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Development and validation of a procedure to isolate viable bone marrow cells from the vertebrae of cadaveric organ donors for composite organ grafting

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Abstract

Background aims—Donor-derived vertebral bone marrow (BM) has been proposed to promote chimerism in solid organ transplantation with cadaveric organs. Reports of successful weaning from immunosuppression in patients receiving directed donor transplants in combination with donor BM or blood cells and novel peri-transplant immunosuppression has renewed interest in implementing similar protocols with cadaveric organs.

Methods—We performed six pre-clinical full-scale separations to adapt vertebral BM preparations to a good manufacturing practice (GMP) environment. Vertebral bodies L4–T8 were transported to a class 10 000 clean room, cleaned of soft tissue, divided and crushed in a prototype bone grinder. Bone fragments were irrigated with medium containing saline, albumin, DNase and gentamicin, and strained through stainless steel sieves. Additional cells were eluted after two rounds of agitation using a prototype BM tumbler.

Results—The majority of recovered cells ($70.9 \pm 14.1\%$, mean $\pm$ SD) were eluted directly from the crushed bone, whereas 22.3% and 5.9% were eluted after the first and second rounds of tumbling, respectively. Cells were pooled and filtered (500, 200 μ m) using a BM collection kit. Larger lumbar vertebrae yielded about 1.6 times the cells of thoracic vertebrae. The average product yielded $5.2 \pm 1.2 \times 10^{10}$ total cells, $6.2 \pm 2.2 \times 10^8$ of which were CD45⁺ CD34⁺. Viability

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was $96.6 \pm 1.9\%$ and $99.1 \pm 0.8\%$, respectively. Multicolor flow cytometry revealed distinct populations of CD34⁺ CD90⁺ CD117^{dim} hematopoietic stem cells ($15.5 \pm 7.5\%$ of the CD34⁺ cells) and CD45⁻ CD73⁺ CD105⁺ mesenchymal stromal cells ($0.04 \pm 0.04\%$ of the total cells).

Conclusions—This procedure can be used to prepare clinical-grade cells suitable for use in human allotransplantation in a GMP environment.

Keywords

good manufacturing practice; organ transplantation; vertebral bone marrow

Introduction

Cadaveric bone marrow (BM) was first proposed as a source of hematopoietic progenitor cells for transplantation in the 1960s (1). In the early 1980s, the US Navy Tissue Bank initiated a cadaveric BM procurement and cryopreservation program and developed methods for harvesting vertebral bodies (VB) for the large-scale preparation of BM cells (2). Our team made use of this resource for pre-clinical validation of one of the earliest protocols to deplete allogeneic BM of lymphocytes for prevention of graft-versus-host disease (3). After the discovery that small numbers of immune cells of donor origin persist in the circulation of long-term surviving recipients of solid organ transplants (4,5), the Starzl group at the University of Pittsburgh (Pittsburgh, PA, USA) initiated a program to promote graft tolerance through infusion of vertebral BM harvested from the organ donor (6). This approach was applied to a variety of organ transplant types with varying success (6–11). An independent meta-analysis of 21 published manuscripts (12) found that microchimerism was detected in the majority of patients, and the effect of microchimerism and BM infusion on rejection episodes was time-dependent and varied with the transplanted organ. The major conclusions drawn by the Pittsburgh group were: (i) the prime window for induction of graft tolerance (i.e. the clonal exhaustion–deletion of alloreactive immune cells) is in the early post-grafting period (13); and (ii) this is best accomplished by infusion of donor BM in the peri-transplant period under the coverage of bolus immunosuppression with potent agents such as single-dose Campath 1H and tapering doses of tacrolimus, with the ultimate goal of achieving a low-dose maintenance regimen, or weaning the patient off immunosuppression altogether. This approach has come to be known as the Pittsburgh Protocol. Reducing chronic immunosuppressive therapy and its attendant adverse effects is highly desirable for all indications of allotransplantation, but is particularly important for applications such as hand and face transplantation, which have the potential to enhance quality of life greatly but are not lifesaving. In anticipation of performing hand transplants in conjunction with cadaveric vertebral BM infusion and the Pittsburgh Protocol, we sought to develop and validate a preparative procedure that could be implemented according to United States Food and Drug Administration (FDA) current good manufacturing practices (cGMP). We present a detailed procedure for harvesting VB and stabilizing the vertebral column, a procedure for preparation of a BM product from VB, and the outcome of BM cell isolation with regard to cell yield, cell types, viability, sterility, relation to body weight and sample hold-time. We also discuss multiple issues that arose during protocol development and their resolution.

Methods

Donors

Approval for retrieval of vertebrae from six brain-dead organ donors was granted by the Center of Organ Recovery and Education (CORE), the local organ procurement organization and the University of Pittsburgh Committee for Oversight on Research Involving the Dead (CORID). The inclusion criteria for donor selection were brain death and age between 18 and 65 years.

VB harvest

VB harvest was initiated after cross-clamping, cold flush of organs with Custodiol preservation solution (Essential Pharmaceuticals, Newtown, PA, USA) and thoracic and abdominal organ retrieval. Both iliopsoas muscles were transected at their insertion at the vertebrae. The soft tissue was then removed carefully from the VB using a Versajet system (Smith & Nephews, London, UK). The Versajet produces and then aspirates a thin high-velocity stream of saline and is commonly used for wound debridement. Nine vertebrae [thoracic (T)8–lumbar (L)4] were cleared from the surrounding connective tissue using this technique, exposing the angle between VB and lumbar or thoracic processes/ribs. Intervertebral discs were then incised between L4 and L5, L2 and L1, T11 and T10 and T8 and T7. Using an oscillating saw with a blade mounted at 90° angle to the handle, the vertebral segments of L4–L2 were incised laterally to disconnect VB from processes and the vertebral arch. The spinal cord as well as the remaining portions of the vertebrae (vertebral arches, transverse process and spinal processes) remained in the donor. VB segments were retrieved in groups of three (L4–L2, L1–T11 and T10–T8), rinsed with a saline-betadine (1:1) solution and double-bagged in plastic bags suited for organ transportation with 2 L Custodiol preservation solution, such that the inside bag remained sterile. The bagged VB were labeled and stored in a Styrofoam cooler on wet ice for transportation. In the first three cases, VB were collected after the field had been contaminated by injury of the colon during excision of the bladder and/or prostate. In the subsequent three cases VB were retrieved prior to gross contamination of the Operating Room (OR) field. Immediately after retrieval and packing, VB were stored on ice in a plastic container at a temperature of 1–4°C for 33 ± 12 h (mean ± SD) before processing.

Isolation of BM cells

The isolation of BM cells was performed at the University of Pittsburgh School of Medicine (UPMC, Pittsburgh, PA, USA) Hematopoietic Stem Cell Laboratory, a foundation for Accreditation of Cellular Therapy (FACT)-accredited good manufacturing practice (GMP)-compliant facility equipped with a class 10 000 clean room. After aseptic unpacking in a class II biologic safety cabinet outside the clean room, VB were immersed in sodium hypochlorite (0.6% in normal saline) or hydrogen peroxide (3% in normal saline) for 5 min followed by immersion in sterile saline. VB were then transferred to the clean room, where intervertebral disks and remaining soft tissue were removed. The VB were cut into 1–2-cm³ cubes using a mallet, osteotome and stainless steel ratchet pruner (catalog number RP-902; Florian Tools, Southington, CT, USA) (Figure 1A). Bone fragments were irrigated with medium containing normal saline, albumin (0.5 g/dL), DNase (350 U/mL; Pulmozyme,

Genentech, San Francisco, CA, USA), MgCl_2 (2.5 mM), sodium heparin (10 U/mL) and gentamicin (50 $\mu\text{g/mL}$), and crushed in a prototype instrument designed to crush bone cubes (bone grinder, Biorep Technology, Miami, FL, USA) housed in a class II biosafety cabinet (Figure 1B). The crushed bone was strained serially through stainless steel sieves (W. S. Tyler, St Catharines, ON, USA; 425 μm , 180 μm), rinsed with medium and the cell-rich filtrate collected (Figure 1C). Rinsed bone fragments were resuspended in 250 mL medium and tumbled for 20 min using a prototype BM tumbler (Biorep Technology) (Figure 1D). After tumbling the bone fragments were sieved and the strained cell-rich filtrate pooled. The tumbling steps were repeated and all cells collected from a total of three washes were pooled and filtered (500 μm , 200 μm) using a BM collection filter set (4R-21-07; Baxter, Deerfield, IL, USA). Thereafter the product was processed using conventional clinical laboratory methods. The filtered product was transferred in 600-mL transfer pack bags and centrifuged at $700 \times g$ at ambient temperature, and resuspended to 140 mL in saline 5% human serum albumin.

Cell counts

Nucleated cell counts were obtained using a (Beckman Coulter, Fullerton, CA, USA) AcT Diff 2 hematology analyzer, according to the manufacturer's instructions. These were made separately on cells recovered at each step of processing, from each individual VB segment. The final cell recovery results (Table II) were based on absolute counts of CD45^+ cells made after cells recovered from the wash steps and individual VB segments were pooled.

Flow cytometry for enumeration of CD45^+ and CD34^+ cells

Flow cytometry was performed using a commercially available single-platform lyse/no wash assay (Stem-Kit; Beckman Coulter) according to the manufacturer's instructions. Stem-Kit measures absolute numbers of CD45^+ and CD34^+ cells by comparison of the number of measured events with that of an internal bead standard of known concentration. Viability is measured by exclusion of 7-aminoactinomycin D (7-AAD). Data were acquired on a four-color Beckman Coulter Epics XL cytometer, which was calibrated daily with Flow-Check and Flow-Set beads (Beckman Coulter). CD34 reference cells (low, normal and high controls) (Streck Laboratories, Omaha, NE, USA) were run with each sample as a positive control of known CD34 content. Data were acquired immediately and analyzed in real time using System II software (Beckman Coulter) and a modification of the Sutherland method (14,15).

Flow cytometry for characterization of BM progenitor cell subsets and mesenchymal stromal cells

The mononuclear cell pellet from Ficoll/Hypaque-separated BM was incubated with mouse serum (2 μL) for 5 min at room temperature to block non-specific antibody binding. Cells were then stained with monoclonal antibodies against CD105 –fluorescein isothiocyanate (FITC; Fitzgerald Industries Int., Concorde, MA, USA), CD73 –Phycoerythrin (PE), CD45 –Allophycocyanine-Cyanine7 (APC-Cy7) (BD Biosciences, San Jose, CA USA), CD34 –Energy Coupled Dye (ECD) (Phycoerythrin - Texas Red), CD90 –PE-Cy5, CD117 –PE-Cy7 (BeckmanCoulter) and CD133 –Allophycocyanine (APC) (Miltenyi Biotec Inc., Auburn,

CA USA) for 20 min on ice. Following staining, the cells were washed and fixed for 20 min with 2% methanol-free formaldehyde and permeabilized with saponin (phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA), 0.1% saponin) for 10 min at room temperature. Prior to acquisition, 1.6 μL 4',6-diamidino-2-phenylindole (DAPI) (200 $\mu\text{g}/\text{mL}$) were added per 10^6 cells, as a marker of nucleated cells. Cells with less than diploid DNA content were excluded from the analysis. Five to 10 million cells per sample were acquired on a Beckman Coulter CyAn ADP cytometer and the data were compensated spectrally and analyzed offline using VenturiOne analytical software (Applied Cytometry Systems, Sheffield, UK).

Statistics

Analysis of variance (ANOVA) was used to compare cell yields from different vertebral segments, with Bonferonni correction for multiple contrasts. Linear regression was used to evaluate the correlations between cell yield and demographic parameters (donor age, height and weight). All analyzes were performed and graphs created using SYSTAT software version 12 (16).

Sterility cultures

Sterility cultures were performed by the UPMC Microbiology Laboratory at (Shadyside Hospital, Pittsburgh, PA 15213). Aerobic and anaerobic bacterial cultures (BACTEC; BD-Diagnostic Systems, Sparks, MD 21152) and inhibitory mold agar slants (BBL; BD-Diagnostic) were incubated until positive, or discarded as negative after 14 days. Positive cultures were evaluated further and an antibiogram performed.

Results

Procurement of VB

The first case revealed several practical problems to be resolved.

- A satisfactory method was needed to stabilize the cadaver physically after removal of nine VB.
- Because VB procurement followed harvest of thoracic and abdominal organs as well as prostate for a research trial, the surgical field was contaminated when the colon was injured during prostate procurement.
- Soft tissue attached to the VB tended to clog the bone grinder used for bone crushing in the cell-processing laboratory.

These problems were solved during subsequent cases as follows. The insertion of a length of $\frac{3}{4}$ -inch polyvinylchloride schedule 40 pipe to replace the harvested vertebrae resulted in full stability of the vertebral column (Figure 2). The pipe was cut approximately 3 inches longer than the space occupied by the VB, notched at both ends and inserted with gentle traction on the legs.

The abdominal organs were retrieved 'on block' without contamination of the abdominal cavity. The VB were also swabbed with betadine upon removal, and gentamicin (50 $\mu\text{g}/\text{mL}$)

was added to the transport medium after the first 3 cases. In the cell-processing laboratory a procedure (described below) was developed to decontaminate the surface of the VB immediately prior to processing.

The Versajet abrasion system proved useful for rapid and complete removal of the soft tissue surrounding the VB. Clearing the angle between the VB and appendages and incision of the VB from the lateral aspect allowed for precise dissection of the VB at the insertion of the vertebral arch. Further debridement at the back table of the OR helped to reduce processing time and prevent clogging of the bone-crushing apparatus.

Cell isolation

The bone grinder and BM tumbler were constructed by Biorep Inc. in consultation with the University of Miami Transplant Program (Director C. Ricordi; Miami, FL, USA). They were supplied as prototypes without instructions or assurance concerning suitability for the present use. In addition to the soft tissue issue described above, the first cases provided the opportunity to resolve the following issues:

- promoting good tissue practice (GTP) consciousness, i.e. preventing the transmission of disease through proper documentation of infectious disease and behavioral markers
- harmonizing open-bench surgical practices and closed-system laboratory practices for maintaining aseptic techniques
- how to ensure surface sterility of VB prior to processing in the GMP clean room environment
- determining the best instruments to divide the VB and the optimal size for processing in the bone-crushing apparatus
- determining the number of cycles required to tumble and rinse the bone fragments in order to maximize cell yield
- determining the yield per VB and comparing yields of thoracic and lumbar VB
- the effect of donor age and size on total cell yield and CD34 content
- the mesenchymal stromal cell (MSC) and endothelial stem cell content of the product.

GTP was instituted by the FDA and codified in 21 CFR 1271, subpart D, to protect patients from the introduction, transmission and spread of communicable diseases through human cell- and tissue-based products. Provisions include rules for product labeling, and donor screening and testing. Because we plan to store cryopreserved VB products in Liquid Nitrogen (LN₂) liquid phase with other cellular products that are subject to the most stringent GTP regulations, it was imperative to ensure that the laboratory receives complete donor infectious disease marker information prior to or concurrent with sample accession, and to enforce labeling requirements (donor name, United Network for Organ Sharing (UNOS) identifier, donor ABO/Rh, collection date and time, medium, additives, intended

recipient name, intended recipient social security and medical record numbers, ‘do not X-ray, place near heat or delay delivery’, ‘store at 0–6°C’).

A ‘cultural’ issue, harmonizing surgical and laboratory methods of aseptic technique, required practice on the part of the surgical team performing the VB dissection and the laboratory staff, who worked side-by-side in a class 10 000 clean room. VB dissection, bone crushing, tumbling and sieving were performed using typical operating-room aseptic techniques, in which the operator wears sterile gloves and gown, operates within a sterile field, and avoids touching non-sterile objects. Cell processing was performed by experienced clinical cell-processing specialists who, although gowned and gloved, practiced clinical laboratory aseptic techniques, in which the technician avoids touching anything in the sterile field, performs manipulations in a biologic safety cabinet wherever possible, and processes cell suspensions in a closed system. These six training cases provided a valuable rehearsal in which the interactions between the two teams could be choreographed.

After the soft tissue and disks were removed from the VB, they had to be divided into several pieces before they could be processed in the bone grinder. This was accomplished by making initial divisions with an osteotome and mallet, a procedure that is difficult to control. VB were then cut into neat cubes (1–2 cm³; Figure 1A) with a stainless steel ratchet pruner (Florian Tools), which we found could be repeatedly autoclaved. Florian Tools is in the process of prototyping shears with a larger jaw and larger handles, to accommodate an intact VB.

We tested two methods to ensure surface sterility, immersion in 10% household bleach (0.6% sodium hypochlorite) and immersion in 3% hydrogen peroxide. Both solutions were diluted in normal saline, and both treatments were performed for 5 min. As nine VB were collected in groups of three, one group was exposed to bleach ($n = 3$), one to peroxide ($n = 4$) and one to saline only ($n = 8$). The VB groups were then processed separately so that the cell viability of the final products could be compared.

Neither treatment adversely affected the viability of recovered BM cells (Table I), but there was no evidence that either was effective for decontamination. The VB segments from the first three cases were contaminated in the OR with typical bowel (*Escherichia coli*, *Enterococcus faecalis*) and skin (*Propionibacterium* sp.) organisms. When prostate recovery was completed after VB to avoid contamination, VB were sterile on delivery to the laboratory and the product remained sterile for the remaining three cases.

Optimal cell dosing in the context of composite organ grafting has not been established. In the absence of data, we wished to develop a procedure capable of routinely providing a cellular product sufficient for hematopoietic engraftment (at least 2×10^6 CD34⁺ cells/kg recipient ideal body weight) (17). Although there is no reason to assume a priori that CD34⁺ cells are required for prevention of solid organ rejection, the viable CD34 count provides an excellent surrogate for the quality of a BM product, and therefore a reasonable metric for comparison between VB-derived products. We therefore asked two related questions: what is the optimal number of cycles for rinsing and tumbling the bone fragments produced by the bone grinder, and what is the cell yield per vertebral segment?

Figure 3 shows the cell yield after initial rinsing (sieving) of the bone fragments produced by the bone grinder and after two subsequent steps of tumbling and rinsing. More than two-thirds of nucleated cells ($70.9 \pm 14.1\%$, mean $\pm$ SD) were washed out from the crushed bone fragments without any manipulation, whereas $22.3 \pm 11.7\%$ and $5.9 \pm 2.9\%$ were eluted after the first and second cycles of tumbling, respectively. We concluded that two cycles of tumbling were sufficient.

Figure 4 shows the CD34 recovery from each segment, expressed as a proportion of the total CD34⁺ cells recovered in each product. The yield increased progressively from the smaller thoracic to the larger lumbar vertebrae. Overall, lower lumbar vertebrae yielded about 1.6 times the cells of thoracic vertebrae. This was not surprising and correlated with the difference in size and weight.

The pooled cell yield (wash steps and segments) is shown in Table II. The white blood count (WBC) yield per product was $5.2 \pm 1.2 \times 10^{10}$ cells (mean $\pm$ SD), with $96.6 \pm 1.9\%$ viability. The CD34 yield was $6.2 \pm 2.2 \times 10^8$ with $99.1 \pm 0.8\%$ viability, an amount sufficient to provide a graft dose of 8.8×10^6 CD34/kg to a 70-kg recipient. Although there was a graphical trend for CD34 yield to decrease with age, this relationship was not statistically significant ($P = 0.29$, linear regression). Neither donor height nor weight was a predictor of cell yield or viability.

The mean hold-time between VB harvest and processing was $27:52 \pm 11:52$ (h:min, mean $\pm$ SD), and was uncorrelated with cell yield or cell viability, showing that ischemia times of up to 40 h were acceptable for VB retrieval and BM cell isolation.

Detailed flow cytometric characterization of VB mononuclear cells

The arithmetic mean values and percentages for the entire series are tabulated directly on Figure 5. A mean of 5.94 million events was acquired. Sources of artifacts, such as cell doublets and clusters, hypo-diploid (apoptotic) cells, autofluorescent cells and subcellular debris, interfere with detection of rare events (18,19) and were eliminated from the analysis. Clean non-autofluorescent singlet events with DNA content $2N$ represented $75.6 \pm 13.3\%$ of total events acquired. Detailed analysis of CD45⁺ CD34⁺ hematopoietic cells revealed that the majority co-expressed CD133 and CD117, with a distinct subpopulation of primitive CD90⁺ CD117^{dim} cells. A subset representing 0.8% of CD45⁺ CD34⁺ cells co-expressed mesenchymal markers CD73 and CD105, whereas MSC, characterized as CD45⁻ CD73⁺ CD105⁺, represented 0.68% of CD45⁻ cells and $0.04 \pm 0.04\%$ of total clean events. The majority of MSC co-expressed CD90.

Discussion

Minimization of long-term immunosuppression through the induction of donor-specific tolerance is a primary goal in solid organ and composite tissue allotransplantation. Donor-derived BM products (6), mobilized hematopoietic stem cells products (20) and culture-expanded allogeneic MSC (21) have been proposed to promote graft tolerance. Vertebrae are known to be an excellent source of BM. Cells isolated from cadaveric vertebrae have been characterized as a rich source of hematopoietic progenitor cells (22) and MSC (23).

The only disadvantages to using donor vertebrae as a source of tolerogenic cells is that the cadaver is unstable after removal of the vertebrae, and vertebrae require significant physical manipulation for cell isolation. To address the first issue, we developed a modified surgical procedure that allows for dissection of VB while leaving the vertebral arches and appendages *in situ*. This procedure, together with a simple method of inserting a scaffold for the resected VB (Figure 2), represents a simple and safe method for the retrieval of donor BM.

Having retrieved VB, we sought to develop a method to ensure the sterility of the cellular product derived from them. In our first three attempts, VB were contaminated with gut and skin flora in the operating room (Table I). We attempted to decontaminate the products by a short immersion in bleach or hydrogen peroxide. Although this procedure was unsuccessful in sterilizing the product, there is a suggestion that it greatly reduced the level of contamination, as treated, but not untreated, products were successfully used for *in vitro* experiments under penicillin, gentamicin coverage and were injected into immunodeficient mice without detectable untoward effects (data not shown). As neither cell recovery nor viability was affected by VB decontamination, future experiments will determine the efficacy of longer VB immersion and stronger disinfectant. Importantly, the improved technique in the operating room for the last three cases resulted in sterile products that did not require disinfection.

Performing cell isolation in accordance with GMP required practice and close collaboration between the surgical and cell processing teams. The exacting requirements for sample acceptance initially seemed superfluous to our surgical team. They questioned the importance of timely arrival of infectious disease marker data considering that, in the planned clinical protocol, the patient would be receiving a hand transplant from the same donor 2 weeks prior to infusion of the VB marrow product. They also reasoned that detailed labeling was unnecessary as we would never process more than one product on any given day. However, our surgical team complied readily once they understood that the laboratory is legally and ethically bound to uphold these regulatory requirements and that deviations could affect the status of other cryopreserved products in their custody. Much of the initial processing was performed on the clean room bench top, including division of VB before processing, and rinsing and sieving of crushed bone fragments. The actual bone crushing and post-sieving processing was performed in a class II biosafety cabinet.

Cell recovery was excellent, with an average WBC recovery of 5.2×10^{10} cells/product and viability in excess of 96%, despite a lag time between VB harvest and processing of up to 40 h. Products had hematopoietic stem and progenitors, and MSC content, consistent with that seen in freshly isolated BM aspirates. Although both cell number and viability would predictably be lower after volume reduction, cryopreservation and thawing, the pre-cryopreservation CD34 dose of lowest yield product in this series would have been $4 \times 10^6/\text{kg}$ (assuming a 70-kg body weight), a dose sufficient for full hematopoietic engraftment in a myeloablated subject.

Taken together, the data collected in this series provided the opportunity to optimize and validate a cell-processing procedure to be performed under GMP for subsequent clinical trials.

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Figure 1.

VB processing. The VB were processed in a class 10 000 clean room. Following separation and removal of soft tissue and intervertebral disks, the VB were divided into cubes using a mallet, osteotome and stainless steel ratchet pruner (A). Bone cubes were irrigated with medium as they were fed into the bone grinder (B) and emerged as fine bone fragments that were placed on a sieve set (C) and rinsed with medium. The rinsed fragments were then placed in the BM tumbler with more medium to dislodge remaining cells (D).

Figure 2.

Use of plastic pipe to stabilize the cadaver after removal of nine VB. Heavy-duty polyvinylchloride pipe was notched (inset) and inserted to replace the missing VB. The vertebral arches and spinal processes remained in the donor. The use of rubber O-rings (shown) was discontinued.

Figure 3.

Total cell recovery from each wash step. The data comprise the cells recovered at each step of the procedure expressed as a proportion of the total cells in the pooled product. Tukey box-plots show median and quartile values. Whiskers show the range exclusive of outliers, circles show individual data points.

Figure 4. CD34 recovery from each vertebral segment. The data are expressed as a proportion of total CD34⁺ cells recovered in each product. Tukey box-plots show median and quartile values. Whiskers show the range exclusive of outliers (asterisk), circles show individual data points.

Figure 5.

Detailed flow cytometric characterization of VB mononuclear cells. Analysis of a representative sample is shown. The analytical gate for each histogram is shown in square brackets. Numerical results are arithmetic means $\pm$ SD for all six samples. Removal of sources of artifact (top row): singlets (mean $5\,316\,530 \pm 2\,584\,685$ events acquired), determined by forward scatter pulse analysis, represented 91.9% of all events; 85.3% of all singlets had DNA content $\geq 2N$ (GT2N) and 99.7% of these had light scatter properties consistent with intact cells (clean scatter). Autofluorescent events were defined using a compound gate of events falling on the diagonal streaks of fluorescence (FL1) versus FL2, FL1 versus FL3 and FL2 versus FL3. Autofluorescent events had high forward angle light scatter (FS) and intermediate side scatter (SS), or intermediate FS and high SS. They represented a mean of 3.3% of cells within the clean scatter gate, and were eliminated from subsequent analyzes. Taken together, 24% of the events represented potential sources of artifact and were removed from the numerator and denominator of subsequent analyzes. Heme CD34 classifiers (middle row) were detected among clean scatter, non-autofluorescent events according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol, where CD34⁺ hematopoietic progenitor cells (heme CD34) are defined as CD45⁺ CD34⁺, with restricted CD45 expression and restricted light scatter properties. Heme CD34 outcomes are further parameters measured on this population. The majority of CD45⁺ CD34⁺ cells were CD133⁺ (67.1%) and CD117⁺ (80.3%), with a distinct population of CD90⁺ CD117^{dim} primitive hematopoietic stem cells (13.7%). A small subset (0.8%) of CD45⁺ CD34⁺ cells, 79.8% of which were CD90⁺, co-expressed mesenchymal markers CD73 and CD105. MSC, classified (bottom row) as CD45⁻ (non-heme), CD73⁺ CD105⁺, represented 0.68% of CD45⁻ cells. The majority of MSC (63%) co-expressed CD90. The right-most panel in the bottom row, gated on all non-autofluorescent clean scatter events, shows the cut-point defining CD90⁺ events.

Table I

Viability of recovered BM cells is not affected by emersion of VB in bleach or peroxide.

Case	Segment	Treatment	Sterile, pre	Sterile, post	WBC via (%)	CD34 via (%)		WBC via (%)		CD34 via (%)	
						Mean	SD	Mean	SD	Mean	SD
VBPC1	L4_L3_L2	None	ND	No	96.8	99.4					
VBPC1	L1_T12_T11	None	ND	No	87.9	99.1					
VBPC1	T10_T9_T8	None	ND	No	79.9	97.2					
VBPC2	L1_T12_T11	None	No	No	95.2	99.0					
VBPC2	T10_T9_T8	None	No	No	94.8	98.8					
VBPC3	L1_T12_T11	None	No	No	96.2	99.1					
VBPC4	T10_T9_T8	None	Yes	Yes	98.0	99.8					
VBPC5	T10_T9_T8	None	Yes	Yes	90.4	98.0	92.4	6.1	98.8	0.8	
VBPC3	T10_T9_T8	Bleach	No	No	96.3	99.4					
VBPC4	L4_L3_L2	Bleach	Yes	Yes	98.1	99.6					
VBPC5	L4_L3_L2	Bleach	Yes	Yes	97.1	99.2	97.2	0.9	99.4	0.2	
VBPC2	L4_L3_L2	H ₂ O ₂	No	No	94.8	97.5					
VBPC3	L4_L3_L2	H ₂ O ₂	No	No	94.0	99.2					
VBPC4	L1_T12_T11	H ₂ O ₂	Yes	Yes	98.3	99.3					
VBPC5	L4_L3_L2	H ₂ O ₂	Yes	Yes	96.3	99.4					
VBPC6	ALL	H ₂ O ₂	Yes	Yes	98.8	99.6	96.4	2.1	99.0	0.9	

WBC and CD34 viabilities (via) were determined flow cytometrically by 7-AAD exclusion. Viability was indistinguishable statistically between the treatment groups by ANOVA. ND, not determined.

Table II

Demographics, cell yield and quality.

Case	Sex	Age (years)	Height (cm)	IBW	Hold time	Process time	Total time	WBC ($\times 10^{10}$)	WBC via (%)	CD34 ($\times 10^6$)	CD34 via (%)	Sterile
VBPC1	M	63	180	75	28:54	6:07	35:01	4.6	96.8	6.7	99.4	No
VBPC2	M	18	183	78	38:28	4:43	43:11	7.5	94.8	8.2	97.5	No
VBPC3	M	45	188	82	30:26	6:31	36:57	5.2	94.0	5.2	99.2	No
VBPC4	F	20	168	59	22:21	5:16	27:37	5.1	98.1	5.7	99.6	Yes
VBPC5	M	48	183	78	39:47	5:02	44:49	4.5	97.1	2.8	99.2	Yes
VBPC6	M	18	173	69	7:20	5:10	12:30	4.4	98.8	8.8	99.6	Yes
	Mean	35.3	179.2	73.4	27:52	5:28	33:20	5.2	96.6	6.2	99.1	
	SD	19.3	7.4	8.3	11:57	0:41	11:55	1.2	1.9%	2.2	0.8	

IBW, ideal body weight; hold time, the time between cessation of cardiac function and initiation of cell processing (h:min); process time, the time from sample accession in the laboratory to time of the last cell count; WBC, total CD45⁺ cell recovery; WBC via: WBC viability; CD34, the total CD34⁺ CD34⁺ cell recovery; sterile (yes, no), the outcome of 14-day bacterial and fungal cultures.