Ficoll-Paque™ versus Lymphoprep™: a comparative study of two density gradient media for therapeutic bone marrow mononuclear cell preparations

Aims: Contradictory outcomes from recent clinical trials investigating the transplantation of autologous bone marrow mononuclear cell (BM-MNC) fraction containing stem/progenitor cells to damaged myocardium, following acute myocardial infarction, may be, in part, due to the different cell isolation protocols used. We compared total BM-MNC numbers and its cellular subsets obtained following isolation using Ficoll-Paque™ and Lymphoprep™ – two different density gradient media used in the clinical trials. Materials & methods: Bone marrow samples were taken from patients entered into the REGENERATE-IHD clinical trial after 5 days of subcutaneous granulocyte colony-stimulating factor injections. Each sample was divided equally for BM-MNC isolation using Ficoll-Paque and Lymphoprep, keeping all other procedural steps constant. Isolated fractions were characterized for hematopoietic stem cells, endothelial progenitor cells, T lymphocytes, B lymphocytes and NK cells using cell surface markers CD34+, CD133+VEGFR2+, CD45−CD3+, CD45−CD19+ and CD45−CD16−CD56+, respectively. There were no significant differences in the absolute numbers and percentage cell recovery of various mononuclear cell types recovered following separation using either density gradient media. Cell viability and the proportion of various cell phenotypes investigated were similar between the two media. They were also equally efficient in excluding unwanted red blood cells, granulocytes and platelets from the final cell products. Conclusion: We demonstrated that the composition and quantity of cell types found within therapeutic BM-MNC preparations for use in clinical trials of cardiac stem cell transplantation are not influenced by the type of density gradient media used when comparing Ficoll-Paque and Lymphoprep.

KEYWORDS: acute myocardial infarction, Ficoll-Paque™, Lymphoprep™, bone marrow progenitor cells, density gradient separation, mononuclear cell fraction

Myocardial infarction is the leading cause of congestive heart failure and death in developed countries [1]. An estimated 10 million people in Europe alone suffer with heart failure [2] and this number is set to increase over the coming decades. Experimental evidence suggests that bone marrow-derived stem/progenitor cells may contribute to the regeneration of infarcted myocardium and could result in recovery of cardiac function [3].

Several clinical studies have been performed involving the transfer of the autologous bone marrow mononuclear cell (BM-MNC) fraction to the damaged myocardium of patients following acute myocardial infarction [4–8] with conflicting results. Whereas the Autologous Stem Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial found no demonstrable improvement in left ventricular systolic function of the cell-treated group over the control group [5], the Reinfusion of Enriched Progenitor Cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) study associated intracoronary infusion of autologous BM-MNCs with improved recovery of left ventricular contractile function [6].

Isolation of the BM-MNC fraction from whole bone marrow [9] requires a density gradient medium, a polysaccharide solution of a specific density, which acts as a liquid filter to remove undesired components (i.e., red blood cells [RBCs], platelets and granulocytes) while retaining BM-MNCs. The BM-MNC fraction contains a heterogeneous population of cell types including hematopoietic stem cells (HSCs) identified by a CD34 marker, monocytes (which are CD14+), lymphocyte subsets characterized by CD3+ (T cells), CD19+ (B cells) and CD16−CD56− (natural killer [NK] cells) cell surface markers and endothelial progenitor cells (EPCs), which can be defined by CD133+VEGFR2+ positivity.

Despite current evidence of cardiac improvement following reinfusion of BM-MNCs in various clinical trials [10] and animal experiments [11], it is still unclear which of the cell type(s) within this fraction is responsible for the effect. Several mechanisms have been suggested, including in situ transdifferentiation of transplanted cells into cardiomyocytes, neoangiogenesis and paracrine effects. The role of HSCs and...
Materials & methods

Study population
Bone marrow samples were obtained from patients entered into the Randomized Controlled Trial to Compare the Effects of G-CSF and Autologous Bone Marrow Progenitor Cells Infusion on Quality of Life and Left Ventricular Function in Patients with Heart Failure Secondary to Ischemic Heart Disease (REGENERATE-IHD) clinical trial and used for further in vitro analysis. In brief, this trial is designed to determine whether autologous bone marrow-derived stem/progenitor cells improve cardiac function and symptoms in patients with established heart failure. Patients included in the trial have a history of ischemic heart disease with resulting symptomatic heart failure (New York Heart Association class II–IV), despite optimal medical therapy with no further treatment options. Patients undergo a bone marrow aspiration following 5 days of granulocyte colony-stimulating factor mobilization treatment. The clinical trial was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. Bone marrow aspirations from 14 patients were used for this study.

Bone marrow harvest
Following written consent, patients were rolled into a left lateral position and the posterior superior iliac spine was identified. The overlying area was cleaned thoroughly with Hydrex Pink (pink chlorhexidine gluconate 0.5% w/v in 70% v/v DEB, Ecolab Ltd, UK). A total of 10 ml of 2% lidocaine hydrochloride injection BP (2% w/v 20 mg in 1 ml) was infiltrated into the skin puncture site. A 15-gauge bone marrow aspiration needle (Sternum Temno, Allegiance Healthcare Corporation, USA) was introduced at 90° to the bone surface. A total of 5 ml of bone marrow was aspirated into a 10 ml Luer-Lok syringe containing 1 ml heparin sodium (20,000 IU in 20 ml, LEO Laboratories Ltd, UK). After each aspiration, the bone marrow needle was either rotated clockwise 180° or advanced further into the marrow cavity to ensure a new reservoir of bone marrow was accessed. A single bone puncture site yielded three to four sets of bone marrow aspirate before the needle was removed and introduced into a new site. A total of 50 ml of bone marrow was aspirated equally into 10 × 10 ml heparin-treated syringes from three separate sites over the right iliac crest. The bone marrow aspirates were then kept at room temperature whilst awaiting transfer to the stem cell laboratory.

Isolation of BM-MNCs
Bone marrow aspirates from the heparin-treated syringes were pooled into a single transfusion bag (Baxter, IL, USA). The entire volume (50 ml) was passed through a blood component transfusion set with a 200 μm filter (Baxter SA, Lessines, Belgium). A total of 25 ml of this volume was then layered on 25 ml of Ficoll-Paque (density gradient: 1.077 ± 0.001 g/ml, GE Healthcare, Uppsala, Sweden) and the remaining 25 ml of bone marrow layered on 25 ml of Lymphoprep (density gradient: 1.077 ± 0.001 g/ml, Axis-Shield, Oslo, Norway). Both preparations were centrifuged in a Sorvall RT Legend centrifuge (Thermo Fisher Scientific, Leicestershire, UK) at 2500 rpm for 30 min with the brake off. The BM-MNC layer from each preparation was extracted and spun again at 2500 rpm for 10 min with the brake off. The resulting cell pellets were resuspended and underwent two cycles of washing in 0.9% saline (Baxter, Norfolk, UK). The final cell pellets were resuspended in 2 ml autologous serum. Serum was obtained from 6 × 7 ml plain red-top vacutainers (BD, Plymouth, UK) of peripheral blood, which had been allowed to clot and centrifuged in a Sorvall RT Legend centrifuge (Thermo Fisher Scientific) at 2500 rpm for 5 min to yield autologous serum. Each cell suspension was passed through a 100 μm filter (BD Biosciences, MA, USA), which was rinsed through with 0.5 ml autologous serum. A total of 250 μl was extracted from each final cell suspension for this analysis.

EPCs in these mechanisms is being explored in numerous studies. However, much less is known about the contribution of other BM-MNC cell types, such as lymphocytes, to the regenerative process. For example, lymphocytes produce TNF-α, which upregulates vessel-wall expression of VCAM-1 [12], which is required for homing of stem/progenitor cells to injured tissue via very late antigen-4 [13].

It has been suggested that the contradictory outcomes from the clinical trials may partly be due to the cell isolation protocols used [14], with different density gradient media giving different cell yields. Here, we sought to compare the two different media used in the clinical trials (Ficoll-Paque™ vs Lymphoprep™) to test whether, in addition to total BM-MNC number, there are significant differences in cellular subsets within this fraction obtained after isolation.
Flow cytometry analysis of BM-MNCs

All flow cytometry analyses were performed using a BD FACSCanto™ Flow Cytometer with BD FACSDiva™ v 5.0.3 software (BD Biosciences).

For the identification of HSC populations, cells were incubated with fluorescein isothiocyanate (FITC)-labeled antibody against human CD45 (BD Biosciences, Erembodegem–Aalst, Belgium) and phycoerythrin (PE)-labeled antibody against human CD34 (BD Biosciences) for 15 min at room temperature.

Endothelial progenitor cells were analyzed by initially incubating samples with mouse serum IgG (Sigma, Dorset, UK) for 15 min at 4°C to block nonspecific binding/specific binding via FcR. Following this, cells were incubated for 15 min at 4°C with a cocktail of antibodies comprising allophycocyanin (APC)-labeled antibody to CD133 (Miltenyi Biotec, Surrey, UK) and PE-labeled antibody to VEGFR-2 (R&D Systems, Abingdon, UK) to characterize EPCs and FITC-labeled monoclonal antibodies to CD2, CD13 and CD22 (Beckman Coulter, High Wycombe, UK) to identify and therefore eliminate inclusion of lineage-negative nonprogenitor cells. To ensure exclusion of nonviable cells in the final EPC count, cells were also incubated with a PerCP-Cy5-labeled 7AAD stain (BD Biosciences). Cells were then incubated for 15 min at room temperature with 2 ml of Pharm Lyse™ buffer (BD Biosciences) to lyse RBCs. Samples were washed once in phosphate-buffered saline and 20 µl of Accucount flow cytometry beads (Saxon Europe, Kelso, UK) were added before analysis.

Total lymphocytes were identified by incubating for 15 min at room temperature with MultiTEST™, multiclonal, four-color direct immunofluorescence reagent (BD Biosciences) containing antibodies CD45 PerCP/CD16+CD56 PE/CD3 FITC/CD19 APC to identify lymphocytes and the major subsets of NK, T and B cells, respectively.

The total white blood cell (WBC) count and BM-MNC count were derived from data obtained by full blood count analysis of the final cell suspensions using a LH750 Beckman

Figure 1. Enumeration of white blood cell and bone marrow mononuclear cell within the bone marrow fraction isolated using different density gradient media. Comparison of (A) absolute WBC (n = 14) and BM-MNC counts (n = 14); and (B) proportion of BM-MNC population within the total WBC count in the bone marrow-derived cell fraction isolated using either Lymphoprep™ or Ficoll-Paque™ density gradient separation (n = 14).

BM-MNC: Bone marrow mononuclear cell; WBC: White blood cell.
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Coulter counter (Beckman Coulter). Viability was ascertained based on data from the 7AAD gated cells from the EPC fluorescence-activated cell-sorting analysis.

Statistical analysis

Unless otherwise stated, continuous variables are presented as means ± standard deviation. Statistical comparisons were made by the parametric paired t-test or nonparametric paired Wilcoxon signed-rank test. A p-value of less than 0.05 was assumed to indicate statistical significance. All reported p-values are two-sided. Statistical analyses were performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

Results

Results were obtained from bone marrow aspirations from 14 patients; however, owing to logistical reasons, not all samples received the complete range of cellular analyses.

Characterization of BM-MNC

CD34+ cells, CD133+ cells & EPCs

The proportion of various BM-MNC subpopulations obtained using each media is illustrated in Table 2. We looked specifically at the CD34+ and CD133+VEGFR2+ subpopulations within the BM-MNC fraction as they have previously been implicated in myocardial repair. Our results demonstrate that the proportion of CD34+ subpopulation in BM-MNCs is similar between the two density gradient media used (p = 0.81). However, there was a nonsignificant increased proportion of EPCs present in BM-MNCs separated using Ficoll-Paque (p = 0.05).

| Table 1. Mean percentage cell recovery following density gradient separation. |
|---|---|---|---|
| Cell type | n | Lymphoprep™ (mean ± SD) | Ficoll-Paque™ (mean ± SD) | p-value |
| WBC | 14 | 18.05 ± 12.58 | 20.08 ± 11.14 | 0.12 |
| BM-MNC | 14 | 57.56 ± 105.60 | 48.85 ± 94.98 | 0.39 |
| CD34+ | 14 | 71.68 ± 83.92 | 81.01 ± 108.20 | 0.95 |
| CD133+ | 13 | 69.65 ± 74.67 | 90.20 ± 114.50 | 0.13 |
| CD133+VEGFR2+ | 10 | 34.12 ± 32.62 | 34.82 ± 21.80 | 0.50 |
| Lymphocytes | 10 | 47.92 ± 29.45 | 53.74 ± 25.63 | 0.23 |
| T cells | 10 | 50.46 ± 31.45 | 55.63 ± 24.66 | 0.38 |
| B cells | 10 | 55.62 ± 38.73 | 59.76 ± 34.46 | 0.19 |
| NK cells | 10 | 41.25 ± 33.43 | 45.90 ± 31.82 | 0.40 |

BM-MNC: Bone marrow mononuclear cell; NK: Natural killer; SD: Standard deviation; WBC: White blood cell.

| Table 2. Proportions of cellular subpopulations within the mononuclear fraction after separation with Lymphoprep™ and Ficoll-Paque™. |
|---|---|---|---|
| | Lymphoprep™ | Ficoll-Paque™ | p-value |
| CD34+BM-MNC (n = 14) | 0.063 ± 0.053 | 0.073 ± 0.078 | 0.81 |
| CD133+VEGFR2+BM-MNC (n = 13) | 0.000074 ± 0.000075 | 0.000147 ± 0.000227 | 0.05 |

BM-MNC: Bone marrow mononuclear cell.
Within the BM-MNC fraction, the total number of CD34+ HSCs obtained was similar between the two methods, with a mean count of $0.94 \pm 0.58 \times 10^6$ obtained following Lymphoprep separation and $0.96 \pm 0.52 \times 10^6$ with Ficoll-Paque (p = 0.84) (Figure 2A). A total of 13 bone marrow aspirates were processed and analyzed for CD133+ cell and EPC (CD133+/VEGFR2+) enumeration (Figures 2B & C, respectively). Although there was no difference in CD133+ cell counts between the

![Figure 2. Enumeration of different subpopulations within the bone marrow-derived mononuclear cell fraction isolated with either Lymphoprep™ or Ficoll-Paque™. Cellular subpopulations were defined by (A) hematopoietic stem cell marker CD34+ (n = 14), (B) early progenitor cell marker CD133+ (n = 13) and (C) endothelial progenitor cell markers CD133+/VEGFR2+ (n = 13). (D) Quantification of total number of lymphocytes and its major constituent subsets found within the mononuclear fraction obtained following Lymphoprep or Ficoll-Paque separation (n = 10). NK: Natural killer.](image)
two methods (p = 0.14), there was a trend for higher EPC counts obtained using Ficoll-Paque, with a mean of 12.64 ± 9.05 × 10^2 cells compared with 8.80 ± 4.90 × 10^2 cells using Lymphoprep. However, this did not reach statistical significance (p = 0.08).

**Lymphocyte subsets: B cells, T cells & NK cells**

Analysis of lymphocyte subsets was performed on ten sets of bone marrow samples. Results showed that there was no significant difference between the total number of lymphocytes isolated using Lymphoprep (21.34 ± 15.75 × 10^6 cells) and Ficoll-Paque (24.00 ± 15.49 × 10^6 cells) (p = 0.37). Moreover, this lack of difference was observed across the various lymphocyte subsets analyzed (Figure 2D). The number of B cells obtained using Lymphoprep compared with Ficoll-Paque was 2.12 ± 1.75 × 10^6 and 2.43 ± 1.89 × 10^6, respectively (p = 0.28). Similarly, T-cell yield using Lymphoprep was 14.08 ± 10.59 × 10^6 cells compared with 15.73 ± 10.09 × 10^6 cells using Ficoll-Paque (p = 0.39). NK cell counts of 3.31 ± 3.00 × 10^6 and 3.52 ± 2.49 × 10^6 were obtained using Lymphoprep and Ficoll-Paque, respectively (p = 0.69).

**RBCs, platelets & granulocytes**

Density gradient centrifugation of bone marrow samples primarily aims to exclude RBCs, platelets and granulocytes from the final mononuclear fraction. Data from 14 sets of bone marrow samples processed using Lymphoprep and Ficoll-Paque showed no significant differences between the numbers of RBCs, platelets and granulocytes present in the isolated cell fractions. The mean number of RBCs detected after Lymphoprep separation was 0.54 ± 0.38 × 10^8 versus 0.55 ± 0.34 × 10^8 following Ficoll-Paque separation (p = 0.81). The total number of platelets present after Lymphoprep and Ficoll-Paque separation was 2.23 ± 1.14 × 10^8 and 2.50 ± 0.91 × 10^8, respectively (p = 0.08). Similarly, 1.21 ± 0.69 × 10^8 granulocytes were found after Lymphoprep separation compared with 1.33 ± 0.76 × 10^8 granulocytes following Ficoll-Paque separation (p = 0.39) (Figure 3).

**Viability analysis**

A total of 12 sets of bone marrow aspirates underwent viability assessment via 7AAD staining as part of EPC enumeration. The mean viability after Lymphoprep preparation was 95.9 ± 3.8% compared with 94.9 ± 4.6% using Ficoll-Paque preparation (Figure 4). This difference was not statistically significant (p = 0.12).

**Discussion**

Our study demonstrates that there is no difference in the absolute numbers and percentage of cell recovery for various mononuclear cell types recovered after processing of bone marrow using Ficoll-Paque or Lymphoprep. In addition, the proportion of various cell phenotypes investigated within the BM-MNC fraction was not significantly different between separation by Ficoll-Paque or Lymphoprep. The two density media are also equally efficient at excluding unrequired RBCs, granulocytes and platelets from the final cell products. Cell viability obtained following processing was not influenced by the type of density gradient media used either.

However, we observed a trend for higher absolute EPC counts and EPC:BM-MNC ratio using Ficoll-Paque separation. EPC enumeration via flow cytometry is an inherent rare-event analysis.
The total number of EPCs is calculated indirectly using flow cytometric beads of a known concentration. Therefore, differences between relatively rare cell events recorded can become artificially amplified when calculating absolute cell numbers. This could account for the apparent skewing of the Ficoll-Paque EPC data shown in Figure 2C and subsequent raised EPC:BM-MNC ratio, which could explain the trends observed. As such, these results should be considered with caution.

It is not unexpected that our analysis has demonstrated that Ficoll-Paque and Lymphoprep are equally effective in isolating BM-MNCs. A comparison of the two density gradient media based on the manufacturer’s data provided revealed that both consist of a polysaccharide solution of 5.7% (w/v) with a density of 1.077 ± 0.001 g/ml. However, although both also include sodium diatrizoate, Lymphoprep with 9.1% (w/v) contains 0.1% more of the compound than Ficoll-Paque, which has 9 g of sodium diatrizoate per 100 ml. Although this difference appears minimal, it is unknown whether such a negligible amount would have an effect on the ability of the media to isolate cells. Another distinguishing feature is that Ficoll-Paque contains 0.0231 g of disodium calcium EDTA per 100 ml, while Lymphoprep does not contain any of this anticoagulant. It is conceivable that the additional anticoagulant effect of EDTA within Ficoll-Paque could improve cell yield following processing owing to a decreased risk of clotting and subsequent encapsulating of the cells within those clots. However, our results indicate that this effect is unlikely to have occurred.

The two largest randomized controlled clinical trials published to date on the use of bone marrow stem/progenitor cells in cardiac repair following acute myocardial infarction have reported conflicting outcomes despite similar trial design and patient characteristics. The REPAIR-AMI trial involving 204 patients investigated the effect of intracoronary infusion of autologous BM-MNCs 3–7 days after reperfusion therapy for acute myocardial infarction [6]. It demonstrated a significant increase in global left ventricular ejection fraction of 5.5% in cell-treated patients compared with 3.0% in placebo-treated patients. On the other hand, patients who received intracoronary autologous BM-MNC infusion had no significant improvement in left ventricular function compared with the nontreated control group in the ASTAMI trial [5]. It has been proposed that the different bone marrow-derived stem/progenitor cell isolation protocols used in these studies may account for the contrasting results [14]. The analysis performed by Seeger et al. included a direct comparison between these protocols – Ficoll-Paque in REPAIR-AMI and Lymphoprep in ASTAMI – and concluded that a significantly higher number of BM-MNCs were obtained using the Ficoll-Paque protocol in REPAIR-AMI as compared with that obtained using the Lymphoprep protocol in ASTAMI. It is important to note that other than the type of density medium used, there were many other key processing steps which differed between the two protocols. These include washing procedures, washing media and cell reconstitution media used and overnight storage conditions – factors that may all affect cell number, viability and functionality.

Our study has demonstrated that the type of density media used is unlikely to cause the differences in cell numbers observed by Seeger et al., and therefore may not account for the contradictory outcomes in the clinical trials. Further investigations should therefore focus on examining each of the other key procedural steps in the protocol systematically to identify the main factors influencing cell numbers obtained. This may be an extremely important factor in clinical trials involving the use of stem cells in any organ/tissue regeneration should a positive outcome be, in part, due to a dose-dependant response.
addition, analysis of any effect on cell function by each of these procedural steps would also be desirable as this is an important factor in stem cell regenerative capability.

As the exact cell type or combination of cell types within the BM-MNC fraction responsible for cardiac functional improvement has not been fully elucidated, it is essential that cell isolation using different types of density gradient media does not affect the number and proportion of various cellular components within the BM-MNC fraction obtained. Our results clearly demonstrate that no such differences are observed between Lymphoprep and Ficoll-Paque and therefore it is safe to assume that either density gradient media would be equally effective when used in clinical trials provided similar protocols are used.

**Conclusion**

In conclusion, we demonstrated that in the case of Lymphoprep and Ficoll-Paque, the type of density gradient media used in the isolation of the BM-MNC fraction for use in clinical trials does not affect the composition and quantity of cell types found within.

**Financial & competing interests disclosure**

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

**Ethical conduct of research**

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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**Executive summary**

- Clinical trials investigating the transplantation of autologous bone marrow-derived mononuclear cell fraction to injured myocardium following acute myocardial infarction have reported conflicting results.
- Different cell isolation protocols for processing bone marrow may account for this difference.
- We compared the effects of two different types of density gradient media, Lymphoprep™ and Ficoll-Paque™, used in the clinical trials on bone marrow-derived mononuclear cells obtained.
- Cellular composition and cell numbers within the bone marrow-derived mononuclear cell fraction is not significantly different.
- Further studies should examine other cell isolation procedural steps to identify the main factors influencing quality and quantity of cell yield.

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**Bibliography**
