Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction

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Aims
The recently published REPAIR-AMI and ASTAMI trial showed differences in contractile recovery of left ventricular function after infusion of bone marrow-derived cells in acute myocardial infarction. Since the trials used different protocols for cell isolation and storage (REPAIR-AMI: Ficoll, storage in X-vivo 10 medium plus serum; ASTAMI: Lymphoprep, storage in NaCl plus plasma), we compared the functional activity of BMC isolated by the two different protocols.

Methods and results
The recovery of total cell number, colony-forming units (CFU), and the number of mesenchymal stem cells were significantly reduced to 77±4%, 83±16%, and 65±15%, respectively, when using the ASTAMI protocol compared with the REPAIR protocol. The capacity of the isolated BMC to migrate in response to stromal cell-derived factor 1 (SDF-1) was profoundly reduced when using the ASTAMI cell isolation procedure (42±8% and 78±3% reduction in healthy and CAD-patient cells, respectively). Finally, infusion of BMC into a hindlimb ischaemia model demonstrated a significantly blunted blood-flow-recovery by BMC isolated with the ASTAMI protocol (54±6% of the effect obtained by REPAIR cells). Comparison of the individual steps identified the use of NaCl and plasma for cell storage as major factors for functional impairment of the BMC.

Conclusion
Cell isolation protocols have a major impact on the functional activity of bone marrow-derived progenitor cells. The assessment of cell number and viability may not entirely reflect the functional capacity of cells in vivo. Additional functional testing appears to be mandatory to assure proper cell function before embarking on clinical cell therapy trials.

KEYWORDS
Cell therapy; BMC; Isolation protocols; Invasion capacity; REPAIR-AMI

Introduction
Reperfusion therapy has significantly improved survival and prognosis of patients with acute myocardial infarction. However, the development of post-infarction heart failure, particularly in patients with a large myocardial infarction, remains a major challenge.¹,² Cell therapy may provide a novel therapeutic option to modify left ventricular remodeling processes and prevent post-infarction heart failure. Experimental studies have extensively documented that the infusion of different subsets of bone marrow-derived progenitor cells, circulating endothelial progenitor cells, or tissue-residing stem cells improved neovascularization and cardiac function.³ Clinical studies at present predominantly used bone marrow mononuclear cells (BMC) isolated from bone marrow aspirates by density gradient centrifugation.⁴⁻¹⁰ Intracoronary infusion of these BMC significantly increased global or regional ejection fraction and/or reduced infarct size and endystolic volumes in patients with acute myocardial infarction as demonstrated in initial pilot trials⁴,⁵ and in randomized studies.⁸,¹⁰,¹¹ The recently published randomized, double-blind, placebo-controlled multicentre REPAIR-AMI trial with 204 patients confirmed the results achieved in the pilot studies and documented a significant improvement of ejection fraction in BMC-treated patients.⁸ However, another recently published randomized study, the ASTAMI trial, which compared the effect of BMC therapy with a non-treated control group in 100 patients, did not reveal any effect of BMC treatment on functional contractile recovery in patients after acute myocardial infarction.⁹ Although the overall study designs and the patient characteristics appear to be similar to the REPAIR-AMI trial, slightly different protocols were used to isolate the BMC.⁸,⁹,¹²,¹³ Therefore, we compared the two...
different cell isolation protocols with respect to the recovery of cells and the functional activity of the isolated BMC in vitro and in vivo.

Our study demonstrates that the recovery of BMC was significantly reduced, when the cells were isolated according to the ASTAMI compared with the REPAIR-AMI protocol. Moreover, BMC isolated by the ASTAMI protocol showed a significantly reduced invasion capacity in response to the chemotactic cytokine stromal cell-derived factor 1 (SDF-1) and an abolished capacity to augment neovascularization in an experimental hindlimb ischaemia model. The overnight storage of the cells in 0.9% NaCl with plasma in the ASTAMI protocol compared with the use of X-vivo 10 medium with serum in the REPAIR-AMI protocol appears to be the major factor leading to an impairment of cell function despite unaffected viability.

**Methods**

**Isolation of BMC**

Bone marrow aspirates were obtained from healthy volunteers or patients with angiographically known CAD. Patients with a history of myocardial ischaemia documented by the classic symptoms of chest pain, ECG alterations, or elevation of creatine kinase or troponin T within the previous 3 months were excluded. Further exclusion criteria were the presence of active or chronic infection, surgical procedures or trauma within the last 3 months, or evidence for malignant diseases. 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. Aspirates were divided and the same volume was used for isolation of BMC according to the published ASTAMI or REPAIR-AMI study protocols.4,8,9,12

Briefly, in the Ficoll protocol (used in the REPAIR-AMI trial), bone marrow aspirates were diluted with 0.9% NaCl (1:5), were filtrated (100 μm), and mononuclear cells were isolated by density gradient centrifugation using Ficoll (Cambrex; 800 g, 20 min, without break). Mononuclear cells were washed three times with 45 mL PBS (800 g), were counted, and used for the experiments or incubated overnight at room temperature in X-vivo 10 medium (Cambrex) containing 20% autologous serum (Figure 1). In the Lymphoprep protocol (used in the ASTAMI trial), bone marrow was diluted with 0.9% NaCl (1:5), filtrated, and isolated by density gradient centrifugation using Lymphoprep<sup>TM</sup> (Axis Shield, 800 g, 20 min, without break). Mononuclear cells were washed three times with 45 mL 0.9% NaCl containing 1% autologous heparin-plasma (250 g), counted, and used for the experiments or incubated at 4°C in 0.9% NaCl containing 20% autologous heparin-plasma and 50 IU heparin. An illustration of protocols is provided in Figure 1.

**Flow cytometry analysis of BMC**

Bone marrow aspirates and BMC before and after overnight storage were analysed by FACS. For the identification of populations, we used directly conjugated antibodies against human CD45 (FITC-labelled, BD Pharmingen), CD34 (PE-labelled; BD Pharmingen), CD133 (APC-labelled; Miltenyi), CXCR4 (APC-labelled, BD Pharmingen), and KDR (PE-labelled, R&D Systems).

**Colony-forming unit assay**

BMC (1 × 10<sup>6</sup> per dish) were seeded in methylcellulose plates (Methocult GF H4534; StemCell). Plates were studied under phase-contrast microscopy, and colony-forming units (CFU; colonies >50 cells) were counted after 14 days of incubation at 37°C by independent investigators. CFUs were examined in duplicates.

**Assessment of invasion capacity of BMC**

A total of 1 × 10<sup>6</sup> BMC 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 supplemented with 20% foetal calf serum and singlequots. For some experiments, 100 ng/mL SDF-1 was added in the lower chamber. After 24 h of incubation at 37°C, transmigrated cells were counted by independent investigators. Invasion assays were run in duplicates.

**Assessment of mesenchymal stem cell colonies**

A total of 1 × 10<sup>6</sup> BMC were resuspended in 3.5 mL Mesencult (StemCell), plated on a T25 culture flask, and cultured at 37°C. The medium was changed every second day. After 2 weeks, adherent cells were fixed with a mixture of methanol/acetic acid (4:1) for 30 min, washed, and stained with 1% crystal violet. Colonies were counted macroscopically. All assays were run in triplicates.

**Hindlimb ischaemia model**

The neovascularization capacity of BMC was investigated in a murine model of hindlimb ischaemia by use of 8- to 10-week-old athymic NMRI nude mice (Jackson Laboratory) weighing 18-22 g. The proximal portion of the femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were ligated with 7-0 silk suture. All arterial branches between the ligation were obliterated with an electrical coagulator. The overlying skin was closed with three surgical staples. After 24 h, 1 × 10<sup>6</sup> BMC/mouse were injected intravenously.

**Limb perfusion measurements**

After 7 and 14 days, we measured ischaemic (right)/normal (left) limb perfusion ratio with a laser Doppler perfusion imager system (Laser Doppler Perfusion Imager System, MoorLDI-Mark 2, Moor Instruments). The average perfusions of the ischaemic and non-ischaemic limb were calculated on the basis of coloured histogram pixels. To minimize variables including ambient light and temperature, calculated perfusion was expressed as the ratio of ischaemic to non-ischaemic hindlimb perfusion.

**Statistical analysis**

If not stated otherwise, data are shown as mean ± SEM. Statistical comparisons were made by the non-parametric Wilcoxon 2-sample test (paired analysis) or the non-parametric Mann–Whitney U test. Statistical significance was assumed at a value of P < 0.05. All statistical analysis was performed with SPSS (Version 11.5, SPSS Inc.).

**Results**

**Comparison of cell recovery**

The total number of BMC recovered from the same bone marrow aspirate immediately after density gradient centrifugation was significantly reduced, when the Lymphoprep protocol was compared with the Ficoll protocol (Figure 2A). The lower recovery of cells isolated with the Lymphoprep protocol was reflected by a significantly reduced total number of CD34<sup>+</sup> and CD133<sup>+</sup> haematopoietic stem cells (HSC) (Figure 2B and C; day 0). Likewise, the number of HSC giving rise to colonies (CFUs) and of mesenchymal stem cell colonies (MSC) after isolation of BMC from equal amounts of bone marrow aspirates from the same donor was significantly reduced, when the Lymphoprep protocol...
was used for cell isolation (Figure 2D and E; day 0, respectively). Viability of the isolated BMC was examined by trypan blue dye exclusion directly after density gradient centrifugation as well as after overnight storage. Viability of the BMC was 99% in both isolation protocols directly after isolation (data not shown) as well as after storage (Table 1).

Next, we determined the influence of the overnight incubation. In the ASTAMI trial, all patients received the BMC 1 day after bone marrow aspiration was performed. Therefore, BMC were incubated with 0.9% NaCl, 20% heparin-plasma, and 50 IU heparin at 4°C. In contrast, in the REPAIR-AMI study, BMC were incubated overnight in X-vivo 10 medium with 20% autologous serum at room temperature. Therefore, we determined HSC, CFU, and MSC after overnight incubation of BMC with the respective protocols. However, the overnight incubation protocols did not additionally affect the recovery of HSC, MSC, and CFU (Figure 2B-E; day 1), indicating that the reduced cell recovery between the two protocols is predominantly caused by differences in the initial centrifugation steps.

**Determination of cell function**

Recent studies demonstrated that the migratory capacity of BMC predicts the functional improvement after cell transplantation in a hindlimb ischaemia model as well as in a clinical pilot trial. Therefore, we determined the basal migration and the migratory response of BMC after overnight storage towards the chemoattractant cytokine SDF-1. Basal migration as well as SDF-1-induced migration were higher when the Ficoll protocol was used for cell isolation compared with the Lymphoprep protocol in both healthy volunteers (Figure 3A) as well as CAD-patients (Figure 3B). Of note, this difference was achieved when identical numbers of cells were seeded in the Boyden chambers indicating that the Lymphoprep protocol not only reduced the recovered number of cells, but additionally had a direct effect on the functional activity of the isolated cells. When the additional lower recovery was taken into account, the absolute number of migrating cells was even more profoundly reduced, when CAD-patient-derived cells were isolated (78% reduction, P = 0.028) (Figure 3D).

Strikingly, when CAD-patient-derived cells were isolated according to the Lymphoprep protocol, the migratory responsiveness towards the chemoattractant SDF-1 was completely abolished (Figure 3B/D). Since previous experiments demonstrated a close correlation between SDF-1-induced migratory capacity and blood flow recovery after ischaemia, we then tested the capacity of the isolated BMC to augment neovascularization in vivo. Therefore, 1 × 10^6 BMC from the same donor were isolated and stored according to the Ficoll or the Lymphoprep protocol and were intravenously infused in nude mice 24 h after induction of hindlimb ischaemia. Infusion of BMC isolated with the Ficoll protocol significantly augmented neovascularization as assessed by the recovery of perfusion measured by Laser Doppler (Figure 3E). In contrast, the infusion of the same number of BMC isolated with the Lymphoprep protocol resulted in a significantly blunted blood flow recovery (Figure 3E). Importantly, this difference

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### Table 1: Results at a glance

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Recovery of BMC (10^6)</th>
<th>Viability (%)</th>
<th>CD45^-/CD34+ BMC (10^3)</th>
<th>CFU</th>
<th>MSC</th>
<th>CXCR4+ BMC</th>
<th>Basal invasion (×10^2)</th>
<th>SDF-1 invasion (×10^3)</th>
<th>Perfusion in hindlimb ischaemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll protocol</td>
<td>25.5 ± 13</td>
<td>99 ± 0</td>
<td>6.8 ± 4.8</td>
<td>5270 ± 3919</td>
<td>230 ± 123</td>
<td>1357 ± 851</td>
<td>1244 ± 1307</td>
<td>2195 ± 1287</td>
<td>48 ± 23</td>
</tr>
<tr>
<td>Lymphoprep protocol</td>
<td>19.1 ± 7.6</td>
<td>99 ± 0</td>
<td>4.4 ± 3.6</td>
<td>3891 ± 2425</td>
<td>161 ± 160</td>
<td>447 ± 292</td>
<td>448 ± 384</td>
<td>822 ± 501</td>
<td>26 ± 7.5</td>
</tr>
<tr>
<td>P-value</td>
<td>0.027</td>
<td>1.0</td>
<td>0.043</td>
<td>0.023</td>
<td>0.015</td>
<td>0.02</td>
<td>0.04</td>
<td>0.02</td>
<td>0.012</td>
</tr>
</tbody>
</table>

The upper part shows a side-by-side comparison of the two different isolation protocols out of 10 mL bone marrow aspirate after overnight storage. Data are mean ± SD of patients and controls.
was obtained, although the same numbers of BMC were transplanted, further documenting that the Lymphoprep protocol not only is associated with a reduced cell recovery, but additionally affects the functional capacity of BMC to augment neovascularization in vivo.

**Effect of cell isolation on CXCR4 expression**

Basal and SDF-1-mediated migration as well as the rescue of neovascularization after ischaemia depend on the expression of the CXCR4 receptor on progenitor cells.\(^{14,16-18}\) Therefore, we determined the influence of the isolation and storage protocols on the surface expression of the CXCR4 receptor on the isolated progenitor cells by FACS analysis. Total CXCR4 expressing BMC (37 ± 8% of REPAIR, \(P = 0.002\)) were significantly reduced in cells isolated with the Lymphoprep protocol compared with Ficoll protocol after overnight storage. In addition, mean CXCR4-receptor-expression (area under curve) was significantly reduced (79 ± 12% of Ficoll, \(P = 0.012\)) after overnight incubation with the Lymphoprep compared with the Ficoll protocol.

**Influence of overnight incubation conditions**

To further determine the specific influence of the overnight incubation protocols, we isolated the BMC by Ficoll density

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**Figure 2** Effect of different isolation protocols on cell number, surface expression-markers, CFU, and MSC immediately after cell isolation as well as after overnight storage. (A) Total number of isolated BMC out of 10 mL bone marrow aspirate immediately after density gradient centrifugation, \(n = 6\) donors, data are shown as mean ± SEM. (B) Total number of BMC expressing CD34 after isolation (day 0) and after overnight storage (day 1), \(n = 5\) donors, data are shown as mean ± SEM. (C) Total number of BMC expressing CD133 after isolation (day 0) and after overnight storage (day 1), \(n = 5\) donors, data are shown as mean ± SEM. (D) Number of CFU out of 10 mL bone marrow aspirate after isolation (day 0) and overnight storage (day 1), \(n = 6\) donors, each measured in duplicates, data are shown as mean ± SEM. (E) Number of MSC out of 10 mL bone marrow aspirate after isolation (day 0) and overnight storage (day 1), \(n = 6\) donors, each measured in triplicates, data are shown as mean ± SEM.
gradient centrifugation according to the Ficoll protocol and then used different media for overnight incubation. Compared with the Ficoll protocol (storage in X-vivo 10 medium plus 20% serum), the use of 0.9% saline instead of the medium significantly reduced basal and SDF-1-induced migration (Figure 4). Likewise, the migration capacity was significantly suppressed, when heparin-plasma was used in combination with X-vivo 10 medium (Figure 4) indicating that the overnight incubation with 0.9% saline or 20% heparin-plasma significantly contributes to the profound impairment of the functional capacity of BMC.

Figure 3  Effect of different isolation protocols on invasion capacity after overnight storage of BMC. (A) Invasion capacity of 1 × 10^6 BMC in healthy volunteers (n = 3 donors, each invasion measured in duplicates, data are shown as mean ± SEM). (B) Invasion capacity of 1 × 10^6 BMC in CAD-patients (n = 3 donors, each invasion measured in duplicates, data are shown as mean ± SEM). (C) Invasion capacity out of 10 mL bone marrow aspirate in healthy volunteers (n = 3 donors, each invasion measured in duplicates, data are shown as mean ± SEM). (D) Invasion capacity out of 10 mL bone marrow aspirate in CAD-patients (n = 3 donors, each invasion measured in duplicates, data are shown as mean ± SEM). (E) Relative perfusion in a hindlimb ischaemia model 7 and 14 days after injection of 1 × 10^6 overnight stored BMC out of the two different isolation protocols (n ≥ 8, data are shown as mean ± SEM).

Discussion
The present study demonstrates that the recovery of cell numbers, haematopoietic, and mesenchymal colony-forming cells, and the functional activity of the isolated BMC is significantly affected by the isolation protocol used. Although the isolation protocols used in the REPAIR-AMI and ASTAMI trials appear similar at a first glance, the recovery of cell numbers isolated from the same volume of bone marrow aspirate was significantly lower, when the Lymphoprep protocol was applied. The data of the present study are in accordance with a lower number of cells
infused in patients in the ASTAMI trial\(^9\) (median: \(68 \times 10^6\)) compared with the REPAIR-AMI trial\(^8\) (median: \(198 \times 10^6\)), although an identical volume of 50 mL bone marrow aspirate was harvested for cell isolation in both studies. In line with a reduced recovery of total BMC, the number of haematopoietic and mesenchymal CFU was lower, when using the Lymphoprep protocol. In addition to the reduction of the cell numbers, the Lymphoprep cell isolation protocol was associated with a profoundly impaired capacity of the recovered BMC to migrate in response to SDF-1. Moreover, basal cell migration was significantly suppressed. The cytokine SDF-1 is released in response to hypoxia and acts as a potent chemo-attracting factor to recruit circulating progenitor cells.\(^{19-21}\) Therefore, this in vitro migration assay mimics the capacity of circulating cells to react to the endogenous chemoattractant provided by the infarcted tissue. Previous studies have demonstrated that the migratory capacity of progenitor cells measured ex vivo correlates with the neovascularization improvement of the infused cells in a hindlimb ischaemia model.\(^{14}\) Moreover, a higher migration of EPC and BMC ex vivo was associated with a greater reduction of infarct size in patients treated with the respective cells in the clinical pilot trial TOPCARE-AMI.\(^{15}\)

To determine a potential underlying mechanism, we measured the expression of the SDF-1 receptor, CXCR4, on BMC. The striking reduction of cell migration was associated with a significant reduction of CXCR4-expressing cells after overnight storage of BMC according to the Lymphoprep protocol. Importantly, BMC isolated from heterozygous CXCR4 mice, which exhibit an approximately 50% reduction of CXCR4 receptor surface expression, entirely failed to augment neovascularization after infusion in a hindlimb ischaemia model indicating that even a half maximal expression of CXCR4 is sufficient to profoundly impair cell function.\(^{18}\) Moreover, inhibition of CXCR4 by blocking antibodies reduced the basal migration and the responsiveness towards VEGF and SDF-1\(^{18,22}\) indicating that CXCR4 plays a crucial role in progenitor cell migration. The significant reduction of CXCR4 expressing cells, therefore, may well rationalize the impaired response towards SDF-1 of BMC isolated by the Lymphoprep protocol.

Analysis of the individual steps in the protocols indicates that two steps are critical. The use of the Lymphoprep separation protocol compared with the Ficoll gradient centrifugation lead to ~30% reduction of recovery of total cell number, which also translates into a similar reduction of the numbers of CD34\(^+\) haematopoietic progenitor cells, clonally expanding HSC, and MSC. The additional overnight incubation protocol did not further reduce the recovery of the different progenitor cell populations. However, the overnight incubation protocols significantly affected the migratory capacity of the isolated BMC. Preclinical studies have confirmed that the storage of BMC conditions used in the REPAIR-AMI trial in \(X\)-\(vivo\) 10 medium and 20% serum for up to 24 h does not impair cell function in vitro and does not affect the capacity of the cells to improve neovascularization in an animal model.\(^{13}\) However, when cells isolated with the REPAIR-AMI protocol were stored in 0.9% NaCl or when 20% serum was exchanged to 20% heparin-plasma, cell function was significantly reduced after 24 h of incubation.

In most of the previous clinical trials, BMC were isolated using the Ficoll centrifugation method.\(^7-10\) Only the BOOST trial used an entirely different gelatine polysuccinate sedimentation technique.\(^{11}\) Moreover, cells were administered on the day of preparation without overnight incubation in the TOPCARE-AMI and BOOST trials as well as in the studies by Perin \textit{et al.}\(^6\) and Janssens \textit{et al.}\(^10\) Only Strauer \textit{et al.}\(^5\) and Fernandez-Aviles et al.\(^7\) incubated the isolated cells overnight. However, the latter studies incubated the cells in medium (\(X\)-\(vivo\) 15; RPMI1640\(^7\)). Additionally, both studies used lower concentration of plasma (2%),\(^5,7\) and Strauer \textit{et al.}\(^5\) additionally included a heat inactivation step.\(^3\) Although it is impossible to judge the different isolation protocols based on the available information in the literature, it appears that most of the pilot trials demonstrating a benefit of cell therapy in patients with acute myocardial infarction clearly applied distinct protocols for cell isolation as compared with the ASTAMI trial. Therefore, it is conceivable that different cell isolation and incubation protocols may have contributed to the failure of the ASTAMI trial to demonstrate a beneficial effect of cell therapy on functional recovery in patients with acute myocardial infarction.

However obviously, the present study cannot exclude that other factors besides the quality of the cell preparations may have influenced the outcome of the clinical trials.

Taken together, the present study demonstrates that different isolation protocols have a profound impact on the function of BMC to augment neovascularization after ischaemia. Even small changes in isolation protocols can have a negative impact on functional activity of isolated cells and, therefore, affect clinical outcome. Assessing cell number and progenitor cell viability is insufficient to predict the functional capacity of isolated cells. Additional \textit{in vitro} assays and careful testing of cell preparations \textit{in vivo} appear to be mandatory before embarking on future clinical trials aiming at the recovery of contractile function in patients with acute myocardial infarction.

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Conflict of interest: Some of the authors of this study were also authors of the recently published REPAIR-AMI trial that showed positive effects of BMC in acute myocardial infarction.

References


