franklin Lakes, New Jersey), in vitro assays to measure cell activity, or contamination of the cell preparation by platelets or erythrocytes affects the recovery of left ventricular ejection fraction (LVEF) in the multicenter, double-blind, randomized, placebo-controlled REPAIR-AMI (Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial including 204 patients with AMI, of whom 101 patients have been treated with BMCs. At 4-month follow-up, BMC-treated patients showed significantly improved LVEF compared with the placebo group, and the treatment was associated with a reduced number of composite clinical end points at 1 year (14,15). Here we report that, among the various parameters tested, the contamination of the isolated BMCs with red blood cells (RBCs) was a significant independent predictor of reduced recovery of LVEF in the BMC group. Experimental studies confirmed these findings by demonstrating that the addition of RBCs impairs BMC cell function in vitro and in vivo.

Methods

Study population and protocol. Patients with an acute ST-segment elevation myocardial infarction successfully reperfused with stent implantation with a residual significant LV regional wall motion abnormality (LVEF ≤45% by visual estimate at the time of reperfusion therapy) were included in the REPAIR-AMI trial. A total of 204 patients were randomized to receive either intracoronary infusion of placebo medium or BMCs. In 187 patients (92 in the placebo and 95 in the BMC group), paired analysis of LV angiograms at baseline immediately before BMC/placebo administration (4.3 ± 1 day after AMI reperfusion) and 4-month follow-up were available (for detailed baseline characteristics see Schächinger et al. [14]). Cell processing and quality controls were performed in a central core laboratory, and patients were randomized to receive intracoronary infusion of placebo medium or BMCs into the infarct-related artery during low-pressure balloon occlusion. The BMCs were infused 17 ± 11 h after bone marrow collection and were stored in X-vivo 10 medium (Lonza, Walkersville, Maryland) + 20% autologous serum. In the placebo group, bone marrow was aspirated, processed, and functionally tested as in the BMC group, but patients received only X-Vivo 10 medium supplemented with 20% autologous serum, never having contained any cells. Further details of cell preparation and administration have been described previously (16,17). The ethics review board of each individual participating center approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki.

The BMCs for the experimental in vitro and in vivo validation and confirmation experiments were obtained from patients with ischemic cardiomyopathy undergoing intracoronary infusion of BMCs within an ongoing registry, having the same inclusion and exclusion criteria as the previously published randomized TOPCARE-CHD (Transplantation of Progenitor Cells and Recovery of LV Function in Patients with Chronic Ischemic Heart Disease) trial (18,19). The Ethics Review Board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

LV angiography. Left ventricular angiograms were obtained in identical standard projections at the baseline procedure (immediately before intracoronary cell infusion) and at 4-month follow-up. Quantitative analysis of paired LV angiograms was performed by an experienced investigator (BA) in a central core laboratory, blinded to the treatment modality of the individual patients with the software CMS version 6.0 (Medis, Leiden, the Netherlands), as previously described (14). The LVEF was calculated with use of the area-length method.

Isolation of BMCs and viability assessment. Briefly, in the REPAIR-AMI trial, bone marrow aspirates were diluted with 0.9% sodium chloride (1:5) and filtrated (100 μm), and mononuclear cells were isolated by density gradient centrifugation with Lymphocyte Separation Medium (Lonza, 800 *g*, 20 min, without brake). Mononuclear cells were washed 3 times with 50 ml phosphate-buffered saline (800 *g*) and counted; viability was assessed by a dye exclusion stain with trypan blue. Cells were resuspended in X-vivo 10 medium (Lonza), supplemented with 20% autologous serum, and released for immediate use. Trial centers outside of the greater area of Frankfurt received the cell preparations the next day.

Isolation of erythrocytes (RBCs). With a leukocyte depletion filter for whole blood donations (Leucoflex LCR5, Macopharma, Langen, Germany), erythrocytes were eluted with 10 ml X-vivo 10 medium (Cambrex) from 5-ml bone

marrow aspirate obtained from patients with ischemic heart failure. Enumeration of RBCs and the depleted total nucleated cells was performed with an automated differential blood count (Sysmex XT 1800i [Sysmex, Mundelein, Illinois], linearity of analysis: total nucleated cells: 0 to 100.0 $\times$ $10^3/\mu l \pm 300$; RBC: 0 to 8.00 $\times$ $10^6/\mu l \pm 30.000$; HCT: 0.0% to 60 $\pm$ 1%).

For all coincubation experiments, the defined amount of RBCs was added to the bone marrow preparation in X-vivo 10 medium containing 20% autologous serum and was stored for 24 h at room temperature to mimic the settings used in the REPAIR-AMI trial (16). The RBCs and BMCs used were from the same patient.

Flow cytometry analysis of BMCs. After Ficoll density gradient centrifugation, BMCs were analyzed by FACS. For the enumeration of bone marrow cell populations, we used directly conjugated antibodies against human CD45 (FITC-labeled, BD Pharmingen, San Diego, California), CD34 (PE-labeled, BD Pharmingen), CD133 (APC-labeled, Miltenyi, Bergisch Gladbach, Germany), and KDR (PE-labeled, R&D Systems, Minneapolis, Minnesota). Gating was performed according to the International Society of Hematotherapy and Graft Engineering guideline (20).

For analysis of the mitochondrial membrane potential, BMCs were stained with the lipophilic cation 5,5′,6,6′tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Invitrogen, Carlsbad, California) according to the manufacturer's instruction. Briefly, 24 h after storage, JC-1 was added to the cells and incubated for 20 min at 37°C. After washing with phosphate-buffered saline, FACS analysis was immediately performed (FACS Canto II, Becton Dickinson).

Assessment of hematopoietic colonies. The BMCs (1 $\times$ 10^5/dish) were seeded in methylcellulose plates (Methocult GF H4534, StemCell, Vancouver, British Columbia, Canada). Plates were studied under phase-contrast microscopy, and colony-forming units (CFUs) (colonies >50 cells) were counted after 14 days of incubation at 37°C by an independent investigator. CFUs were examined in duplicates.

Assessment of invasion capacity of BMCs. A total of 1 $\times$ 10^6 BMCs were resuspended in 250 μl X-vivo 10 medium and placed in the upper chamber of a modified Boyden chamber filled with Matrigel (BioCoat invasion assay, 8 μm pore size, Becton Dickinson). Then, the chamber was placed in a 24-well culture dish containing 500 μl endothelial basal medium. For stimulation of invasion, 100 ng/ml stromal cell-derived factor (SDF)-1 was added in the lower chamber. After incubation for 24 h at 37°C, transmigrated cells were counted by independent investigators. Invasion assays were run in duplicates.

Cell-matrix adhesion. Cell-matrix adhesion was performed as previously described (21). Ninety-six-well plates were coated overnight at 4°C with 2.5 μg/ml soluble recombinant human ICAM-1 (R&D Systems) in coating buffer (150 mmol/l sodium chloride, 20 mmol/l Tris hydrochloride, 1 mmol/l magnesium chloride, pH 9.0) and then blocked for 1 h at room temperature with 3% (w/v) heat-inactivated (2 h, 56°C) bovine serum albumin (Sigma, St. Louis, Missouri). Human BMCs were stained with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-actoxymethyl-ester (BCECF, Invitrogen; 2.5 μM) and stored overnight with different concentrations of RBCs as indicated in X-vivo medium containing 20% serum. Then, cells were seeded at 1.1 $\times$ 10^5 cells/well in 100 μl in the coated wells for 10 min at 37°C. After washing with warm RPMI 1640, adherent cells were quantified in triplicates with a fluorescence plate reader (Synergy HT, Biotek, Bad Friedrichshall, Germany).

Hindlimb ischemia model. The neovascularization capacity of the BMC was investigated in a murine model of hindlimb ischemia by use of 8-week-old athymic Balb/C nude mice (Jackson Laboratory, Bar Harbor, Maine) weighing 18 to 22 g. The proximal portion of the femoral artery including the superficial and the deep branch as well as the proximal portion of the saphenous artery were obliterated and removed with an electrical coagulator. The overlying skin was closed with 7-0 silk suture. After 24 h, 1 $\times$ 10^6 BMC/mouse containing the indicated number of RBCs were injected intravenously. Injection of 5 $\times$ 10^6 RBCs obtained after leukocyte depletion of the bone marrow served as control.

Limb perfusion measurements. After 21 days, we measured ischemic (right)/normal (left) limb perfusion ratio with a laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System, moorLDI-Mark 2, Moor Instruments, Wilmington, Delaware). The perfusions of the ischemic and nonischemic limb were calculated from mean value multiplied by the number of pixels of the region below the inguinal ligament. To minimize variables including ambient light and temperature, calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

Statistical analysis. If not stated otherwise, data are shown as mean $\pm$ SEM. Statistical comparisons were made by the nonparametric Wilcoxon 2-sample or Friedman's tests (paired analyses) and the nonparametric Mann-Whitney *U* or Kruskal-Wallis tests for between-group analyses. For univariate analysis, nonparametric Spearman correlation was applied. The multivariable analysis was done by a parametric linear regression model. Because this approach assumes the normality of the dependent variable, we applied the Box-Cox transformation for the dependent variable in the multivariate analysis (22). The stepwise linear regression model was performed with the transformed approximately normal distributed dependent variable. Statistical significance was assumed, if $p < 0.05$. All reported p values are 2-sided. All statistical analysis was performed with SPSS (version 17.0, SPSS, Inc., Chicago, Illinois).

Results

Impact of cell characteristics on functional recovery of patients treated with BMCs. To analyze the effect of various cell quality and functional parameters on LV con-

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Table 1 Baseline Cell Characteristics of the Final Cell Preparation and Impact on Contractile Recovery Within the REPAIR-AMI Trial

	Median (25th/75th Percentile)	
	Placebo Patients (n = 103)	BMC Patients (n = 101)
Total mononuclear cells isolated ($\times 10^6$)	210 (110/336)	198 (130/284)
Viability (%)	99 (98/99)	99 (98/99)
Invasion basal	82 (49/125)	81 (46/139)
SDF-1–induced invasion	161 (90/210)	163 (98/209)
CFU capacity	29 (20/43)	27 (19/40)
$CD34^+$ $CD45^+$ (%)	1.43 (1.01/2.04)	1.35 (0.96/1.91)
$CD133^+$ $CD45^+$ (%)	1.10 (0.80/1.50)	1.00 (0.69/1.40)
$CD45^+$ KDR^+ (%)	0.06 (0.30/0.80)	0.06 (0.03/0.11)
RBC ($\times 10^9$)	0.30 (0.10/0.40)	0.20 (0.10/0.30)
Hematocrit (%)	0.3 (0.2/0.5)	0.2 (0.2/0.4)
Platelets ($\times 10^6$)	150 (100/330)	140 (100/200)
Neutrophils (%)	38 (32/47)	36 (27/49)
Lymphocytic cells (%)	43 (36/50)	42 (34/53)
	Univariate Analysis for ΔLVEF	
	Placebo (n = 92)	BMC (n = 95)
Total mononuclear cells isolated ($\times 10^6$)	r = −0.05; p = 0.7	r = −0.08; p = 0.5
Viability (%)	r = −0.17; p = 0.1	r = 0.15; p = 0.2
Invasion basal	r = 0.07; p = 0.5	r = −0.05; p = 0.6
SDF-1–induced invasion	r = 0.06; p = 0.6	r = 0.02; p = 0.8
CFU capacity	r = −0.01; p = 1.0	r = −0.09; p = 0.4
$CD34^+$ $CD45^+$ (%)	r = −0.09; p = 0.4	r = −0.10; p = 0.3
$CD133^+$ $CD45^+$ (%)	r = −0.09, p = 0.4	r = −0.09, p = 0.4
$CD45^+$ KDR^+ (%)	r = −0.15, p = 0.2	r = −0.04, p = 0.7
RBC ($\times 10^9$)	r = 0.13, p = 0.2	r = −0.25, p = 0.02
Hematocrit (%)	r = 0.10, p = 0.3	r = −0.22, p = 0.04
Platelets ($\times 10^6$)	r = 0.02, p = 0.8	r = −0.23, p = 0.03
Neutrophils (%)	r = 0.12, p = 0.3	r = 0.06, p = 0.6
Lymphocytic cells (%)	r = 0.03, p = 0.7	r = −0.10, p = 0.4

Cell characteristics of the placebo group are shown for comparative reasons only; these patients did not receive the cell preparation but a cell-free placebo preparation.

BMC = bone marrow mononuclear progenitor cell; CFU = colony-forming unit; LVEF = left ventricular ejection fraction; RBC = red blood cell; REPAIR-AMI = Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction; SDF = stromal cell-derived factor.

tractile recovery measured as absolute change in LVEF, we performed a univariate analysis (Table 1). None of the cell characteristics, which include the total number of cells and the subpopulations of hematopoietic progenitor cells, correlated with the contractile recovery in the BMC-treated patients. Because in the placebo group BMCs were isolated accordingly but were not infused in the patients, analysis of the placebo group offered the unique opportunity to exclude the possibility that the contamination of the final cell product with RBCs is affected by patient-specific factors influencing LVEF recovery.

Moreover, we quantified the cell intrinsic function of progenitor cells by measuring the hematopoietic CFU capacity. Likewise, we determined both the basal as well as SDF-1–induced migratory capacity of BMCs, which was shown to correlate with cell recruitment and homing after intravascular administration of BMCs (13). However, none of these functional properties of the administered BMCs was associated with the extent of LV contractile recovery as measured by changes in LVEF (Table 1). Only the number of contaminating RBCs and the hematocrit of the final cell product were significantly and inversely correlated with the recovery of LVEF, whereas no significant association was detected for the number of contaminating neutrophils and lymphocytic cells. In addition, the number of contaminating platelets also showed a significant inverse correlation with LVEF recovery by univariate analysis (Table 1). To identify whether RBC contamination is an independent predictor of LVEF recovery at 4 months, we performed a multivariate analysis including classical determinants of LV contractile recovery after AMI, like end-systolic volume, peak creatinine kinase levels, time of symptom onset to treatment, age, and the presence of diabetes as well as the previously identified predictors of LVEF recovery, namely a reduced LVEF at baseline and the time to treatment (14). As shown in Table 2, the number of contaminating RBCs together with a reduced baseline LVEF and maximum creatinine kinase levels remained as the only independent predictors for the recovery of LVEF. In contrast, the contaminating platelets were not an independent predictor of LVEF recovery (Online Tables 1A and 1B). Thus, univariate and multivariate analysis revealed that the contamination of the final cell product with RBCs is an independent predictor of reduced recovery of LVEF in BMC-treated patients with AMI. Of note, the negative impact of RBCs on the recovery of LVEF was only detected in the BMC group but not in the placebo control group, which did not receive the cell product (Fig. 1A). Thus, the impact of RBC contamination is confined to patients receiving BMC administration but does not reflect a patient-intrinsic association. Moreover, the number of RBCs in the bone marrow aspirate (before density gradient centrifugation) did not correlate with the recovery of LVEF in the BMC group (p = 0.73), indicating that the composition of the bone marrow aspirate before processing did not affect functional recovery. These data suggest that the number of contaminating RBCs in the

Table 2 Multivariate Analysis for LVEF Improvement After 4 Months in the BMC Group

	p Value	Standardized Coefficient
Age	0.57	0.06
Diabetes	0.22	0.13
Maximum creatinine kinase levels	0.02	0.27
Time symptom to stent	0.22	−0.13
Baseline end-systolic volume	0.39	0.12
Baseline LVEF	0.001	0.48
Time to BMC administration	0.76	−0.03
RBC ($\times 10^9$) contamination of the final cell product	0.01	0.27

Significance (analysis of variance) = 0.003.

Abbreviations as in Table 1.

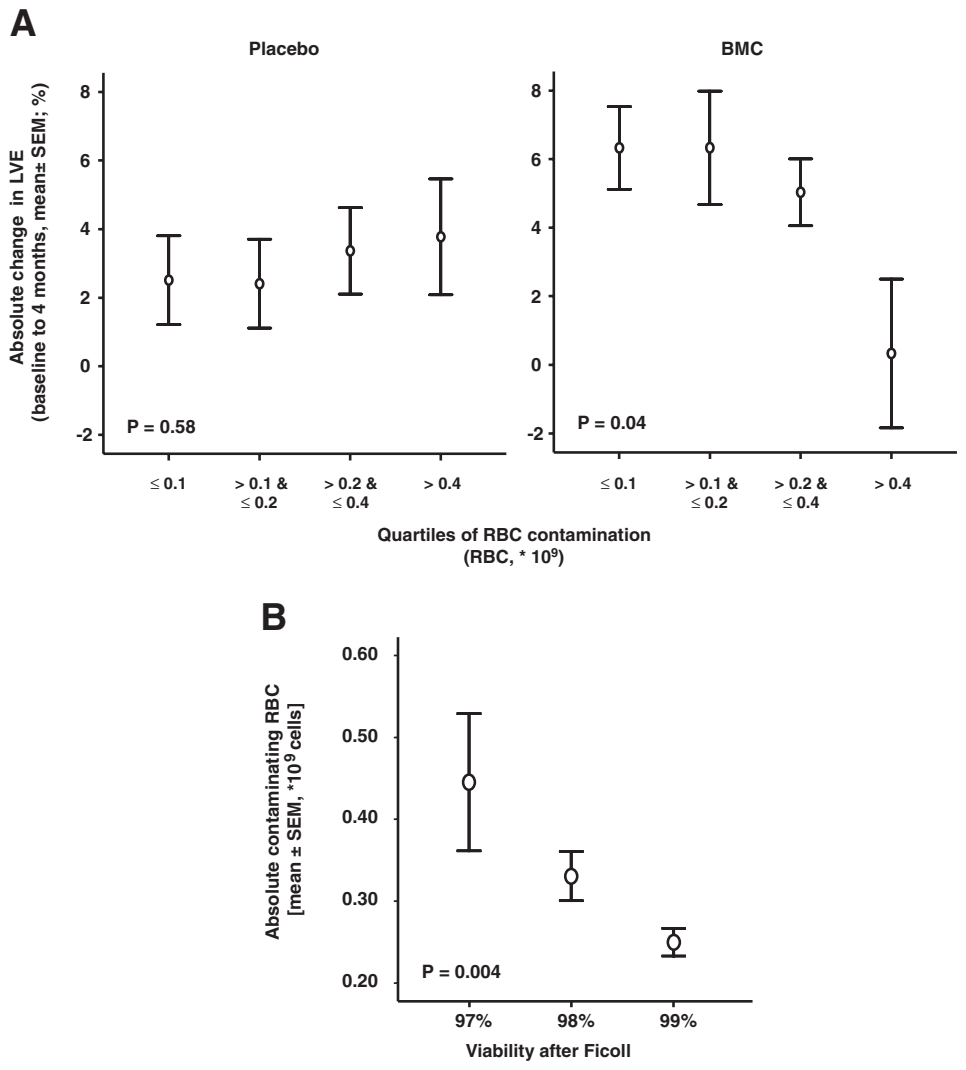

Figure 1 Cell Quality Results of the REPAIR-AMI Trial

(A) Association between left ventricular ejection fraction (LVEF) improvement at 4 months and red blood cell (RBC) contamination for the placebo and the bone marrow-derived progenitor cell (BMC) group within the REPAIR-AMI (Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial. The x-axis shows quartiles of RBC contamination. Statistical comparison between groups was performed with the Kruskal-Wallis test. **(B)** The RBC contamination influences BMC viability within the REPAIR-AMI trial. Statistical comparison between groups was performed with the Kruskal-Wallis test.

purified BMC population might have influenced the functionality of the cells used for cell therapy.

Therefore, in the next step, we analyzed the effect of RBC contamination on functional parameters of isolated BMCs in the REPAIR-AMI trial. Importantly, increasing numbers of contaminating RBCs were not only associated with a reduced viability of the isolated cells, as measured by trypan blue exclusion assay, but also correlated with a significant impairment of invasion capacity and CFU capacity of the isolated BMCs (Table 3, Fig. 1B), indicating that RBCs directly affect cell viability and cell functionality.

RBC contamination impairs BMC function in vitro and in vivo. To prospectively test the hypothesis that contamination with RBCs influences the functionality of BMCs used for cell therapy, we coincubated isolated BMCs with increasing numbers of RBCs for 24 h in vitro. The highest number used in the experiments reflects the median concentration of contaminating RBCs observed in the clinical trial. The RBCs dose-dependently reduced the viability of BMCs (Fig. 2A), consistent with the statistical evaluation of the clinical trial. Furthermore, the invasion capacity of BMCs at baseline and after stimulation with SDF-1 was significantly reduced by RBC addition. In fact, the highest dose of RBC contamination completely abolished the migratory capacity of isolated BMCs toward SDF-1 (Fig. 2B). Moreover, direct incubation of RBCs with freshly isolated BMCs in the migration assay significantly inhibited SDF-1-induced invasion (50 ± 19%, $p < 0.05$). Incubation of BMCs with

Table 3 Influence of RBC Contamination on Cell Characteristics Within the REPAIR-AMI Trial

	Univariate Analysis (RBC/MNC)
Viability (%)	r = −0.23; p = 0.001
Invasion basal	r = −0.21; p = 0.003
SDF-1–induced invasion	r = −0.27; p < 0.001
CFU capacity	r = −0.16; p = 0.03
$CD34^+$ $CD45^+$ (%)	r = 0.13; p = 0.06
$CD133^+$ $CD45^+$ (%)	r = −0.04, p = 0.5
$CD45^+$ KDR^+ (%)	r = −0.06, p = 0.4

MNC = mononuclear cell; other abbreviations as in Table 1.

RBCs for 24 h further reduced the formation of granulocyte/macrophage-CFUs, a prototypical marker for functionally competent hematopoietic progenitor cells (Fig. 2C), but did not affect the adhesion of the BMCs (Fig. 2D).

The integrity and function of mitochondria is essential for stem cell competence (23), the migration of progenitor cells (24), and cell survival (25). Therefore, we tested whether the addition of RBCs might influence mitochondrial function of BMCs by measuring the mitochondrial membrane potential with JC-1 staining. As demonstrated in Figure 3, contamination with RBCs dose-dependently reduced the quantitative mitochondrial membrane potential of BMCs, indicating that RBCs impair the mitochondrial function of BMCs.

To test whether RBCs affect the functional benefit of cell therapy in vivo, we injected BMCs that had been co-incubated with or without RBCs for 24 h into nude mice after induction of hindlimb ischemia. Indeed, laser Doppler-derived blood flow recovery was significantly higher in mice treated with BMCs compared with the group that received BMCs after co-incubation with RBCs (Fig. 4). Taken together, these data confirm that RBC contamination dose-dependently impairs the in vitro and in vivo functions of isolated bone marrow-derived mononuclear cells.

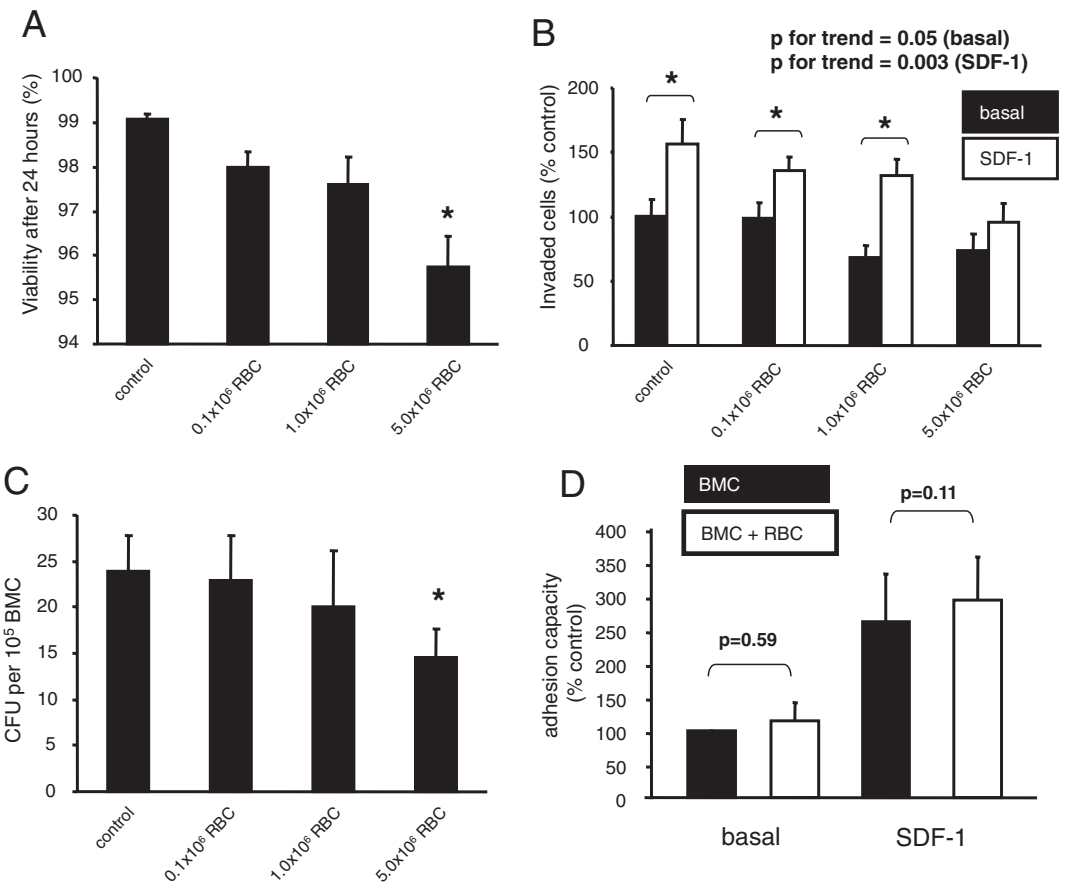

Figure 2 Functional Activity of BMCs With RBC Contamination

(A) After isolation, RBCs were added to 1×10^6 BMCs in the indicated concentrations, and cells were stored according to the REPAIR-AMI protocol. After 24 h, viability was assessed with a trypan blue dye exclusion assay. Data are mean ± SEM of n = 4; ∗p < 0.05 versus control. **(B)** Invasion capacity of BMCs after storage with the indicated concentrations of RBCs for 24 h under basal conditions as well as toward a stromal cell-derived factor (SDF)-1 gradient was assessed with modified Boyden chambers. Data are mean ± SEM of n ≥ 4; ∗p < 0.05 versus basal migration. **(C)** Number of colony-forming units of 1×10^5 BMCs after 24 h storage with the indicated numbers of RBCs. Data are mean ± SEM of n ≥ 4; ∗p for trend <0.05 versus BMCs alone. **(D)** Adhesion capacity of BMCs on ICAM-1 (R&D Systems, Minneapolis, Minnesota) after storage with 5×10^6 RBC/1×10^6 BMCs for 24 h. Data are mean ± SD of n = 3 experiments measured in triplicates. Abbreviations as in Figure 1.

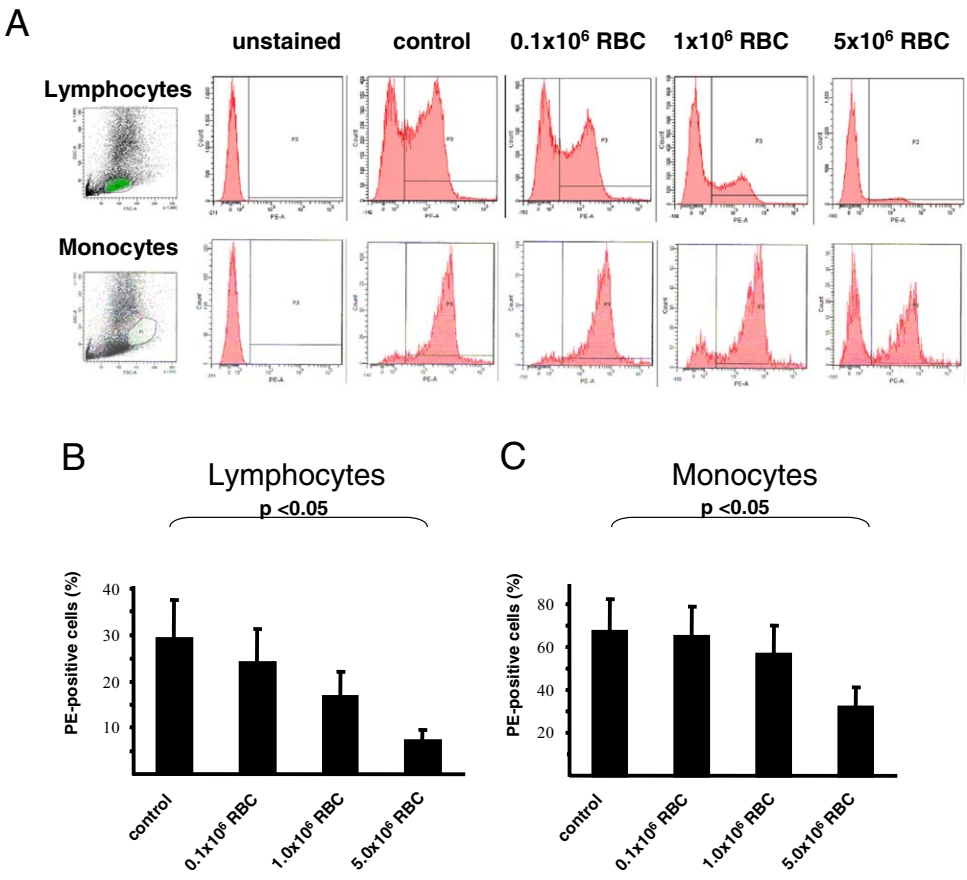

Figure 3 **Membrane Potential of BMCs**

(A) Representative pictures showing histograms of JC-1–stained (Invitrogen, Carlsbad, California) BMCs after incubation of BMCs with the indicated concentration of RBCs for 24 h. The fluorescence PE-channel depicts JC-1 dimers at higher membrane potential in the lymphocyte or monocyte gate. **(B and C)** Quantification of PE-positive BMCs in the lymphocyte or monocyte gate after incubation with RBCs as indicated. Data are mean ± SEM of n = 4 experiments. Similar results were obtained when the PE/FITC ratio was analyzed ($p < 0.05$). Abbreviations as in Figure 1.

RBCs impair BMC function via a secreted factor. Finally, we performed further experiments to gain mechanistic insights into RBC action on BMC function. Because hemolysis of RBCs is well-known to result in high levels of free heme, causing cellular injury (26), we tested the hypothesis of whether the release of hemoglobin might cause BMC dysfunction. However, supernatants of RBCs after hypotonic lysis did not affect BMC invasion (Fig. 5A) indicating that the release of hemoglobin by RBCs is unlikely to induce migratory defects of BMCs. Next, we tested whether a secreted RBC-derived factor is sufficient to impair BMCs. Therefore, RBCs were incubated in X-vivo medium for 24 h, and the supernatant was subsequently incubated with BMCs. As shown in Figure 5B, supernatants of intact RBCs indeed inhibited SDF-1–induced invasion of BMCs. Furthermore, we addressed whether the effect might be caused by shed microparticles and, therefore, centrifuged the RBC supernatant to pellet microparticles. However, RBC-derived microparticles did not affect BMC migration, whereas the microparticles-free supernatant still prevented BMC migration (Fig. 5B), indicating that a secreted factor but not microparticles mediate the migration impairment of BMCs induced by RBCs.

Discussion

Post-hoc analysis of the REPAIR-AMI trial indicates that contamination of the autologous cell product with RBCs impairs functional improvement of LVEF in patients after cell therapy. The number of contaminating RBCs was not only associated with reduced recovery of LVEF by univariate and multivariate analysis but also correlated with the viability, invasion, and CFU capacity of the applied BMCs. This retrospective statistical analysis, indicating that RBC contamination negatively influences the improvement of LVEF after cell therapy, was further prospectively validated and confirmed by experimental studies showing that ex vivo incubation of BMCs with RBCs dose-dependently reduced the viability and functional activity of the BMCs in vitro and impaired the therapeutic benefit of the applied cells in

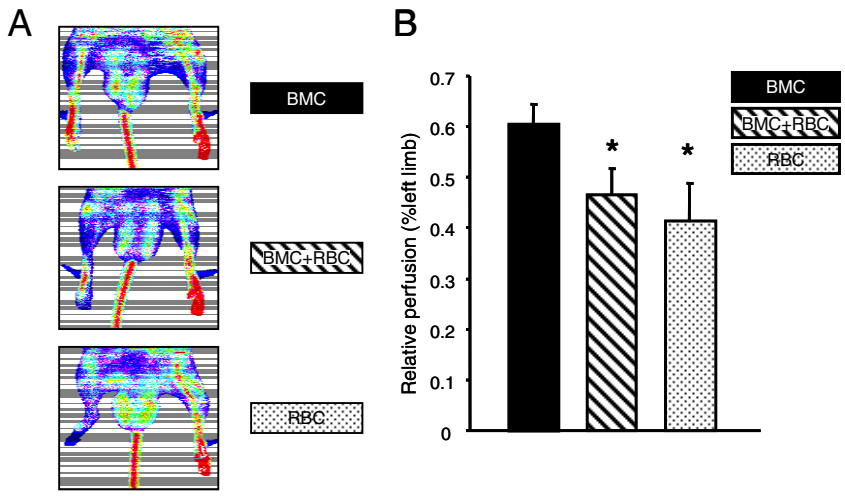

Figure 4 **Impairment of In Vivo Functions of BMCs**

Representative pictures **(A)** and quantitative analysis **(B)** of relative perfusion in a hindlimb ischemia model 21 days after injection of RBCs or BMCs stored 24 h according to the indicated conditions. Data are mean ± SD of n ≥ 8 mice. *p < 0.05 versus BMCs alone. Abbreviations as in Figure 1.

an animal model in vivo. Thus, RBCs indeed dose-dependently impair BMC functions involved in functional recovery after ischemia.

It is well-established that risk factors for coronary artery disease impair the functionality of patient-derived BMCs with respect to their capacity to induce neovascularization (9). Moreover, previous clinical pilot trials suggested that the measurement of cell functionality by detecting the invasive and migrating capacity defines the benefit of cell therapy (12,18). Whereas these parameters did not seem to be significant univariate determinants in the present study in patients with AMI, the invasive capacity in response to the

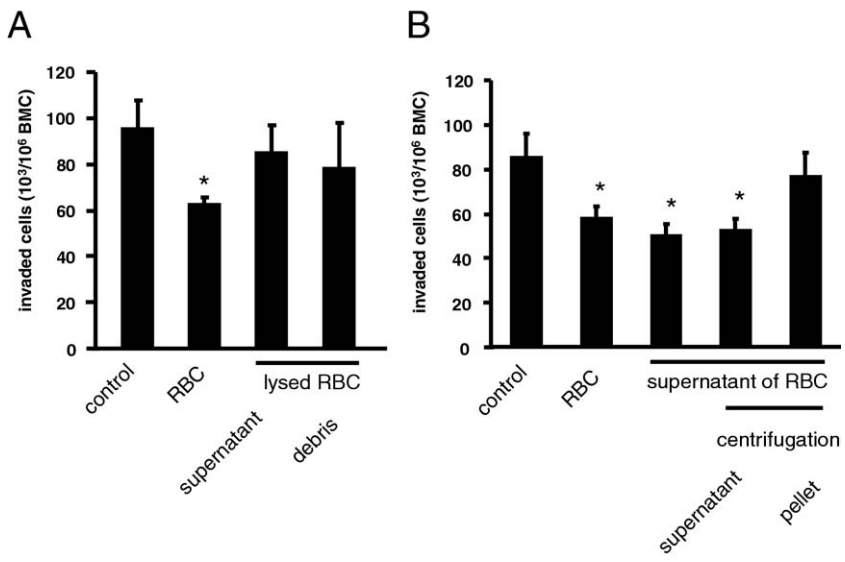

Figure 5 **Mechanistic Insights Into the BMC Impairment Mediated by RBCs**

(A) RBCs (5×10^6 RBC/1×10^6 BMC) were pelleted and lysed with 50 μl of water for 25 s. After the addition of 350 ml X-vivo-10 medium, the sample was centrifuged for 10 min at 800 *g*. The resulting supernatants or the pellet (resolved in 400 μl medium) were added to the BMCs in the invasion chamber. The SDF-1–induced invasion was determined by using a modified Boyden chamber assay. Data are mean ± SEM of n ≥ 2 experiments measured in duplicates. *p < 0.05 versus control. **(B)** RBCs were incubated in X-vivo-10 medium for 24 h at room temperature, and the conditioned medium ("supernatant") was either directly added to BMCs or centrifuged at 20,500 *g* for 45 min. After centrifugation, the supernatant or the resolved pellet was added to BMCs, and BMC invasion was determined by using modified Boyden chamber assays with SDF-1 as stimulus. Data are mean ± SEM of n = 2 experiments measured in duplicates. *p < 0.05 versus control. Abbreviations as in Figures 1 and 2.

physiologically important cytokine SDF-1 (27) was significantly associated with improved LVEF recovery in multivariate statistical analysis. Importantly, normalization of SDF-1–induced invasion by the contaminating RBCs provided a highly significant predictor of global LV contractile recovery (Online Table 2, Online Fig. 1). Thus, combining intrinsic patient-determined functional parameters of BMCs with extrinsic effects imposed by the cell isolation procedure indeed discloses a cause-and-effect relationship between the cell product and LVEF recovery after cell therapy in patients with AMI.

Interestingly, in the REPAIR-AMI trial, we could not detect an association between the applied cell numbers and contractile recovery. This might be because 85% of the patients in the BMC group received BMC numbers exceeding 10^8 cells, which was identified as the minimum cell number necessary for beneficial effects in a recent meta-analysis by Martin-Rendon et al. (28). Thus, to detect a potential direct cell number-related dose–response relationship, it seems to be necessary to prospectively administer predefined numbers of BMCs varying at least by a factor of 100-fold difference.

Mechanistically, our results suggest that addition of RBCs affects the mitochondrial membrane potential of BMCs. Mitochondria act as important signaling organelles in endothelial cells (29) and are required for the migratory capacity of endothelial progenitor cells (22). Moreover, it is well-established that mitochondrial function and metabolism are important determinants of stem cell self-renewal and differentiation (30,31). The mitochondrial marker dye rhodamine 123, which measures mitochondrial activity and membrane polarity, shows an age-related relationship between the mitochondrial dye efflux and the ability of human hematopoietic stem cells to repopulate the hematopoietic system of irradiated animals (32,33). Importantly, administration of progenitor cells with a reduced mitochondrial membrane potential induced by hypoxia was associated with a significantly impaired neovascularization capacity in the hindlimb ischemia model (34). By contrast, increasing the mitochondrial membrane potential of progenitor cells via an AMP-activated protein kinase-dependent mechanism was associated with enhanced migration and functional reendothelialization (24). Thus, the functional integrity of the mitochondria seems to be essential not only for stem and progenitor cell function and survival but also for regulating the vascular repair capacity of administered cells.

The question remains: by which signaling mechanism might RBCs affect viability and mitochondrial function of isolated BMCs? Hemolysis of RBCs results in high levels of free heme causing cellular injury (26). Moreover, free hemoglobin was shown not only to directly induce apoptosis in cultured endothelial cells (35) but also to scavenge nitric oxide (NO). Nitric oxide plays a crucial role for stem cell maintenance, differentiation, and neovascularization capacity (36–39) and increases mitochondrial function in endothelial cells (29), thus raising the hypothesis that reduced bioactive NO levels in the BMC suspension might have contributed to impaired benefit of BMCs associated with mitochondrial dysfunction. However, supernatants of lysed RBCs containing free hemoglobin did not affect BMC functions, and the addition of NO donors did not rescue the impaired migratory capacity of BMCs induced by RBCs (data not shown). In contrast, supernatants of intact RBCs were shown to significantly reduce BMC invasion, indicating that an actively secreted factor mediates BMC impairment. Further subfractionation of these RBC supernatants excluded a role of microparticles. On the basis of these results, one might speculate that secreted lipids or proteins might mediate the effects of RBCs on BMCs.

In summary, the results of the present study demonstrate for the first time an association between cell quality and contractile recovery within a large randomized, multicenter clinical trial, suggesting a bioactivity response relationship very much like a dose–response relationship in drug trials. Therefore, future studies are warranted to establish rapid and reliable tests for quality control of the cell product when using autologous BMCs. On the basis of the presented data, testing for contaminating RBCs might be used as an ad hoc quality control parameter for future clinical trials.

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Key Words: myocardial infarction ■ progenitor cells ■ REPAIR-AMI trial.

APPENDIX

For supplementary tables and a supplementary figure, please see the online version of this article.