Density Gradient Centrifugation Compromises Bone Marrow Mononuclear Cell Yield

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Introduction

Bone marrow transplantation was originally established to treat hematological malignancies [1] and is nowadays widely used in different branches of regenerative medicine. The bone marrow is a capable source of autologous cells with distinct regenerative properties, which can be quickly harvested and are thus applicable for both chronic and acute diseases. Preclinical and clinical safety, feasibility and efficacy have been reported, inter alia, for ischemic limb injury [2,3], cerebral ischemia [4,5] and in particular for myocardial infarction [6,7] for which by now more than 30 placebo controlled randomized trials have been accomplished [8].

In the majority of studies, aspirated bone marrow was further processed in order to isolate the mononuclear cell fraction (BMNC), a heterogeneous population containing differentially matured B-cells, T-cells and monocytes, as well as rare progenitor cells such as hematopoietic stem cells (HSC), mesenchymal stromal cells (MSC), endothelial progenitor cells (EPC) and very small embryonic-like cells (VSEL). It has been constantly described that this cell mixture promotes distinct angiogenic properties [2], mediates vascular repair, expresses several cytoprotective growth factors and cytokines [9] and restores pathologically altered genes after ischemic heart injury [10]. However, which component or combination of components exactly determines the efficacy of BMNCs is not entirely understood, hence impeding full realization and further advancement of the therapeutic concept [11]. Some groups suggested that conflicting results in large-scale clinical trials [12,13] are, at least to some extent, due to different cell isolation protocols and a subsequently altered BMNC composition [14]. In fact, it has been proven that efficacy and functionality of BMNCs are significantly influenced by red blood cell contamination [15], the content of apoptotic cells [16], different washing steps [14] and even by the centrifugation speed [17].

Another decisive point seems to be the choice of the density gradient medium. Most preclinical and clinical studies used Ficoll-Paque (hereafter indicated as Ficoll) as density medium in order to enrich the mononuclear cell population as well as the rare progenitor cells therein [18]. However, it is a well-known problem that Ficoll-based density gradient centrifugation (DGC) causes a significant reduction of BMNCs to only 15–30% of the initial content [17,19]. This is critical since the efficacy of autologous BMNC transplantation is likely dose-dependent [20], and little data is available on a possible asymmetry of the cell loss [21,22]. Recently, it was described that Ficoll DGC even depleted cells with a high regenerative potential, such as MSC [23] and VSEL [24], and irreversibly impaired cell function by decreasing expression of chemokines receptors [25,26]. Accordingly, the objective of this study was to determine whether and
how Ficoll DGC affects the yield and composition of the cell graft compared to alternative methods such as adjusted Percoll DGC [27] and immunomagnetic bead separation of granulocytes [28]. Our findings indicate that the common method to isolate BMNC in both preclinical and clinical research can be considerably improved by replacing Ficoll with adapted Percoll or preferably by immunodepletion of unwanted constituents of bone marrow.

Methods

Rat Bone Marrow Harvest and Lysis of Erythroid Cells

Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rat bone marrow was obtained from 12-week-old male Sprague-Dawley rats. Femurs and tibiae were aseptically opened and bone marrow was harvested by repeated flushing with phosphate buffered saline (PBS). In order to dissolve remaining cell aggregates, the solution was resuspended using a 20 G cannula and sieved through a 100 μm cell strainer. Erythroid bone marrow cells were lysed by short-term incubation (30 seconds) with hypotonic ammonium chloride buffer (0.155 M NH4Cl, 10 mM KHCO3 and 0.01 mM Na2EDTA) followed by repeated washing steps with PBS containing 3% fetal calf serum (PBS/3% FCS). Viability and cell count were determined by the trypan blue exclusion method using a hemocytometer.

Rat Mononuclear Cell Isolation by Density Gradient Centrifugation

Bone marrow cells from 5 donor rats were separated using Ficoll-Paque 1.084 (which is used to isolate rat mononuclear cells (MNC; GE Healthcare, Munich, Germany) with 3 technical replicates per sample. A total of 10E7 bone marrow cells were resuspended in 10 mL HBSS/3% FCS and carefully layered upon 7.5 mL of Ficoll separation medium. Ficoll gradients were centrifuged for 40 min at 400 g without brake. The bone marrow mononuclear cell (BMNC) layer was then collected, washed in PBS/3% FCS, counted and prepared for flow cytometric analysis.

Next, we aimed to establish an appropriate density gradient for isolating BMNC using Percoll medium (GE Healthcare, Munich, Germany). Concentrated Percoll was diluted in 1.5 M NaCl to an isotonic Percoll stock solution (SIP) with a density of 1.1228 g/mL. We then prepared a series of different separation media (1.071, 1.073, 1.075, 1.080 and 1.084 g/mL) by mixing SIP with varying volumes of Hank’s balanced salt solution (HBSS) containing 3% FCS. Likewise, an invariant bottom layer was adjusted to 1.095 g/mL. For each of the separation gradients, 10E7 bone marrow cells from two donor rats were resuspended in 5 mL Percoll medium with the highest density (1.095 g/mL). This layer was then carefully covered with 5 mL of the respective lower concentrated Percoll dilutions and finally capped with 5 mL of HBSS/3% FCS. Discontinuous gradients were centrifuged for 40 min at 480 g without brake. After centrifugation, the cell layers located between HBSS/separation medium or in between the two Percoll layers were separately collected and washed twice in PBS/3% FCS. Cells were counted and prepared for flow cytometric analysis. The Percoll medium density leading to the highest BMNC yield along with the lowest polymorphonuclear cells (PMN) contamination (1.080 g/mL) was then chosen for according BMNC isolation from 5 donor rats with 3 technical replicates per sample.

Rat BMNC Isolation by Immunomagnetic Depletion of PMNs

A total of 10E8 bone marrow cells obtained from 6 donor rats (with 3 technical replicates per sample) were incubated with 5 ng/ml phycoerythrin conjugated anti-rat granulocytes antibody (clone RPI; BD Pharmingen, Heidelberg, Germany) for 15 min at 4°C. After washing in cold PBS/0.5% FCS, bone marrow cells were further incubated with 50 μL magnetic anti-phycoerythrin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 12 min at 4°C. Non-adsorbed microbeads were eliminated by an additional washing step. Finally, bone marrow cells were resuspended in 500 μL PBS/0.5% FCS. Magnetic separation was performed on LD columns using a magnetic QuadroMACS separator according to manufacturer’s instructions (Miltenyi Biotec). Both discharge and the fraction that was magnetically trapped were separately collected, counted and prepared for flow cytometry.

Flow Cytometric Characterization of rat BMNCs

Cellular composition of lysed rat bone marrow and separated BMNCs following different isolation protocols were analyzed by flow cytometry. For labelling, 2.5 x 10E5 cells were incubated with a mixture of monoclonal antibodies (Table 1) for 20 min at 4°C. Erythroid cells were labeled by biotinylated anti-erythroid-antibody which was secondly conjugated with streptavidin PerCP (BD Pharmingen, Heidelberg, Germany). After incubation, cells were washed and resuspended in 300 μL PBS/3% FCS. Flow cytometric acquisition and analysis was performed using a FACS Canto II equipped with FACS Diva software (BD Biosciences, Heidelberg, Germany). Cellular subpopulations were identified by specific antigen expression (Table 1) and categorized according to the gating strategy displayed in Figure 1.

Determination of Progenitor Frequency in Rat Samples

The frequency of hematopoietic or non-hematopoietic progenitors was assessed by granulocyte-macrophage (CFU-GM) and fibroblast (CFU-F) colony forming unit assays. For CFU-GM, 1.5 x 10E4 vital cells were seeded in duplicates with 1.1 mL Methocult medium (GF R3774, Stem Cell technologies, Grenoble, France) into 35 mm suspension dishes and further cultivated at 37°C, 5% CO2 and 95% humidity for 14 days. Proliferating CFU-GM colonies were counted and the frequency was calculated and normalized to leukocytes counts. CFU-GM frequency was analyzed from 8 (lysed bone marrow) or 4 (Percoll, Ficoll and MACS) donor rats with 2 technical replicates per sample.

Accordingly, to determine CFU-F frequency, 10E7 vital cells from 8 (lysed bone marrow) or 4 (Percoll, Ficoll and MACS) donor rats with 2 technical replicates per sample were plated into 35 mm culture dishes in Dulbecco’s modified Eagle’s medium containing 4.5 g/L glucose (PAA Laboratories, Colbe, Germany), 1% FCS plus 1% Penicillin/Streptomycin and maintained at 37°C, 5% CO2 and 95% humidity for 21 days. Medium was initially changed after 5 days and twice a week thereafter. After 3 weeks, cells were fixed in ice-cold methanol and stained with Giemsa. Fibroblast colonies were counted and frequencies were calculated.

Pretreatment of Human Bone Marrow Aspirates

Next, we aimed to validate the protocol of BMNC isolation by immunodepletion in human samples. Human bone marrow aspirates from 3 donors were purchased from Lonza Walkersville (Lonza, Walkersville, MD; FDA-registered company for the processing of human cells, tissue and cellular and tissue based
products in accordance with the US Code of Federal Regulations (21 CFR Par 1271)). Human bone marrow was diluted 10-fold with PBS/3% FCS and 5 mM EDTA and sieved through a 30 μm cell strainer (pluriSelect, Leipzig, Germany) to exclude fat and bone fragments. After centrifugation for 5 min at 440 g, human bone marrow was resuspended in PBS/3% FCS and 5 mM EDTA to the original volume.

Each of the three bone marrow samples was then split into two experimental groups: (i) whole human bone marrow and (ii) lysed human bone marrow. For lysis, human bone marrow cells were incubated with a 4 fold volume of hypotonic ammonium chloride buffer (0.155 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂EDTA) for 10 min at room temperature followed by repeated washing steps with PBS/3% FCS. Viability and total cell count were determined by the trypan blue exclusion method in a hemocytometer. Leukocytes were separately stained and counted with Turk’s solution.

Immunodepletion of PMNs from Human Bone Marrow

First, PMNs were depleted from lysed human bone marrow by means of magnetic-activated cell sorting (MACS). One mL of lysed bone marrow was incubated with 50 μL CD15 whole blood MicroBeads (for human PMNs; Miltenyi Biotech) for 20 min at 4°C. Non-adsorbed MicroBeads were eliminated by washing with PBS/0.5% FCS. The labeled cell suspension was then resuspended in 2 mL of PBS/0.5% FCS. Subsequently, magnetic separation was performed on LS columns using a QuadroMACS separator according to the manufacturer’s instructions (Miltenyi Biotech). LS columns were successively loaded with 0.5 mL of CD15-labeled lysed bone marrow cells and 1 mL of PBS/0.5% FCS. Both discharge and the magnetically trapped CD15+ fraction were separately collected, counted and prepared for flow cytometry.

Alternatively, PMNs were depleted from lysed human bone marrow using the PluriBead technology (PluriB) that bases on differentially sized beads (resotted with according antibodies) that can be separated by cell strainers (pluriStrainer, pluriSelect). One mL of lysed bone marrow was incubated with anti-human CD15 whole blood S-pluriBeads (pluriSelect; with a ratio of 2 pluriBeads per 3 target cells) on a pluriPlix shaker (pluriSelect) for 30 min at room temperature. Next, the labeled cell suspension was passed through an S-pluriStrainer (pluriSelect) accompanied by continuous rinsing with 15 mL of washing buffer (pluriSelect). Cells that were trapped in the S-pluriStrainer were detached from the pluriBeads using 1 mL detachment buffer (pluriSelect) for 10 minutes at room temperature and flushed through the strainer with 6 mL of washing buffer. Both fractions were separately collected, counted and prepared for flow cytometry.

In the next step, we considered to replace lysis by immunodepletion of erythrocytes. For immunomagnetic depletion of erythrocytes, whole human bone marrow was incubated with 300 μL of CD235a MicroBeads (Glycophorin A; for human erythroid cells; Miltenyi Biotech) for 20 min at 4°C. Further steps correspond to those described for the immunomagnetic CD15 depletion (above), except for the loading of the LS columns. Due to the significantly higher number of magnetically labeled cells (ratio of leukocytes to erythrocytes in whole bone marrow was 1:200),

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### Table 1. Anti-rat monoclonal antibodies used for flow cytometry.

| Antigen | Fluorochrome | Clone | Manufacturer | B-cells | Mo/DC | PMNs | CD45 other | Erythroid cells | CD45+/Ery- |
|---------|--------------|-------|--------------|---------|-------|------|------------|----------------|-----------|
| CD45R   | FITC         | HIS24 | BD           | +       | -     | -    | -          | -              | -         |
| Granulocytes | PE   | RP-1  | BD           | -       | -     | +    | -          | -              | -         |
| Erythroid | Biotin      | HIS49 | BD           | -       | -     | -    | +          | -              | -         |
| CD45    | APC-Cy7     | OX-1  | BD           | +       | +     | +    | +          | -              | -         |
| CD11b   | Pacific Blue | MRC-OX42 | Abd Serotec | -       | +     | -    | -          | -              | -         |

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1 mL of CD235a-labeled whole bone marrow was loaded onto 3 LS columns. Both discharge and the mobilized CD235a+ cells were separately collected, counted and prepared for flow cytometry. In a second step, discharge was further immunomagnetically depleted of CD15+ PMNs as described above.

Finally, we used the pluriBead technology to combine multiple protocol steps by using differentially sized beads to simultaneously deplete erythrocytes and PMNs. Whole human bone marrow was incubated with anti-human CD235a M-pluriBeads (with a ratio of 1 M-pluriBead per 140 target cells) and CD15 whole blood S-pluriBeads (with a ratio of 2 pluriBeads per 3 target cells) for 30 min at room temperature on a pluriPlix shaker. Further steps correspond to those described for the CD15 pluriBead depletion (above).

Flow Cytometric Characterization of Human BMNC

Cellular composition of human bone marrow cells and subsequently separated cell fractions were analyzed by means of flow cytometry according to the protocols described for the analysis of rat BMNCs (above). Human cellular subpopulations were identified by expression of specific antigens (Table 2).

Statistical Analysis

Statistical differences were analyzed using t-tests (in case of 2 groups) or by one way analysis of variance (ANOVA; in case of more than 2 groups) and Holm-Sidak post hoc test. A p-value of 0.05 or less was considered statistically significant. All data was shown as mean ± standard deviation (SD).

Results

Rat Bone Marrow Cell Yield and Effect of Lysis

Rat bone marrow obtained from femurs and tibiae was pooled and yielded 1.2×10^9±2.4×10^8 cells per animal that were composed of CD45+ leukocytes (62.3±3.7%) and CD45- cells (37.8±3.7%; Figure 2A). The latter population primarily belonged to the erythroid lineage (CD45-/Ery+ cells), but also contained a small number of CD45-/Ery- cells (Figure 2D). As intended, short-term incubation of bone marrow cells with lysis buffer caused a 90% decrease of erythrocyte cells that was accompanied by a significant loss of about 35% of the CD45+ leukocytes (Figure 2A, B). However, we found the leukocyte loss being equally distributed to the major bone marrow subpopulations (Figure 2B, C). Both unprocessed and lysed bone marrow consisted of two-third BMNCs and one-third PMNs. The BMNC fraction was further classified into B-cells (52.1±5.5%), monocytes/dendritic cells (Mo/DC) (5.1±1.4%) and other CD45+ cells (9.4±1.7%; Figure 2C). The small CD45-/Ery- cell population was hardly affected by lysis and increased to approximately 40% of the CD45- cells within lysed bone marrow (Figure 2D).

Establishing an Adapted Density Separation Gradient using Percoll

We used a series of different Percoll separation gradients to identify the density gradient most suitable to separate MNC and PMN from rat bone marrow. The main endpoints were BMNC loss and yield as well as PMN contamination. We found a positive relation between escalating Percoll densities and BMNC yield (Figure 3) with highest BMNC yield at densities 1.080 g/mL and 1.084 g/mL. However, at a density of 1.084 g/mL, we observed a distinct increase of PMN contamination (Figure 3).

Rat BMNC Recovery following Different Isolation Protocols

We next compared BMNC yield and composition after applying either the Percoll gradient selected above (1.080 g/mL), a standard Ficoll gradient or an immunomagnetic depletion (MACS) of PMNs. Pre-isolation, 10^7 lysed bone marrow cell contained about 5.7×10^6 BMNCs, 2.6×10^6 PMNs and 1.7×10^6 CD45- cells (Figure 4A). As expected, all isolation protocols caused a nearly complete depletion of PMNs (Ficoll: 2.5±1.3%, Percoll 11.5±6.1% and MACS 9.5±8.5% recovery of total PMNs in lysed bone marrow) and, to a lesser extent, of the CD45- population, particularly of the erythroid cell population (Figure 4A). Depletion of the CD45- cell population was significantly more severe after Ficoll DGC compared to Percoll DGC and MACS (14.6±8.3% versus 41.2±5.1% and 51.0±6.0% recovery of total CD45- cells in lysed bone marrow). One major result of this study is the significant decrease of the BMNC population after each of the isolation procedures. This cell diminution, however, was especially pronounced after Ficoll DGC, during which almost 75% of the BMNC were lost. The adjusted Percoll gradient yielded two-fold more BMNC than the Ficoll DGC, but still showed

| FC panel | Fluorochrome | Clone | Manufacturer | B-cells | T-cells | Monocytes | PMNs | Erythroid cells | MSC | HSC |
|----------|--------------|-------|---------------|---------|---------|------------|------|----------------|-----|-----|
| CD15     | FITC         | VIMC6 | Miltenyi      | —       | —       | —          | +    | —              | N/A | N/A |
| CD3      | PE           | UCHT1 | BD            | —       | +       | —          | —    | —              | N/A | N/A |
| CD235a   | PE-Cy5       | GA-R2 | HIR2 BD      | —       | —       | —          | —    | +              | N/A | N/A |
| CD14     | PE-Cy7       | M5E2  | BD            | —       | —       | +          | —    | —              | N/A | N/A |
| CD19     | APC          | HIB19 | BD            | +       | —       | —          | —    | —              | N/A | N/A |
| CD45     | APC-Cy7      | 2D1   | BD            | +       | +       | +          | —    | —              | (±) |     |
| CD133    | PE           | AC33  | Miltenyi      | —       | —       | —          | —    | —              |     |     |
| CD105    | PerCP-Cy5.5  | 266   | BD            | —       | —       | —          | —    | +              |     |     |
| CD34     | APC          | AC33  | Miltenyi      | —       | —       | —          | —    | +              |     |     |

Table 2. Anti-human monoclonal antibodies used for flow cytometry.

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a BMNC loss of about 50%. By contrast, immunomagnetic depletion of PMN showed a BMNC deficit of less than 30% (Figure 4A). Proportions of leukocyte subpopulations within the respective BMNC fractions resembled those of lysed bone marrow (Figure 4B) indicating symmetric BMNC loss independent of the isolation protocol.

To investigate the amount and composition of cell loss after isolation, we analyzed the cells within layer 2 after Percoll DGC and the eluate after MACS. As expected, we detected large amounts of PMNs both after Percoll and MACS separation (61.3 ± 18.0% versus 75.8 ± 16.5% recovery of total PMNs in lysed bone marrow). By contrast, the incidence of BMNCs was significantly increased within the waste after Percoll DGC compared to MACS (10.6 ± 1.2% versus 4.4 ± 2.7% recovery of total BMNCs in lysed bone marrow; Figure 4D).

**Determination of Rat Progenitor Frequencies**

Additional experiments were performed to clarify whether or not the different isolation protocols influence the progenitor frequencies within rat BMNCs. To answer this question we used both CFU-GM and CFU-F to assess the proportion of bipotent hematopoietic stem cells (HSC) and of non-hematopoietic progenitors such as mesenchymal stromal cells (MSC). In lysed bone marrow, the CFU-GM frequency was 0.37 ± 0.08% which corresponds to 3.5 × 10^4 HSCs in 1 × 10^7 bone marrow cells (Figure 5A, B). Separation of BMNCs by means of Ficoll, Percoll or MACS depletion led to an enrichment of HSCs resulting in a CFU-GM frequency of 0.63 ± 0.12%. This effect was less pronounced after Percoll DGC and disappeared after immunomagnetic separation (0.3 ± 0.13% and 0.37 ± 0.05%; Figure 5A). However, considering the significant different BMNC yields (Figure 4A), only one-third of all HSCs were harvested after Ficoll DGC. By contrast, after
adjusted Percoll DGC and immunomagnetic separation, the HSC yield was two-fold higher as compared to Ficoll DGC and increased to two-thirds of the total amount in lysed bone marrow (Figure 5B). As described previously [29], CFU-F frequency was extremely low in lysed bone marrow (0.00007%). In contrast, the CD34+ population was further differentiated into CD133- and CD133+ cells by increased size and granularity (Figure 6C; 2). The HSC population was further differentiated into CD133- and CD133+ cells (Figure 6C; 3). Overall, the CD133+ HSCs accounted for approximately 40% of the CD34+ cells in all experimental groups (data not shown).

Flow cytometric quantification of HSCs and MSCs was not feasible in human whole bone marrow, owing to the extremely low rate of progenitor cells (approximately 0.005% (HSCs) and 0.0005% (MSCs) of all events in whole bone marrow). However, after lysis of human bone marrow, we ascertained 10.2±0.2×10E4 HSCs and 1.6±0.2×10E4 MSCs per mL (Figure 6D). The subsequent isolation of BMNCs was responsible for a significant loss of both HSCs and MSCs (on average of 30% for HSCs and of 55% for MSCs). The decrease of HSCs was thereby mostly pronounced after one-step depletion of erythrocytes and PMNs using PluriB (Figure 6D). However, the proportion of progenitors to the amount of total nucleated cells (i.e. frequency) increased after BMNC isolation, simply as a consequence of selective PMN depletion. Thus, the HSC frequencies increased from 0.76±0.04% in lysed bone marrow to 3.51±0.16% (lys+MACS), 2.97±0.05% (lys+PluriB), 3.8±0.06% (MACS+MACS) and 1.58±0.03% (PluriB combination). The MSC frequencies increased from 0.12±0.007% in lysed bone marrow to 0.34±0.01% (lys+MACS), 0.37±0.11% (lys+PluriB), 0.44±0.04% (MACS+MACS) and 0.26±0.04% (PluriB combination).

Discussion

In this study, we provide evidence that the application of density gradient centrifugation (DGC) for isolation of the...
mononuclear cell fraction from rat bone marrow (BMNC) is confounded by a significant loss of the cells of interest. When using Ficoll as density medium, only 25% of the BMNCs being detectable in lysed bone marrow were recovered. These findings are consistent with other studies investigating human bone marrow that show BMNC recovery rates between 15 to 30% after Ficoll DGC [17,19]. Interestingly, we found that DGC using Percoll with an equal density to that of Ficoll (1.084 g/mL) yielded significantly more BMNCs (Figure 3), suggesting that excessive cell loss during Ficoll DGC is a consequence of density medium-related cytotoxicity [30]. Moreover, Ficoll based DGC has been primarily established for separation of blood into its components. In contrast to peripheral blood mononuclear cells (PBMCs), BMNCs exhibit an increased variability of cell maturity and consequently of buoyant density [31]. Hence, BMNCs with densities deviating from those of PBMCs might less likely accumulate within the correct Ficoll density layer. To address this problem, we used a series of different layer densities by diluting Percoll and found that the optimal density range for isolating rat BMNCs is in between 1.080 to 1.084 g/mL.

![Figure 4. Rat BMNC yield following different isolation procedures.](image)

(A) Compared to lysed bone marrow, each of the isolation procedures caused an almost complete depletion of PMNs (C; arrows indicate the remaining RP1+ PMNs). As an unwanted side effect, this was accompanied by a significant loss of BMNCs and CD45- cells (A). BMNC loss was maximal after Ficoll DGC followed by Percoll DGC and MACS separation (*p<0.05). Both Percoll and MACS preserved the CD45- population compared to Ficoll (A; *p<0.05). Further analysis revealed a symmetric cell loss among the BMNC subpopulations (B; C, representative forward/sideward scatter diagrams). (D) For both Percoll and MACS, primarily RP1+ PMNs but also particular BMNC populations were detected within the waste (Percoll: layer 2; MACS: content of the columns). Values are means ± SD for 5 samples. *p<0.05 by one-way ANOVA. doi:10.1371/journal.pone.0050293.g004
bunded above by unwanted polymorphonuclear cell (PMN) contamination and below by decreasing BMNC yields (Figure 3). However, even after adjustment of the density layers, density gradient separation of BMNCs was generally confounded by a cell loss of at least 50%. This is highly relevant for clinical practice, since meta-analyses revealed a clear dose-dependency of autologous BMNC transplantation [20]. A profound cell loss during DGC may hence directly attenuate therapeutic efficacy. Consequently, we looked for an alternative method to separate BMNCs with a higher yield, and decided to immunomagnetically deplete PMNs as it has been described for human blood samples from patients with sepsis or burn [28]. Immunomagnetic separations by either positive or negative selection of special cell populations can be conducted under conditions of good manufacturing practice [32] and have already been applied in several clinical studies [33–35]. In our experiment with rat samples, we show that this approach achieved a significantly higher BMNC yield compared to both DGC-based methods (Figure 4A), while the leukocyte composition was identical. We therefore conclude that immunomagnetic separation could be an appropriate method to isolate bone marrow mononuclear cells for transplantation purposes offering the advantage of higher BMNC doses for patients. To further validate this hypothesis, we performed a proof-of-concept study with fresh human bone marrow samples from three healthy donors. We found that immunomagnetic separation (MACS) of CD15+ PMNs from lysed human bone marrow was feasible and yielded a BMNC recovery of 55%. An alternative method for immunoselection based upon differently sized beads (PluriB) recovered slightly more cells (58%) with comparable effort and almost equal final composition of BMNCs. Generally, human BMNC yields were lower compared to what we observed in our rat study (72%), but we assume that a further optimization of the human proof-of-concept protocol, as it has been extensively done for the rat protocol, would approximate the BMNC yields.

One further important advantage of using immunoselection compared to density gradient centrifugation is the flexibility to adapt the protocol to a supposed mechanism of action by either depletion of adverse or enrichment of beneficial cell populations. We verified this concept in human bone marrow samples by immunoselection of both PMNs and erythrocytes, since it was shown that the content of erythroid cells directly impaired the function of BMNCs [15]. Interestingly, immunomagnetic depletion of erythrocytes resulted in a highly purified CD45+ cell population (ca. 5% erythroid cells; Figure 6A) compared to lysis (ca. 80% erythroid cells) or Ficoll DGC (ca. 70% erythroid cells [15]).

Hematopoietic CD34+ stem cells (HSC) account for approximately 1.5% of the BMNCs and have been discussed to have a crucial impact on the observed therapeutic effects [33]. DGC-based separation of BMNCs was hence often justified to augment these progenitors within the cell graft [27]. In fact, we and others [12] found a significant enrichment of HSCs after Ficoll DGC. An increased resistance against the toxicity of the density medium or a suitable buoyant density (i.e. close to lymphocytes) might explain HSC enrichment after Ficoll DGC. Nevertheless, due to the significant loss of BMNCs, the overall HSC yield was significantly decreased after Ficoll DGC. Enrichment might thus be beneficial in preclinical studies with a fixed, non-autologous BMNC transplantation scheme. However, in a clinical and autologous setting, DGC might rather result in a loss of effector cells. In fact, the amount of CD34+ cells in human whole bone marrow was described to be 1.5×10^5 per mL [36] whereas several clinical trials yielded between 1.4 to 7.2×10^4 CD34+ cells per mL [12,13]. After immunoselection of PMNs from lysed human bone marrow, we found 7.5×10^4 CD34+ cells per mL, which is considerably higher compared to that what was reported recently in large scale clinical studies.

It is still controversially discussed which cell population within BMNCs is actually required for beneficial effects and which is not. Thus, for example, one recent study described the B-cells as being the effective cell population within the BMNCs [37]. In the present study we observed a distinct CD45− cell population within the rat bone marrow cells which clearly did not belong to the erythroid line (Figure 2D). In total the CD45− population was almost completely depleted after Ficoll DGC (15% recovery of total CD45− cells in lysed bone marrow); a finding that is supported by Medina et al. [38] who found many non-erythroid CD45− cells within the usually discarded pellet after DGC. By contrast, in our rat study, both Percoll and MACS separation conserved a large proportion of this population (41% and 57% of lysed bone marrow; Figure 4A). Interestingly, these CD45− cells seem to contain specific stem cell populations such as MSCs, VSEL, epithelial progenitors [38], multilineage inducible cells [39] and endothelial progenitor cells [40] and might thus be relevant for therapeutic effects of BMNCs. In line with these observations, it was recently described that most of the bone marrow MSCs were discarded during Ficoll DGC due to their aggregate nature.

Figure 5. Recovery of rat hematopoietic progenitors. (A) Analysis of the CFU-GM frequency revealed a significant enrichment of hematopoietic stem cells (HSC) by Ficoll compared to MACS. However, when relating the CFU-GM number to the absolute BMNC yield, Ficoll DGC resulted in a significant loss of HSCs compared to both Percoll and MACS separation. Values are means ± SD for 4 samples. *p<0.05 by one-way ANOVA. doi:10.1371/journal.pone.0050293.g005

Figure 6. Recovery of rat hematopoietic and endothelial progenitors. (B) Enrichment of CD34+ hematopoietic progenitors was achieved by Ficoll DGC at the expense of the CD45− cell population compared to Ficoll DGC (ca. 70% erythroid cells [15]).
and that whole bone marrow, but not BMNCs obtained by Ficoll DGC improved functional recovery after experimental myocardial infarction [41]. Collectively, these considerations question whether the concept of stem cell enrichment during Ficoll DGC-based BMNC separation is true and reasonable in the setting of clinical studies.

In conclusion, our findings show that the isolation of BMNCs by density gradient centrifugation causes a distinct and symmetric cell loss that includes a decrease of therapeutically relevant stem cell populations. A higher cell yield can be obtained by using a customized Percoll protocol or by immunodepletion of unwanted constituents such as erythrocytes and granulocytes.
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Author Contributions

Conceived and designed the experiments: CP KM JB DCW. Performed the experiments: CP KM WF LS. Analyzed the data: CP KM DCW. Wrote the paper: CP DCW.