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Cells Isolated from Cadaveric Bone Marrow are Safe for Use in Bone Healing and Effective at Promoting Osteogenic Re-construction

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Abstract

The efficacy and immune-modulation of mesenchymal stem cells is well documented. The issue of obtaining mesenchymal stem cells without patient risk, extensive intra-operative procedure, cell manipulation or exposure of cells to harmful reagents remains an issue. This study was designed to test the viability, composition and osteogenic potential of cells derived from cadaveric bone marrow by a new process. Vertebral bone from cadavers was collected within 24 hours of death, processed by a new procedure of tumbling and collection, and evaluated for viability, marker expression, cell composition, and inflammatory properties at various stages of the isolation process and following cryopreservation. Viability was excellent in all fractions and at all stages of the study. Cell staining and microscopic observation showed increased erythrocyte content in the first tumble of bone for marrow extraction, as well as gross observation of debris.

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Cryopreservation favored the preservation of CD45-/CD105+ and GlycoA-/STRO-1+ mesenchymal stem cells at the expense of platelets, red blood cells, white blood cells and neutrophils. The resulting cell solution contains a percentage of mesenchymal stem cells far above that required for immune modulation. A mixed lymphocyte reaction assay showed no inflammatory response to this cell composition. The cells produced and preserved in this manner are viable, should elicit no immune response, suppress recipient immune responses and osteogenically differentiate.

Keywords: MSC; cadaveric; cryopreservation; immune modulation; fracture repair.

1. Introduction

Due to their inherent osteogenic capacity and immunomodulatory properties, multipotent mesenchymal stem cells (MSCs) are strongly desired for fracture repair. It has been known since 2002 that MSCs suppress T-cell proliferation in mixed lymphocyte cultures [1]. With the inhibition of T-cell allo-responses widely documented [2, 3] an abundance of research has subsequently investigated the use of MSCs with skin [4, 5], cardiac [6], liver transplants [7], islet transplant [8, 9] and for the amelioration of Graft Versus Host Disease (GVHD) [10, 11, 12] and consistently demonstrated a lack of immune response or graft rejection. This has established bone marrow (BM) MSCs as a safe and advantageous component for allograft, with doses as low as 10,000 MSCs shown to be effective [2].

The metrics of dosage, concentration, carrier and effective tissue response to cell compositions all support regenerative applications, but the problem remains that the bone marrow contains a large variety of cell types. If one desires to separate MSCs from this mix of cells, centrifugal separation can segregate cells of specific density ranges, but cannot isolate specific cell types within that density range. Several methods of specific identification and separation have been developed with magnetically coupled antibodies, however the use of antibody-based cell isolation has not been approved for use beyond research applications with the exception of CD34 (cleared by the FDA, January 2014) for treatment of Acute Myeloid Leukemia. In the effort to achieve a pure cell determinant, or cluster of determination (CD), many of the cell types discarded during specific isolation are supportive of the regeneration process and MSC proliferation. The frustration of wasted regenerative potential in conjunction with the desire to retain the immunomodulatory characteristics of MSCs lead us to believe that the isolation of specific cell types may be unnecessary, impractical and perhaps even undesirable. We propose that the presence of supportive cell populations in combination with MSCs yields an attractive allograft that can not only be safely transplanted, but moreover supports effective regenerative potential. Further, this viable cellular allograft can also be attained without significant cell manipulation, without exposure of cells to enzymes that cleave cell attachment (eg. collagenase) or toxic, and devoid of damaging cryopreservants (eg. DMSO) or cryopreservants that contain potentially unsafe serums.

CD105 (endoglin) and STRO-1 are well known markers of mesenchymal stem cells [13, 14, 15, 16]. Additionally, STRO-1+ cells are 100 times more osteogenic than STRO-1- cells [17]. Therefore, we utilized CD105 and STRO-1 as markers for MSCs with predisposition to osteogenic capacity. A process was developed to evaluate the safety and osteogenic efficacy of a cellular suspension obtained by tumbling cadaveric bone

marrow, centrifugal separation followed with ficoll, and preservation in poly-l-lysine-based polyampholyte cryopreservant for potential use as allograft material. The key indicators sought were the percentage of MSCs in cell solution, the imposition of immunomodulation of both donor and recipient, the ability to osteogenically differentiate and the absence of significant deleterious manipulation of the cells.

2. Materials and methods

2.1. Isolation of cellular suspension

Donor spines were collected and surrounding soft tissue and fascia was removed. Individual vertebral bodies, primarily cancellous bone in composition, were dissected free of the intervertebral disks and further trimmed into small pieces. The pieces were further ground to small chunks of 4-10 mm and placed in processing media (low glucose DMEM with 2.5% HSA, 10 U/ml Heparin, 0.08 mg/ml Gentamicin and 0.0025 mg/ml DNAse). The resulting slurry was tumbled 4 times. The solution was collected after each tumble and replaced by more processing media. The collected solution was passed through 500 μm, followed by 180 μm sieves.

2.2. Gross and microscopic evaluation

2.2.1. Cell suspensions

Cells from each tumble (1-4), were ficoll separated and viewed with a Nikon DIAPHOT microscope at 10x magnification with SpotAdvanced Version 4.1 software.

2.2.2. Alkaline phosphatase and mineralization

The alkaline phosphatase reaction was photographed using a Nikon DIAPHOT microscope at 4x magnification with SpotAdvanced Version 4.1 software. Reaction products from six-well plates of alkaline phosphatase secretion and mineralization were recorded with a Samsung Galaxy S5 camera.

2.3. Viability

Pre-and Post-Ficoll samples were stained for viability with propidium iodide. Viability was measured as cells negative for propidium iodide with a Moxi Flow™ cell counter (Orflo technologies, United States). The *Post-thaw samples* were counted and assessed for viability by trypan blue staining and counting with a hemocytometer.

2.4. Flow cytometry

Cells were stained for flow cytometry in FACS buffer (DPBS, 5% BSA, 0.01% sodium azide) with either CD15-AF488, CD45-APC, HLADR-FITC, CD3-APC (BioLegend, U.S.A.), CD37-PE, HLA class1-APC, SSEA4-FITC, GlycoA-PE (R&D Systems, U.S.A.), GlycoA-PE + STRO-1-APC (Novus Biologicals, U.S.A.), CD45-APC + CD105-FITC (R&D Systems) or isotype controls for each fluorophore for 30 minutes at 4-8^{0}C in the dark. The cells were then washed twice with FACS buffer and re-suspended in 4% paraformaldehyde. Flow cytometry data was collected on a BD LSR II flow cytometer (BD Biosciences, U.S.A.). All positive/negative

signals were obtained by comparison with isotype control.

2.5. Complete blood counts

Samples were sent to Sylvester Cancer Center for Complete Blood Count by the Diagnostic Molecular Pathology and Flow Cytometry Laboratory.

2.6. Efficacy assay

Cells were seeded in fibronectin-coated 6-well plates and allowed to attach at 37^0C overnight. The media was changed to osteogenic differentiation media (alpha-MEM, 10% FBS, 1% P/S, 100uM Ascorbic Acid, 10 mM B-glycerophosphate and 10 nM dexamethasone) and plates were incubated at 37^0C, 5% CO2 for up to 21 days. In indicated assays, the cells were allowed to grow to near confluence before transition to differentiation media.

2.6.1. Alkaline phosphatase activity

The media was aspirated and cells were washed twice with DPBS. Cells were fixed in 2% paraformaldehyde for 30 minutes. The cells were again washed two times with DPBS then covered with alkaline phosphatase substrate solution (1.85 mM napthol, 1% n-n'dimethylformamide, 1.68 mM Fast Blue B Salt, 3.5 mM magnesium chloride in 30 ml 100 mM Tris-HCl pH 9.6, final solution adjusted to pH 9.0) then incubated for 30 minutes at 37^0C. The alkaline phosphatase substrate solution was aspirated and cells were washed twice in DPBS and allowed to dry for photography.

2.6.2. Mineralization

The media was aspirated and cells were washed two times with DPBS. Cells were fixed in 2% paraformaldehyde for one hour. The cells were again washed two times with DPBS then covered with Alizarin Red solution (40 mM Alizarin Red-S pH 4.2) and incubated for 20 minutes at room temperature. The alizarin red solution was aspirated and cells were washed twice with DI water and allowed to dry for photography.

2.7. Cryopreservation and thaw

2.7.1. Cryopreservation

Cells were re-suspended in a non-DMSO, polyampholyte cryoprotectant to a concentration of 1.1 x 10^6 cells/ml. One milliliter aliquots were transferred to cryovials and allowed to equilibrate for 30-45 minutes. The vials were then frozen at 10^0C/minute and stored at -80^0C.

2.7.2. Thaw

Cells were quickly thawed in a 37^0C water bath and transferred into 3 ml of physiological saline. Cell counts, viability and flow cytometry samples were taken from this solution.

2.8. MLR reactions

A mixed lymphocyte reaction was contracted to an independent laboratory who used a Roche BrdU ELISA proliferation assay. A total of seven samples were sent. The immune response was measured and converted into stimulation indexes (SI) with an n of 1-3 for each sample.

2.9. Statistical analysis

Data are reported as mean ± s.d. All analyses were performed using Microsoft Excel data analysis for two-tailed Student's T-test. Differences between populations with significance $p<0.05$ were considered statistically significant.

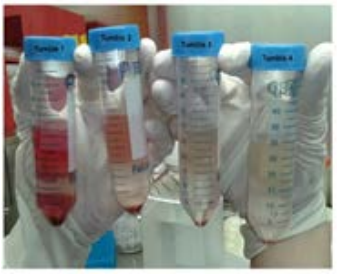
2.10. Use of human subjects

Whole bone marrow was obtained from the vertebral bodies of cadaveric male donors (age range; 22- to 52-years old) following guidelines for informed consent set by the University of Miami, School of Medicine, Committee on the Use of Human Subjects in Research. Vertebrae were removed from normal donors within 24 hours of death and serology was performed to assure safe source and reduced risk of disease transmission.

3. Results

3.1. Phenotypic composition of tumbles used for cell collection

To better understand a composition defining cells desired vs. cells that might be potentially immunogenic, cells



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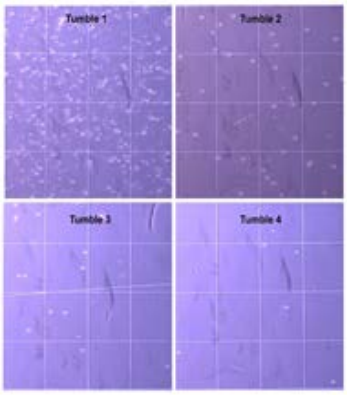

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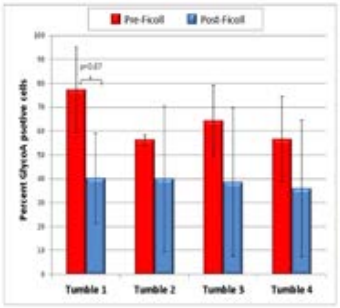

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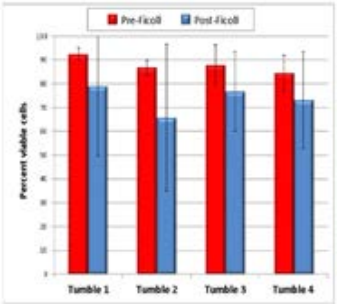

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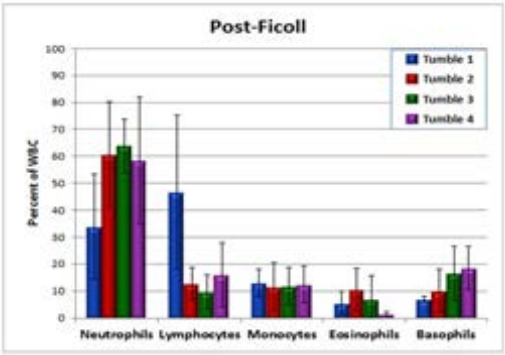
Figure 1: Four tumbles of vertebral bone marrow pieces result in different debris and erythrocyte content but maintain viability. (a) Representative ficoll separations of each tumble for a single donor. The first tumble clearly shows a large RBC pellet as well as a red upper fraction. The MNC ring is not apparent. The other tumbles have sequentially smaller RBC pellets, clearer upper fractions and more apparent MNC rings. (b) Microscopic hemocytometer images of the collected MNC layer of each tumble from a single donor. The first tumble shows more debris and cells that are not clearly round. The remaining tumbles have significantly reduced debris and primarily round healthy cells. (c) Flow analysis of GlycoA positive cells before and after ficoll separation for each tumble. The first tumble has the highest percentage of GlycoA positive cells. All tumbles had reduced percentages of GlycoA+ cells following ficoll separation. (d) The viability of cells by propidium iodide reveals no difference between tumbles before or after ficoll separation. N=3, *P<0.05.

In each of several washes and tumbles were evaluated separately during cell collection. Gross composition, viability and phenotypic composition were analyzed. The suspension from each tumble was separated by density gradient centrifugation. Gross observation of the centrifugally separated samples revealed differences in the content of all fractions (Figure 1a). Most obvious was the extent of hemolyzed material in the upper fraction of Tumble 1 as well as the large red pellet on the bottom, red blood cells (RBCs). An apparent white mononuclear cell ring at the Ficoll interface was minimal, if not absent. These properties diminished with each subsequent tumble. Microscopic evaluation of the mononuclear cell fraction ring revealed more debris and red blood cells in the first tumble, with less debris and more mononuclear cells occurring in each subsequent tumble (Figure 1b). Subsequent cell staining for GlycoA (differentiated erythrocytes) emphasized the high RBC content of Tumble 1, as well as the significant reduction of such cells following ficoll separation (Figure 1c). Propidium iodide

staining of each tumble revealed no significant difference in viability between any of the tumbles or between pre- and post-ficoll separations (Figure 1d). The mononuclear cells from each tumble were stained for typical hematopoietic, granulocyte and immune cell markers, as well as the primitive stem cell marker SSEA4 and mesenchymal stem cell marker CD105. Additionally, the samples were sent to the Sylvester Cancer Center for complete blood differential count (CBC). It is noteworthy that the first tumble (pre-ficoll) was too coagulated for CBC in all cases. Separation of the mononuclear cell fraction from the remainder of the solution made the CBC (post-ficoll) possible. For this reason, all subsequent analysis were only performed on post-ficoll separation samples. The CBC revealed no statistically significant difference between the tumbles in any of the cell populations measured (Figure 2a), although the lymphocytes appear to be drastically reduced after the first tumble. Cell staining (Figure 2b) revealed no significant difference in CD15 (granulocyte marker) expression between the tumbles, providing verification of the CBC data which showed no significant difference in neutrophils and basophils. In addition, the cell staining showed a reduction of Human leukocyte antigen-D related (HLADR) with each tumble but, like the CBC, the results are not statistically significant (Figure 2b). There was no difference in the primitive MSC marker, SSEA4, or MSC marker CD105, between the tumbles (Figure 2c). Donor variability, inherent in this process, played a large part as seen by the error bars in all graphs in Figure 2. Therefore, reducing the undesirable cells and debris from Tumble 1 seems to provide increased safety without affecting any prospective efficacy. For this reason, we eliminated tumble 1 from all subsequent analyses and combined the solutions from tumbles 2-4 for ficoll separation.



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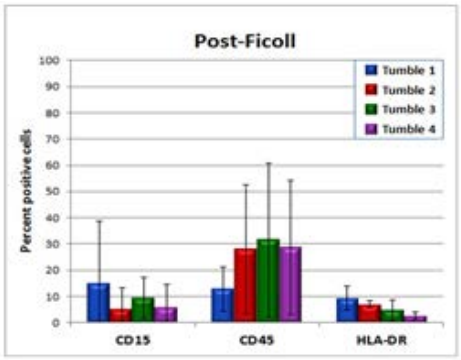

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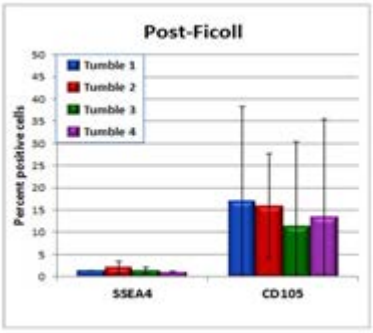

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Figure 2: There is no difference in phenotypic composition between each tumble following ficoll separation. (a) Complete blood count reveals no difference in indicated cell types between tumbles, measured as percent of WBC's. (b) Flow cytometry shows no difference in indicated hematopoietic markers between tumbles, measured as a percent of all cells. (c) There is no difference between tumbles in the percent of mesenchymal stem cells that are SSEA4 positive or CD105 positive. N=3, *$p<0.05$.

3.2. The isolated cells are osteogenic and mineralize

In order to ensure that the cells isolated were osteogenic, cells were placed in osteogenic media and their production of alkaline phosphatase and level of mineralization at various time points were evaluated. Figure 3a shows the production of alkaline phosphatase by the cell suspension. The cells were seeded at 1.1 x 10^6 cells/well and not allowed to expand. They were osteogenically differentiated 24 hours after the time of plating. Colonies formed with the typical swirling pattern of differentiating osteoblasts. The low magnification and microscopic images in Figure 3b show the edges of colonies. Both images illustrate the alkaline phosphatase activity highest at the centers where the cells are more confluent and diminishing outward as the cells are proliferating and differentiating outward. In the following experiment, cells were seeded at the same density and allowed to expand to near confluency, then differentiated with exposure to osteogenic media. There was obvious alkaline phosphatase activity peaking at day 14 and waning at day 21(Figure 3c), as expected when cells transfer from differentiation to mineralization [18, 19]. Alizarin Red staining in Figure 3d shows the samples miner alizing at day 21. This correlates with the decrease in alkaline phosphatase, as it is known that cells will first secrete alkaline phosphatase, then reduce that secretion in favor of mineralization. These data confirm that the cell solution is osteogenic, and will mineralize, illustrating the ability of the cell solution to effectively produce bone.

3.3. The cryopreserved cell suspension is safe for direct implantation

The cells were cryopreserved in a poly-*l*-lysine based preservation media with no dilution or additions. After 30 days of cryopreservation vials from three different donors were sent out for Mixed Lymphocyte Reaction (MLR) testing to evaluate the safety of direct application of the cell solution. A histogram of the resulting Stimulation Indexes (SI) for each test shows that no reaction occurred with any of our cell solutions (Figure 4). Based on these results, the cryopreserved cell solution should elicit no immune response.

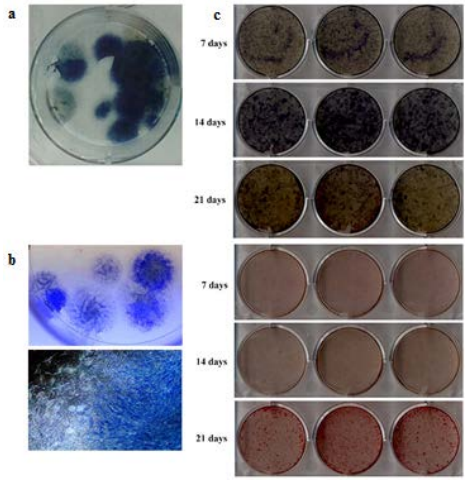

Figure 3: Cells collected by this new method form colonies and are osteogenic. (a) Representative photograph of post ficoll cells grown in osteogenic media for 30 days. No prior expansion was allowed. Alkaline phosphatase staining shows many colonies at varying densities positive for alkaline phosphatase secretion. (b) Representative photograph of post ficoll cells from a different donor grown in osteogenic media for 14 days. No prior expansion was allowed. Low magnification and 4X magnification of alkaline phosphatase staining shows typical swirling pattern within colonies which are positive for alkaline phosphatase secretion. (c) Representative photographs of alkaline phosphatase staining of post ficoll cells from a different donor that were allowed to expand to near confluence, then differentiated in osteogenic media for up to 21 days. (d) Representative photographs of Alizarin Red staining of post ficoll cells from the same donor as in (c) that were allowed to expand to near confluence, then differentiated in osteogenic media for up to 21 days.

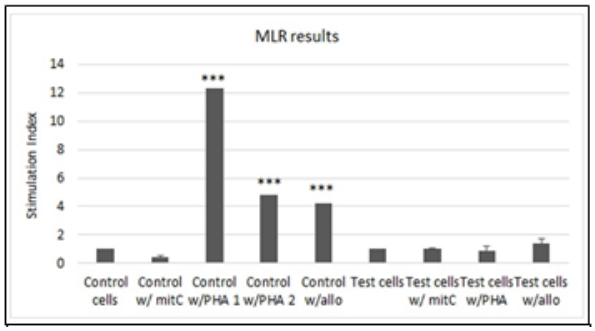

Figure 4: The cell solution does not elicit an immune response. Seven separate frozen donor samples were sent out for mixed lymphocyte reaction (MLR) testing. The MLR performed by Xeno Diagnostics shows no difference in the stimulation index between stimulated test cells and unstimulated control cells. Positive controls (PBMCs) clearly showed significant stimulation. N=3 for six samples, n=1 for the seventh sample. mitC= mytomycin C treated. PHA= phytohemagglutinin treated. Allo= allogenic reaction.

3.4. The cells have reduced blood cells and increased MSC content upon thaw

The final analysis of this study was to ascertain the viability and composition of the cell suspension after cryopreservation. We sought to determine these parameters after 90 days and 6 months of cryopreservation. Viability was not significantly different between cells before freezing (pre-freeze) and those thawed at 90 days

or thawed at 6 months (Figure 5a). The Complete Differential Blood Count (CBC) in Figure 5b shows that although ficoll separation significantly reduced the percentage of platelets and red blood cells, cryopreservation for 90 days further reduced the platelets, RBCs and WBCs. The CBC (Figure 5c) also shows that ficoll separation alone was responsible for significant reduction in lymphocytes and monocytes. In such depletion, the relative number of neutrophils, large unstained cells (LUC) and basophils appeared to increase in proportion to the total number of cells. Following 90 days of cryopreservation, the thawed cells showed significant reduction in neutrophils and an increase in monocytes, eosinophils and basophils compared to pre-freeze samples. Cell staining for flow cytometry showed that after 6 months of cryopreservation, there is no significant change in CD15 or CD3 compared to pre-freeze (Figure 5d). The B-cell marker, CD37 was completely (and significantly) lost. The hematopoietic marker, CD45, was reduced almost to significance ($p=0.08$) and cells expressing HLA class 1 were significantly reduced. Surprisingly, HLADR was significantly increased after 6 months of cryopreservation (Figure 5d). As expected, the percentage of cells expressing GlycoA (differentiated erythrocytes) was significantly reduced after 6 months of cryopreservation (Figure 5e). Interestingly, the percentage of cells negative for hematopoietic marker CD45 and expressing mesenchymal stem cell marker CD105 increased significantly following 6 months of cryopreservation compared to pre-freeze (Figure 5f). Similarly, the percentage of cells that are negative for the erythrocyte marker GlycoA *and* positive for the osteogenic MSC marker STRO-1 increased significantly at 6 months thaw compared to pre-freeze (Figure 5f). The observation supported by this data suggests that freezing in poly-*l*-lysine-based cryopreservant preferentially results in lysis of erythrocytes, lymphocytes and neutrophils, and in consequence augments the percentage of viable MSC's.

4. Conclusion

The production of safe, effective allograft material is challenging. Current regulations preclude the use of antibody-based isolation and separation of specific cell types, and furthermore, highly selective isolation techniques would remove desirable supportive cell populations. In the reflection of bone marrow offering a multitude of potentials in a mosaic of identities, an ideal allograft should contain supportive cell populations yet maintain a high enough percentage of MSCs to abrogate any inflammatory or immune effects to both allograft and recipient. We have isolated a cell solution that is both safe, due to the high percentage of MSCs, and effective due to the presence of several supportive cell populations in coordination with the MSCs.

The elimination of debris, erythrocytes, and considerable amounts of leukocytes and HLADR+ cells by removal of cells from the first tumble establishes a more favorable baseline group of cells for ficoll separation. Observed debris may be due to necrosis, dying cells or platelets, all of which are not desired in this allograft product. This is illustrated by the extensive clotting of the pre-ficoll cells from tumble 1, barring CBC analysis. Fortunately, supportive cells remain in the remaining three fractions, allowing retention for ficoll separation.

Tumbles 2 through 4 in our processing media were combined and separated by density gradient centrifugation with ficoll. The recovered MNC layer secretes alkaline phosphatase at 14 days and begins mineralization at 21 days. This clearly demonstrates the osteogenic capacity of these cells upon introduction to osteogenic factors. Introduction of this cell mixture to an environment rich in bone forming niche cells (e.g. osteoblasts, osteoclasts,

matrix, etc.) such as demineralized bone and the *in vivo* niche would further promote the efficacy of the MSC component, increasing the efficacy of the supporting cells by feedback.

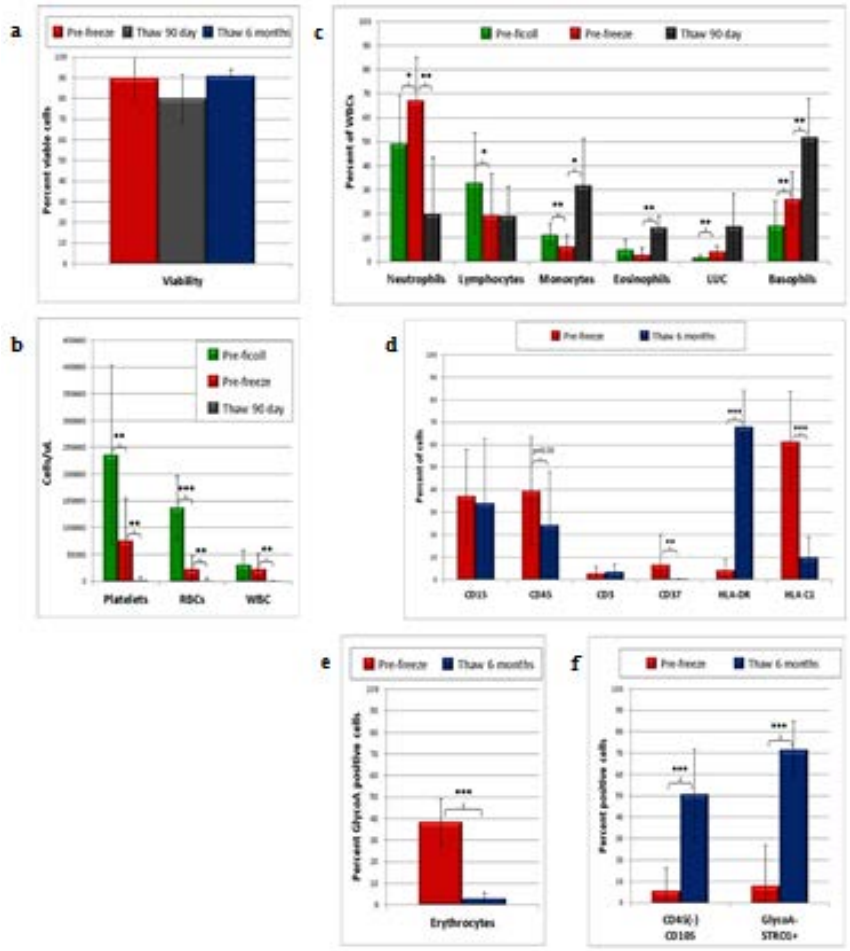

Figure 5: The phenotypic profile of the cell solution following cryopreservation. (a) The viability as measured by trypan blue staining shows high viability following cryopreservation with no difference between pre-freeze (n=42), 90 days frozen (n=9) and 6 months frozen (n=8). (b) Complete blood count shows a significant decrease in the number of platelets, red blood cells (RBCs) and white blood cells (WBC) following ficoll separation, before freeze (n=17) and again after 90 days of cryopreservation (n=6). (c) Complete blood count shows significant changes in some cell types within the WBC count following ficoll separation, before freeze (n=17) and after 90 days of cryopreservation (n=6). (d) Flow cytometry analysis reveals a significant decrease in the percentage of CD37 positive B-cells and HLA class I cells and a significant increase in the percentage of HLADR positive cells after 6 months in cryopreservation (n=8) compared to pre-freeze (n=38). (e) GlycoA staining of cells before freeze (n=35) and after 6 months of cryopreservation (n=8) show the significant decrease in the percentage of erythrocytes in the cell solution. (f) Flow cytometry analysis of MSC markers show that following 6 months of cryopreservation (n=8) the percentage of CD45-/CD105+ and GlycoA-/STRO-1+ cells are significantly increased compared to pre-freeze percentages (n=37). *p<0.05, **p<0.01, ***p<0.001.

The choice of a poly-l-lysine based cryopreservant proved to be an important component as it appears to

preferentially preserve desirable cells. The pH of the poly-l-lysine used may have played a role in its preferential preservation of MSC populations [20]. The poly-l-lysine cryoprotectant is safe for direct *in vivo* injection as evidenced by both *in vitro* studies [21, 22] and mixed lymphocyte reaction testing directly applied to the individual lots of donor allograft material. All thawed populations retained high viability when re-suspended in physiological saline with no centrifugation or washing of cells. As expected, RBCs and platelets were further eliminated during freezing, most likely due to lysis [23]. This is advantageous as platelet rich plasma (the product of factors released from lysed platelets during freeze) is currently being utilized as a healing therapy [24, 25] although The American Academy of Orthopaedic Surgeons concluded in 2011 that its effectiveness is still unproven. The increase in HLADR positive cells was evaluated to ensure safety. Cells that express HLADR on their surface include macrophages, B-cells and dendritic cells [26, 27]. The percentage of cells expressing CD37 (B-cells) was near zero, macrophages (monocytes) were less than 17% of the WBC's, which amounts to 4.8% of total cells. Since the isolate was recovered from the bone marrow, this leaves the balance as immature dendritic cells with low T-cell activation potential. Moreover, MSCs have been shown to suppress immune reactions by modulating dendritic cells [28].

The elimination of B-cells and reduction of T-cells to within one s.d. of zero is ideal. Since these cells were frozen at 1.1×10^6 cells per vial, 50% CD45-CD105+ cells amount to 550,000 MSCs in a single vial. This cell population has been repeatedly shown to be MSCs [13, 29]. It has been reported that MSCs suppress immune reactions [30, 31, 32] avoid allogeneic recognition, interfere with dendritic cell and T-cell function, and generate a local immunosuppressive microenvironment by secreting cytokines [33]. And perhaps most importantly, 10,000 MSCs (10%) sufficiently supports and conveys an immunosuppressive effect to donor cells and recipient cells following transplant [34]. It has also been shown that the immunomodulatory function of human MSCs is further enhanced when the cells are exposed to an inflammatory environment [35]. Since our cells exceed that requirement by 5 times, the cell solution is shown to be safe from immune reaction from both donor and recipient. These data illustrate the safety of the cell solution obtained and preserved in this manner.

The non-erythrocyte (GlycoA-) MSC (STRO-1+) percentage in the cell solution was greater than 70%. This population is highly osteogenic [15, 17] and in combination with the production of alkaline phosphatase and mineralization further illustrates the expected efficacy of this cell suspension. By including supportive cell populations in the cell solution, the efficacy should be further enhanced, without fear of immune reactions.

We have shown that BM allograft material can be isolated in a specific media and cryopreserved without antibody-based isolation, producing an effective and safe bone healing product. The resulting material contains greater than 50% CD45-CD105+ MSCs and greater than 70% GlycoA-STRO1+ MSCs. The percentage of MSCs is more than sufficient to nullify unfavorable immune responses from both donor and recipient. Our data shows excellent reduction of immune cells and erythrocytes upon isolation with continued marked reduction following cryopreservation. These results are further enhanced by the concurrent increase in mesenchymal stem cell concentration. We have shown that the presence of supportive cell populations in combination with high concentration of MSCs yield a well-rounded transplantable allograft that is producible without significant cell manipulation and without exposure of cells to adherence cleaving enzymes commonly used for retrieval of MSCs. There are no toxic, damaging cryopreservants or exposure to cyropreservants with media's that contain

potentially unsafe serums. Taken together, this data illustrates the safety of bone marrow cells which are isolated and preserved in this manner.

This study is constrained to the testing of a singular poly-l-lysine based cryopreservant. Hence, the results are limited to the properties of that preservant. We recommend expansion of the concept and extended testing to include other non-DMSO cryopreservants. Although these data would be best presented as a comparison to DMSO-based industry standards, this formulation is not safe for direct application. Therefore, *in vitro* comparative studies will need to suffice as preliminary data. Animal studies utilizing DMSO-based crypreservants without washing are not recommended due to toxic effects.

Bone allograft is commonly used to enhance healing of non-union fractures and encourage fusion of bone following surgery. The cell solution as a component of a reconstituted cellular allograft described herein introduces options for clinical care for bone defects, surgical imperfections, or traumatic loss of skeletal material. Combination of this solution with bone microparticulate would strongly enhance the healing properties of both the cells and the microparticulate, working in synergy with the patient's bone microenvironment. The application of MSCs to patients have already been shown to be safe in ratios of MSCs to other cells far below that of our solution. Since this cell solution remains viable, is expected to suppress immune responses and will osteogenically differentiate, it is an ideal candidate for testing with bone microparticulate transplanted to non-union fracture or fusion scenarios. There is also the consideration that this cell solution will differentiate effectively into cartilage in the presence of appropriate environmental cues. There are many avenues to be investigated for discovery of the limits and applications of this cell solution, isolated and preserved in this manner.

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