Mobilized Peripheral Blood Stem Cells are Pluripotent and Can be Safely Harvested and Stored for Cartilage Repair

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Purpose: The primary objective of this study was to reproduce and validate the harvest, processing and storage of peripheral blood stem cells for a subsequent cartilage repair trial, evaluating safety, reliability, and potential to produce viable, sterile stem cells. **Methods:** Ten healthy subjects (aged 19-44 years) received 3 consecutive daily doses of filgrastim followed by an apheresis harvest of mononuclear cells on a fourth day. In a clean room, the apheresis product was prepared for cryopreservation and processed into 4 mL aliquots. Sterility and qualification testing were performed pre-processing and post-processing at multiple time points out to 2 years. Eight samples were shipped internationally to validate cell transport potential. One sample from all participants was cultured to test proliferative potential with colony forming unit (CFU) assay. Five samples, from 5 participants were tested for differentiation potential, including chondrogenic, adipogenic, osteogenic, endoderm, and ectoderm assays. **Results:** Fresh aliquots contained an average of 532.9 $\pm$ 166. $\times$ 10^6 total viable cells/4 mL vial and 2.1 $\pm$ 1.0 $\times$ 10^6 CD34+ cells/4 mL vial. After processing for cryopreservation, the average cell count decreased to 331.3 $\pm$ 79. $\times$ 10^6 total viable cells /4 mL vial and 1.5 $\pm$ 0.7 $\times$ 10^6 CD34+ cells/4 mL vial CD34+ cells. Preprocessing viability averaged 99% and postprocessing 88%. Viability remained constant after cryopreservation at all subsequent time points. All sterility testing was negative. All samples showed proliferative potential, with average CFU count 301.4 $\pm$ 63.9. All samples were pluripotent. **Conclusions:** Peripheral blood stem cells are pluripotent and can be safely harvested/stored with filgrastim, apheresis, clean-room processing, and cryopreservation. These cells can be stored for 2 years and shipped without loss of viability. **Clinical Relevance:** This method represents an accessible stem cell therapy in development to augment cartilage repair.

Orthopaedic practitioners have begun to augment surgical procedures and treat degenerative conditions with autologous cell therapies, focusing on stem cells, that is, cells with proliferative and differentiation potential.[1-7] Clinical application studies have suggested that success is dependent on the number of stem cells harvested and applied.[2,3,5,6] The number of stem cells harvested by bone marrow aspiration is variable, depending on patient demographics, aspiration technique, and location of harvest.[1-3] Human granulocyte colony–stimulating factor is a natural physiological glycoprotein that normally upregulates the production and release of functional white blood cells from the bone marrow in varying circumstances. Filgrastim (Neupogen; Amgen, Thousand Oaks, CA) is a human granulocyte colony-stimulating factor (G-CSF) produced by recombinant DNA technology. Filgrastim is approved in the United States for mobilization of

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progenitor cells into the peripheral blood for collection by leukapheresis. Pharmaceutical mobilization, with agents such as filgrastim, followed by peripheral harvest of mononuclear cells with apheresis, has supplanted bone marrow aspirate for the hematologic oncologic clinical practice of stem cell transplant because it allows for improved efficiency of harvest and has established safety.[4-6] Mononuclear cell collection from apheresis consists of monocytes and lymphocytes, and unsorted mononuclear cells collected with an apheresis machine from blood mobilized with filgrastim are often semantically referred to as peripheral blood stem cells (PBSC).

In the setting of tissue injury, cells with stem potential are mobilized from the bone marrow through the peripheral circulation to injury sites.[7] Mobilization with filgrastim hyperstimulates this natural process yet produces a mixture of progenitor cells in the peripheral blood which are more immature than cells cultured from bone marrow, that is, traditional mesenchymal stem cells (MSCs).[8-10] Mononuclear cells fractions of peripheral blood capture these immature cells and is the basis of apheresis harvest. Although it has been long established that a fraction of mononuclear cells, the monocytes, can differentiate into lineages of phagocytes, it is also becoming clear that mononuclear cells also have the potential to differentiate into peripheral fibroblasts that participate in wound healing, as well as cells of mesenchymal origin.[11-18] Differentiation is triggered by differences in oxygen tension between tissues as these cells move between environmental niches. A cell population exists within both mobilized and unmobilized peripheral blood as mononuclear cells that contain proliferative and differential potential.[11,12]

Orthopaedic studies have begun to investigate the potential of the PBSC for orthopaedic implications. Benchtop studies have determined that PBSCs, collected as unsorted mononuclear cells fractions from peripheral blood, have similar in vitro orthopaedic potential as MSCs.[11,19,20] These previous benchtop studies have evaluated mononuclear cells from blood mobilized with filgrastim and blood not mobilized with filgrastim. Cartilage repair models in both small and large animals provide pre-clinical evidence that PBSC improve cartilage healing.[11,19] Human clinical studies have followed including case reports, case series, and 1 randomized controlled cartilage repair trial.[19,21-23] We hypothesized that PBSC cells are proliferative and pluripotent and that they and can be stored for 2 years and shipped without loss of viability. The primary objective of this study was to reproduce and validate the harvest, processing, and storage of PBSC for a subsequent cartilage repair trial, evaluating safety, reliability, and potential to produce viable, sterile stem cells. We hypothesized that the established procedures would produce a viable, sterile, and pluripotent stem cell product.

Methods

Institutional Review Board approval was obtained from the governing hospital system before recruitment and enrollment. Healthy female and male volunteers aged 18 to 50 years were recruited with flyers and word of mouth at the medical campus where the study was performed. Samples were harvested from November 2015 to April 2016. Exclusion criteria included a history of prior adverse events with filgrastim administration; a bleeding or clotting disorder; diabetes; autoimmune disorders; disorders requiring immunosuppression; history of cancer; history of myeloproliferative disorder; hepatitis B; hepatitis C; HIV; patients with an ongoing infectious disease or significant cardiovascular, renal, or hepatic disease; abnormality detected on chest radiography; a platelet count less than 100×10^9/L on screening lab; body mass index (BMI) of 35 or above; or volunteers with a weight of 37.5 kg or less. Females were excluded if they currently were pregnant or attempting to conceive during the study period.

A prescreening visit was completed before enrollment. At the visit a medical history was obtained. Physical examination was performed including vital signs, height, and weight. If the volunteer passed the prescreening visit, informed consent was obtained and screening blood tests were performed including tests for HIV, hepatitis B, hepatitis C, as well as the following lab tests: HbA1c, prothrombin time, partial thromboplastin time, a complete blood count with differential, a complete metabolic profile, and a pregnancy test, for females. A chest X-ray film and electrocardiogram were also obtained. The prescreening visit and all tests were reviewed by a board-certified internal medicine physician to confirm the absence of medical contraindications.

After passing the screening process, the participants presented for 3 mobilization visits on 3 consecutive days, followed by a harvest visit on the fourth subsequent day. The mobilization visits included a blood draw for complete blood count with white blood cell count with differential, vital signs, physical exam, review of systems, and a subcutaneous filgrastim injection into the thigh. A 300 μg injection was administered unless the participant weighed over 100 kg, in which case 600 μg of filgrastim was administered. The dosage of filgrastim outlined for this study is below the recommendation of 10μg/kg/day dosage which is the dose approved by the Food and Drug Administration (FDA) for mobilization before harvest for bone marrow transplantation. In addition, the 3-day protocol is less than the recommended 4-day mobilization protocol recommend for bone marrow transplant harvest. This dosage and dosing regimen were based on clinical experience in previous orthopaedic studies using this cell therapy.[22-24]

Cellular Harvest and Processing

Harvest visits took place 24 hours after the third dose of Filgrastim. On the day of harvest, patients had either 2 peripheral intravenous catheters placed into each antecubital vein or a short-term dialysis catheter (Brevia, Bard Access Systems, Salt Lake City, UT) placed into 1 femoral vein. The 2 peripheral catheters or the 2 lumens from the dialysis catheter were connected to an apheresis machine (Optia, Terumo BCT, Lakewood, CO), and 140 mL of PBSC was collected in 1 product bag, and 30 mL of plasma was collected into another product bag. On completion of the apheresis procedure, the PBSC product and autologous plasma bags were sealed, separated from the collection set, and placed into an insulated container for transport to the processing site (Fig 1).

Processing was performed in a current good manufacturing practice compliant clean room facility. On receipt into the onsite processing facility, a sample was first removed from the fresh PBSC for quality control testing including cell count, viability measurement, sterility, and immunophenotype with flow cytometry. The remaining volume of PBSC product was prepared for cryopreservation by the addition of a cryoprotectant solution. The cryoprotectant solution was prepared in an aseptic manner and combined with the PBSC. A second sample was removed at this point for quality control testing. The PBSC/cryoprotectant combination was than separated into cryovials in 4 mL aliquots. Cryopreservation was initiated with a rate controlled programmed freezing protocol and completed with placement into a secure, monitored, catalogued vapor-phase liquid nitrogen storage tank at −196°C.

Flow Cytometry Analysis

Quantification and qualification were performed on the PBSC product initially at preprocessing, immediately after processing, and at the 24 hour, 1 week, 2 week, 4 week, 3 month, 6 month, 1 year, and 2 year time points. Cell sorting was not performed at any time. Testing included an automated hematocytometer (Sysmex XN-9000, Kobe, Japan) for a white blood cell (WBC) count and flow cytometry (Beckman Coulter FC500, Fullerton, CA) for total viable cell counts, CD34+ cell counts, and a viability percentage. Flow cytometry testing involved first washing and diluting based on the sample concentration and then incubation for 30 minutes with a binding inhibitor. After initial incubation, the samples were incubated for 30 minutes with antibodies for CD34+. Standard isotype control samples were used. Aerobic and anaerobic cultures were performed at each time point to test product sterility. Quantification and qualification were also performed on PBSC samples exchanged with the originating, multicenter trial site in Kuala Lumpur, Malaysia. Ten PBSC samples were shipped in a liquid nitrogen vapor phase cryogenic shipping container from the partnering facility in Malaysia to the study facility, and eight PBSC samples were shipped in a temperature-recording, liquid nitrogen vapor phase cryogenic shipping container to the partnering facility from the study facility. Temperature logs were reviewed and the average temperature of the shipment was recorded at −182°C. Transit time was 3 days. Flow cytometry was performed on the exchanged samples.

Colony-Forming Unit Fibroblast Analysis

Proliferative potential was studied with colony forming unit assays, performed by a laboratory manager with 16 years of assay experience (J.T.) and observed by the facility chief scientific officer (H.P.). PBSC vials were removed from the vapor-phase liquid nitrogen storage tank, and placed into a 37°C water bath for 1 to 2 minutes. Although beneficial to preserve the frozen cells, dimethyl sulfoxide (DMSO) is harmful to cells while growing in culture, so it was separated from the PBSC product. Nine milliliters of Gibco Advanced Dulbecco's modified Eagle medium (DMEM)/F12 media (Grand Island, NY) and 1 mL of the PBSC product was transferred to a 15 mL tube. This diluted the DSMC 10 times, that is, a 10× dilution. The 15 mL tube was centrifuged at 200*g* for 5 minutes. The cells formed a pellet and were separated from the supernatant, which isolated the cells from the DMSO. The pellet was resuspended in 4 mL of media (mixture of DMEM/F12, Performance Plus fetal bovine serum, and Pen-Strep). The resuspended cells were placed into 2 to 3 wells of a 6-well plate at 1 million cells per well. Four milliliters of media was added to each well, and the plate was incubated at 37°C. Standard cell culture conditions were used in a humidified atmosphere containing 5% CO_2 in air (~20% O_2). After 24 hours the plates were gently washed with phosphate buffer solution (PBS), and 4 mL media was added, this removed nonadherent cells leaving plastic adherent cells in the culture dish. The plates were returned to the incubator. After 10 days, plates were removed and cells were fixed in ice-cold methanol for 30 minutes at 4°C. The plates were stained with Crystal Violet, washed 4 times with water, and allowed to air dry. Counting was performed, and colonies were counted when over 50 cells were present (Fig 2).

Cellular Differentiation

Differentiation potential was studied with adipogenic, chondrogenic, osteogenic, endoderm, and ectoderm assays, performed by a laboratory manager with 16 years of assay experience (J.T.) and observed by the facility chief scientific officer (H.P.). For each sample, PBSC were resuspended in culture media after the removal of DMSO as outlined above and placed into culture wells. Plating for each sample included a positive well in

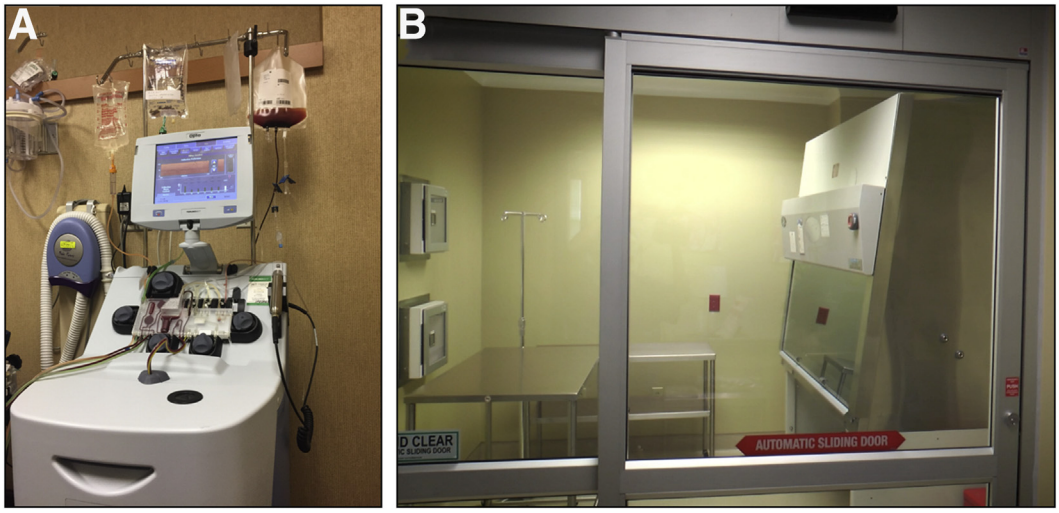


Fig 1. Mobilized peripheral blood stem cells were harvested with an apheresis machine (A) and processed in a clean room (B).

triplicate with differentiation media and a control well without the differentiation media. Confluency of 70% to 80% and a viability greater than 90% was required prior to differentiation procedures. Ectoderm differentiation used a StemPro Neural Differentiation Kit (Thermo Fisher Scientific, Waltham, MA). On day 1, neural induction medium was prepared by mixing 490 mL of neurobasal medium with 10 mL of neural induction supplement and 4 mL was added to each well. One million cells were placed in each well, and the plate was incubated at 37°C with 5% CO_2. Media was changed every 2 days until day 7. On day 7, the cells were rinsed with PBS, and cells were transferred to a conical tube and resuspended in 5 mL of PBS. The sample was centrifuged at 300*g* for 4 minutes. The supernatant was aspirated, and 4 mL of neural expansion medium (a mixture prepared from 49 mL of neurobasal medium, 49 mL of advanced DMEM/F-12, and 2 mL of neural induction supplement) was added, and the cells were incubated for 7 days. Media was changed every 2 days and differentiation concluded at day 14. After completion of the differentiation course, all cultures were washed in PBS and fixed in paraformaldehyde, 4% in PBS (Alfa Aesar, Tewksbury, MA), for 30 minutes at room temperature. Oil Red O isopropanol (Sigma-Aldrich, St. Louis MO) staining was then performed.

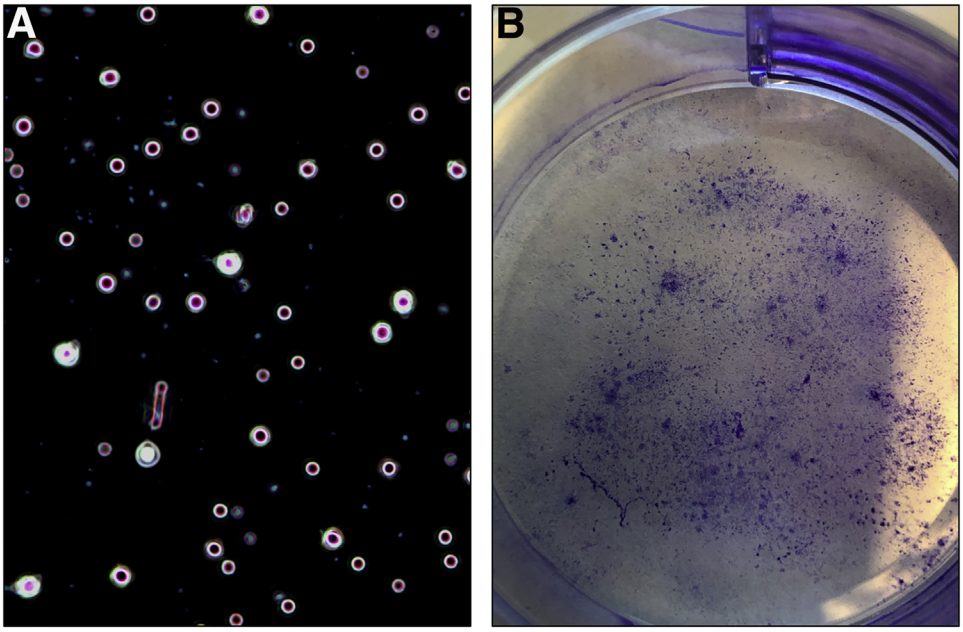


Fig 2. Peripheral blood stem cells were resuspended in culture media (A) after the removal of dimethyl sulfoxide, and plastic adherent cells were cultured in an incubator to quantify colony-forming units (B).

Table 1. Flow Cytometry Results, Mean (SD)

Time	WBC (K/μL)	Total Viable Cell Count ($\times 10^6$/4 mL Vial)	CD34+ ($\times 10^6$/4 mL Vial)	Viability (%)
Fresh	130.6 (40.3)	532.9 (166.6)	2.1 (1.0)	99.2 (0.2)
DMSO	92.8 (28.6)	331.3 (79.4)	1.4 (0.7)	87.6 (7.1)
24 Hours	91.4 (28.2)	296.5 (103.6)	1.2 (0.7)	79.5 (9.2)
1 Week	91.4 (28.2)	287.6 (68.0)	1.2 (0.6)	82.0 (6.4)
2 Weeks	91.4 (28.2)	302.4 (72.6)	1.3 (0.5)	80.5 (7.6)
4 Weeks	91.4 (28.2)	299.7 (78.0)	1.3 (0.6)	80.7 (7.3)
3 Months	91.4 (28.2)	329.0 (72.2)	1.3 (0.6)	83.0 (7.0)
6 Months	91.2 (29.9)	321.5 (92.8)	1.4 (0.7)	83.0 (7.0)
1 Year	91.2 (29.9)	298.8 (58.7)	1.3 (0.7)	81.5 (10.0)
2 Years	91.2 (29.9)	289.5 (92.2)	1.2 (0.7)	77.8 (8.3)

DMSO, dimethyl sulfoxide; SD, standard deviation; WBC, white blood cell count.

Adipogenic differentiation used a StemPro Adipogenesis Kit (Thermo Fisher Scientific), which contains a basal media and adipocyte supplement media. Similar PBSC preparations were prepared as presented above. The adipogenic media was prepared using 90 mL basal media, 10 mL of the supplement media, and 50 μL of gentamicin. The plate was incubated at 37°C with 5% CO_2. Media was changed every 2 days. Wells were incubated for 21 days. After completion of the differentiation course, all cultures were washed in PBS and fixed in paraformaldehyde, 4% in PBS (Alfa Aesar), for 30 minutes at room temperature. Adipogenic staining used Oil Red O isopropanol (Sigma-Aldrich).

Osteogenic differentiation used a StemPro Osteogenesis Kit (Thermo Fisher Scientific) and chondrogenic differentiation used a StemPro Chondrogenesis Kit (Thermo Fisher Scientific). The media was prepared using 90 ml basal media, 10 mL of the supplement media, and 50 μL of gentamicin. The plate was incubated at 37°C with 5% CO_2 for 21 days. After completion of the differentiation course, all cultures were washed in PBS and fixed in paraformaldehyde, 4% in PBS (Alfa Aesar), for 30 minutes at room temperature. Osteogenic staining used an alizarin red powder and chondrogenic staining used an Alcian blue, Millipore stain.

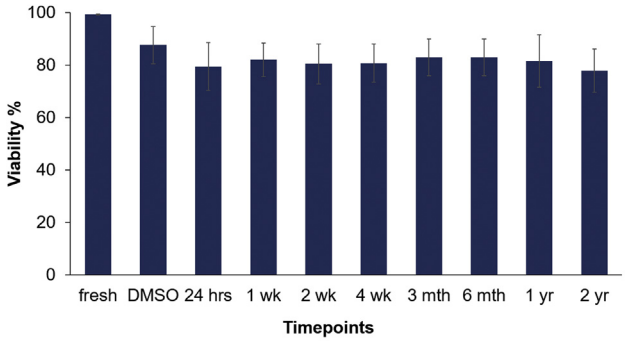
A Gibco Endoderm Induction Assay (Life Technology Corporation, Grand Island, NY) was used for endoderm differentiation; this assay involves serial incubation in 2 medias, DE induction medium A and DE induction medium B. Confluency of 15% to 30% and a viability greater than 90% was required before endoderm differentiation procedures. Plating was performed in 2 mL of an expansion basal media (Essential 8; Thermo Fisher Scientific) and 2 mL of DE induction medium A. Plates were incubated at 37°C with 5% CO_2 for 24 hours. After 24 hours, DE induction medium A was aspirated, and 2 mL of DE induction medium B was added. After an additional 24 hours of incubation, the assayed cells were evaluated.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) software (version 22; SPSS Inc., Chicago, IL). Means and standard deviations were calculated for all variables. Separate within-subjects repeated measures analyses of variance were performed to assess changes in WBC, total viable cell count, percent viability, and CD34+ across the 10 time points (fresh, immediately after processing, and at 24 hours, 1 week, 2 weeks, 4 weeks, 3 months, 6 months, 1 year, and 2 years). If significance was observed, dependent samples *t*-tests were performed to identify the time points where significance occurred. An independent samples *t*-test was performed to assess differences in total cell viability, CD34+, and percent viability of the samples exchanged from the United States site to the international site. An alpha level set a priori at $P \leq .05$ for all analyses.

Results

Twelve volunteers were screened for participation. One participant was excluded on the basis of body mass index, and another participant withdrew after clarification of the time requirements of the study. The final 10 participants included 6 males and 4 females. The average age was 29.5 (range 19-44 years old). The first 4 participants underwent peripheral vein harvest with 2 intravenous antecubital vein catheters. On the first harvest only 100 mL of product was collected, and on the fourth harvest only 80 mL of product was collected. Both of these instances were female participants and involved low flow rates through the peripheral veins indicated by the apheresis machine. The remaining 6 harvests were performed with central venous access using a short-term dialysis catheter, and no further harvest difficulties were encountered. Regarding adverse events, one participant reported flu-like symptoms after the third mobilization day that resolved following apheresis. No adverse events were reported



TP / Values	Fresh	DMSO	24 hrs	1wk	2 wk	4 wk	3 mth	6 mth	1 yr	2 yr
Viability %	99.25	87.65	79.46	82.01	80.47	80.69	82.97	82.96	81.55	77.84
SD	0.16	7.12	9.17	6.41	7.63	7.30	6.96	6.95	9.96	8.29

Fig 3. Bar graph showing average cell viability over time with standard deviation error bar.

Table 2. Comparison of Flow Cytometry Data Following Shipment from United States Test Site to an International Test Site

Participant	WBC (K/μL)	Total Viable Cell Count ($\times 10^6$/4 mL Vial)	CD34+ ($\times 10^6$/4 mL Vial)	Viability (%)
1	101.7	270.8	2.2	66
2	57.6	208.5	1.1	90
3	117	429.8	2.8	79
4	98.1	268	3.9	72
5	74.9	200.7	2.7	72
6	70.8	272.4	1.5	79
7	86.9	227.8	2.6	66
8	122.9	275.7	1.4	60
9	59.5	217	1.3	83
10	49.2	178.6	2.8	87

WBC, white blood cell count.

after the completion of the study. No participants withdrew, and no participant requested that the harvest be halted.

WBC, flow cytometry, and culture data are presented in Table 1. Culture data were limited in the first participant at 1 year and 2 years, and flow/culture data in the fourth participant at 6 months, 1 year, and 2 years because of the incomplete harvests in these participants. One anaerobic culture was lost for participant 3 at the 1-year time point. There were no differences in CD34+ cells harvested or the percentage of viable cells on the basis of sex ($P = .061$ and $P = .927$, respectively). There were no differences observed for the number of CD34+ cells or the percentage of viable cells ($P = .500$ and $P = .964$, respectively) on participant age. No aerobic or anaerobic growth on fresh or cryopreserved sample cultures at time zero (Fresh), PBSC product after DMSO/Plasma, 24 hours, 1 week, 2 weeks, 4 weeks, 3 months, 6 months, 1 year, or 2 years.

Fresh aliquots contained an average of $532.9 \pm 166.6 \times 10^6$ total viable cells /4 mL vial and after processing with DMSO there was a decrease to $331.3 \pm 79.4 \times 10^6$/4 mL vial (Mdiff = 201.6; $P < .001$; 95% confidence interval [CI]: 130.5, 272.7). There was an increase in total viable cells from 4 weeks to 3 months (Mdiff = 29.2; $P = .025$; 95% CI: −53.9, −4.6). At 2 years, total viable cell count was $289.5 \pm 92.2 \times 10^6$ total viable cells/4 mL vial and viability was 77.8% ± 8.3%. After the cells were combined with the cryoprotective medium, DMSO, the percent cell viability decreased from 99% to 88% (Mdiff = 11%, $P = .001$) (Fig 3). WBC concentration decreased after processing with DMSO (Mdiff = 37.8 k/μL; $P < .001$; 95% CI: 27.9, 47.7). There was no difference in concentration of WBC after processing with DMSO to 24 hours ($P = .522$). Fresh aliquots contained an average CD34+ of $2.1 \pm 1.0 \times 10^6$/4 mL vial and decreased to $1.5 \pm 0.7 \times 10^6$/4 mL vial after processing with DMSO ($P = .001$; 95% CI: 0.35, 0.91). A significant decrease in CD34+ was also observed 24 hours after processing with DMSO (Mdiff = 0.20×10^6/4 mL vial; $P = .017$; 95%CI: 0.05, 0.35). Similar results and trend of CD34+ has been observed and reported.[22]

The data from samples shipped to and from the United States test site are presented in Tables 2 and 3. The number of viable cells was similar between samples shipped to and from the United States test site ($P = .059$); however, the percentage of viable cells was greater when samples were shipped from the United States to the international site (MDiff = 8.4%; $P = .039$; 95%CI: −16.2, −0.47). CD34+ was significantly higher in the samples shipped from the international test site to the United States test site (MDiff = 0.98×10^6/4 mL vial; $P = .018$; 95%CI: 0.19, 1.8).

All samples tested with CFU-F assay produced colonies. When evaluating the number of colonies, subjects 1 and 5 were outliers (CFU-F range of 16 to 38) compared to the rest of the samples (CFU-F range, 226-406). If these outliers are included, the mean is 246.5 ± 128.8. If the outliers are removed, the mean is 301.4 ± 63.9.

All 5 samples tested for differentiation potential yielded growth with osteogenic, adipogenic, chondrogenic, endogenic, and ectogenic assays (Fig 4). This confirmed the pluripotent potential of cells from all samples tested. Osteogenic and endogenic samples did not grow as robustly as adipogenic, chondrogenic, and ectogenic.

Discussion

The most important finding of this study was that mobilization with filgrastim and harvest with apheresis produced a peripheral blood mononuclear cell product that is safe, proliferative, and pluripotent, confirming the presence of cells with stem potential, that is, PBSCs. This study reproduced and validated a process for harvest, processing, cryogenic storage, and shipping of PBSC as part of preparations for a multicenter cartilage repair trial. The cell product remained free of bacterial contamination and retained pluripotent stem potential

Table 3. Comparison of Flow Cytometry Data After Shipment from an International Test Site to the United States Test

Participant	WBC (K/μL)	Total Viable Cell Count ($\times 10^6$/4 mL Vial)	CD34+ ($\times 10^6$/4 mL Vial)	Viability (%)
1	UNK	367.4	1.7	89
2	UNK	266.6	0.6	88
3	UNK	335.9	1.3	78
4	UNK	338.8	2.0	81
5	UNK	371.8	0.9	84
6	UNK	213.9	0.6	86
7	UNK	335.1	2.0	80
8	UNK	300.6	0.7	83

UNK, unknown; WBC, white blood cell count.

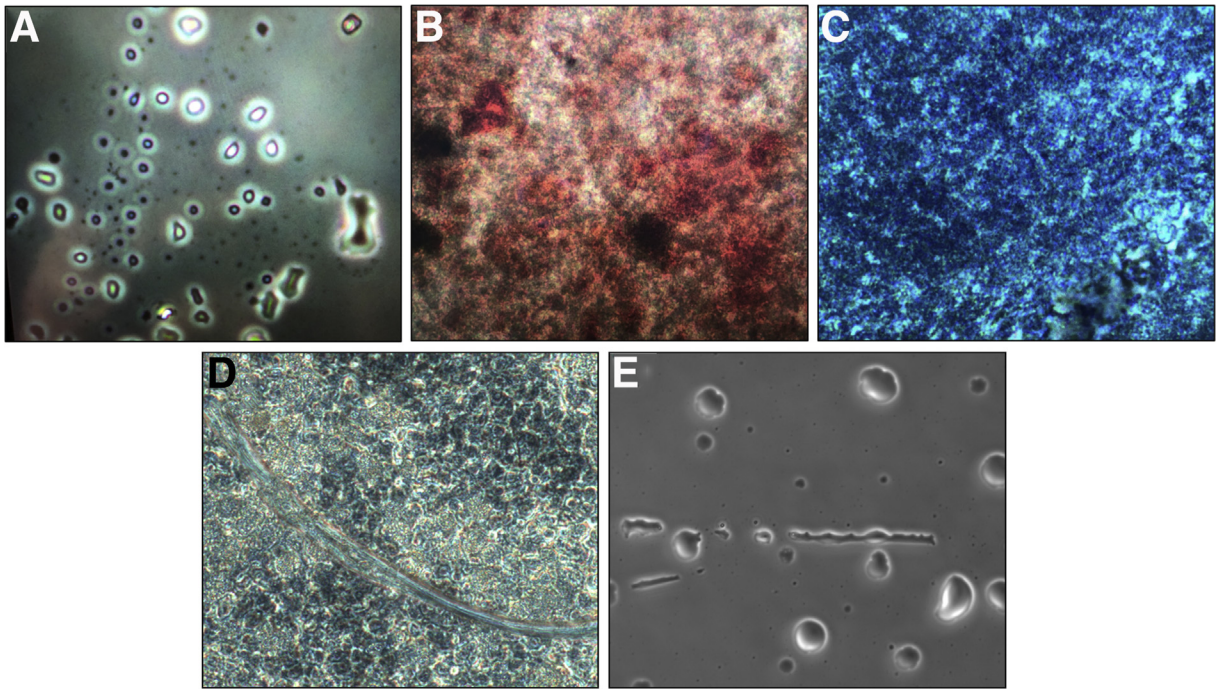


Fig 4. Representative samples from differentiation assays, showing osteogenic (A), adipogenic (B), chondrogenic growth (C), ectoderm (D), and endoderm (E) growth.

out to 2 years. Our findings are similar to previous preclinical and clinical studies regarding PBSCs.[22-24] This study translates PBSCs closer to widespread adoption by validating multicenter reproducibility. Anecdotally, flow rates through peripheral lines were inconsistent in female participants and central line access facilitated harvest for all individuals.

The processing and storage techniques used in this study were first developed to augment arthroscopic cartilage repair at the senior author's (K.S.) facility and involve more than minimal manipulation of human cells.[22,23] Therefore clinical application requires establishing safety and efficacy prior to adoption, and in the United States this requires completion of the FDA Biologics License Application Process. This study was a manufacturing validation study as part of an Investigational New Drug Application in preparation for a multicenter Phase IIb cartilage repair trial, part of the processes toward a Biologics License Application. The 2 manufacturing sites were the senior author's facility (K.S.) and first author's facility (A.A.), both sites of a subsequent multicenter clinical trial. As such, the primary objective of this study was to confirm consistency, viability, and sterility. The hematology oncology profession has used CD34+ for years to evaluate consistency and quantify/qualify bone marrow transplants. CD34+ marker panels are approved by the FDA for this quality control. Although other cell marker panels are available for academic purposes, the FDA has not approved other marker panels for quality control purpose. Although additional maker panels would be of academic value, for practical reasons we did not add additional panels to limit unnecessary costs.

Previously, as part of a cartilage repair study, flow data for PBSCs was reported on 20 consecutive patients.[22] A total of 2.4×10^6/4 mL vial CD34+ cells were present in fresh samples and 1.6×10^6/4 mL vial CD34+ cells were present in frozen samples. Fresh cells demonstrated mean viability of 99% compared to frozen cells of 87%. Similar CD34+ and cell viability values were observed in our study. The hematology/oncology literature provides study for comparison of cell counts in apheresis harvest and is summarized in Table 4.[25-29] CD34+ counts are variable, and the results from both sites fall within the published ranges. Although viability was similar between the 2 manufacturing sites, some variability is expected because of differences in personnel at sites, facilities, equipment, reagents of flow cytometry, and transit time for the exchanged samples. Effective viability and cell count for effective augmentation of orthopaedic indications requires further development. A potential avenue of further study should highlight other cell markers that may be of benefit to orthopaedics as our knowledge within this specific field advances.

Prior preclinical studies have isolated and described PBSCs from both unmobilized and mobilized peripheral blood. Cesselli et al.[8] compared human cultured cells from bone marrow (MSCs) to human PBSCs from mobilized blood collected with apheresis. PBSCs proved more immature than MSCs and were pluripotent as

Table 4. CD34+ Count of Normal Donors Compared to specimens tested from both sites

Source of Data	Calculated CD34+ × 10^6/4 mL		
	Minimum	Maximum	Mean
Korbling et al.,[27] Fresh cells	0.20	0.29	0.25
Anderlini et al.,[25] Fresh cells	0.016	0.44	0.14
Ings et al.,[26] Fresh cells	M: 0.007 F: 0.008	M: 0.49 F: 0.37	M: 0.12 F: 0.11
Martino et al.,[28] Fresh cells	0.10	0.79	0.17
McCullough et al.,[29] Frozen cells	0.24	1.60	0.92
Specimen from International site, Frozen cells	1.1	3.9	2.2
Specimen to international site	0.6	1.7	1.2

M, male; F, female.

opposed to multipotent. Injected subcutaneously into immunocompromised mice, PBSC migrated to distant tissues and integrated into new tissue, acquiring the identity of resident cells. This current study is similar to the evaluation by Cesselli et al.[8] in that it evaluated mobilized blood harvested via apheresis and found PBSC to be pluripotent and proliferative. In addition to PBSC from mobilized blood, PBSC harvested from unmobilized blood have been evaluated for stem potential and compared to mononuclear cells from bone marrow (MSCs).[20] Cells from both sources adhered to plastic, produced colonies in culture, and showed trilineage differentiation which indicated the cells were multipotent.[20] On culture, cells from both sources did not express CD34+ and CD45 but did express CD105, CD166, and CD29. Chondrogenic assays showed equal potential between the two sources. Similarly, the PBSC in this study yielded growth with a chondrogenic assay.

Preclinical cartilage repair models in animals have compared PBSCs to MSCs. In a rabbit model, Fu et al[30] compared cells harvested from mobilized blood without apheresis to cells harvested from bone marrow with culturing and flow cytometry.[30] Morphologic examination, surface marker testing, and in vitro multipotentiality were similar except that the bone marrow cells proved more osteogenic/proliferative and the peripheral blood cells more chondrogenic and adipogenic. Cells from the 2 sources performed similarly well when compounded with decalcified bone matrix and implanted into a cartilage defect, simulating cartilage repair surgery. Similarly, the results of the current study also found PBSC to be more chondrogenic and adipogenic than osteogenic. A second preclinical study for comparison evaluated the multipotentiality and cartilage repair potential of human mononuclear cells from unmobilized blood in a large animal model.[11] The fresh mononuclear cells were positive for markers similar to hematopoietic stem cells such as CD34+, whereas culture of these cells in a hypoxic environment caused them to shed their hematopoietic stem cells markers and express markers consistent with an MSC phenotype. Low oxygen tension caused a transformation of the cells from the peripheral blood to behavior similar to cells from the bone marrow. These findings support the assertion that cells with stem potential are mobilized from the bone marrow in settings of injury and change their cell markers as they move between environments. This also supports the use of CD 34+ as a cell marker for quality control without the necessity of additional panels. A biphasic collagen-glycosaminoglycan was loaded with either cultured mononuclear cells from peripheral blood or bone marrow, and the authors concluded that mononuclear cells from peripheral blood support cartilage healing as well as mononuclear cells from bone marrow.

Clinical trials involving mobilization with filgrastim and apheresis harvest in healthy PBSC donors have proven this to be a safe and tolerated process. In 1 study, 126 patients received filgrastim for mobilization.[31] Adverse events consisted primarily of mild-to-moderate musculoskeletal symptoms reported in 44% of patients. These symptoms were predominantly events of medullary bone pain (33%). Headache was reported in 7% of patients.[31] Information about the long-term follow-up and safety is available from registry data.[32] The Spanish National Donor Registry was developed to record the short- and long-term results of pharmaceutical mobilization and apheresis harvest in normal donors, with data on 736 donors, with 320 donors followed up for 2 years or more.[32] Bone pain (90%) and headache (33%) were the most frequently reported side effects. Changes in blood counts were minimal and mainly affected WBC counts, which returned to normal values within 2 years after mobilization. No patient developed a hematologic malignancy.[32] Additional studies have agreed that the standard regimen of 10 μg/kg/day is safe when administrated to normal subjects with the most common adverse reactions involving bone pain, pyrexia, and headache.[26,33,34] Our study demonstrates that the clinical application of filgrastim is practical with participants tolerating the procedure and harvest without complications with additional benefits of cellular stem potential. Regardless of harvest site, all participants denied significant discomfort, and no volunteers requested the harvest be stopped.

Human clinical studies have evaluated PBSC in the setting of cartilage repair, including case reports, case series, and one randomized controlled trial.[19,21-23] Saw et al.[22] first reported a case series involving arthroscopic marrow stimulation followed by multiple postoperative intra-articular injections in 5 patients, with safety data and histology suggesting good cartilage repair tissue. Development continued with a randomized control trial comparing arthroscopic marrow stimulation followed by 8 postoperative PBSC intra-articular injections over the course of 6 months compared to arthroscopic marrow stimulation followed by 8 postoperative hyaluronic acid

intra-articular injections.[23] A case series combining the cartilage procedure with high tibial osteotomy has also been published, and repair cartilage in this combination procedure when graded with ICRS scoring system approached 95% of a normal articular cartilage score.[21] Similar encouraging results have been seen in two additional case series involving PBSC and one comparative study of open implantation of PBSC to BMC.[19,35,36] The findings of this study demonstrate that the harvesting process is safe and storage capabilities can extend for years to provide a vast array of treatment options for the patient. Further research is needed to determine the threshold of PBSCs and percentage of viable cells that are clinically meaningful for cartilage repair.

One component of the process for consideration is the need for a central line and cost. The most common risks of insertion of femoral venous access include infection, bleeding, vascular injury, hematoma formation, and damage to nearby nerves, arteries, or muscles. Other risks, although rare, include pseudoaneurysm formation, bowel penetration, bladder puncture, psoas abscess, septic arthritis, arrhythmia, deep vein thrombosis, and venous air embolism. The critical care medical literature has evaluated the safety of central lines in the critically ill determining that the incidence of central venous catheter infection and colonization is low overall and that mobilization of these patients with physical therapy carries a low risk of mechanical or thrombotic complications.[37,38] We encountered no adverse events with the central venous access. Although peripheral venous access is suitable for stem cell harvest via apheresis, some series have documented the need for central venous access in 20% of volunteers.[39] We attribute the higher incidence to a more variable population than those typically volunteering for stem cell harvest for oncology purposes. Hypocalcemia is a risk during apheresis. This can cause muscle spasms, tetany, laryngospasms, and the potential for cardiac arrhythmias. This risk is mitigated by supplementation of calcium before and during apheresis. Cost is also a limiting factor to widespread use of mobilization, harvest, and storage of PBSCs for orthopaedic indications. In the United States, Filgrastim is $1.1 dollars per microgram. Mobilization for this study required 10 μg/kg as a dosage and four doses. For the average 70 kg individual, this translates into $3080.

Limitations

Limitations of this study include the limited flow cytometry cell marker panel, that only assessed CD34+. Other marker panels were not performed to limit unnecessary costs. A second limitation is that our differentiation assays did not allow for quantification of our finding. Our methods allowed for either the presence of cell differentiation or the absence of cell differentiation.

Conclusion

Peripheral blood stem cells are pluripotent and can be safely harvested/stored with filgrastim, apheresis, clean-room processing, and cryopreservation. These cells can be stored for 2 years and shipped without loss of viability.

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