Characterization of Supra-Concentrate of Human Bone Marrow Stem Cells for the Treatment of Spine and Musculoskeletal Disorders

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STATEMENT OF THE PROBLEM

The use of autogenous stem cells is one of the pillars of regenerative medicine. The past one and a half decades have witnessed the discovery and characterization of a new type of adult stem cells named Very Small Embryonic-like stem (VSEL) cells. These cells exhibit **pluripotency**, they are localized in many tissues, including bone marrow and mobilized peripheral blood, which makes them easily accessible to the clinician. However, there are two major impediments to implementing their use in a clinical setting. First, current protocols that use blood or marrow as the source of therapeutic concentrates, such as platelet rich plasma (PRP) or bone marrow concentrate (BMAC), respectively, have not been characterized in terms of human VSEL (hVSEL) cell content. And second, there are no protocols to produce a concentrate of autologous hVSEL cells that can be implemented and in a clinical setting.

STUDY OBJECTIVES

1. To characterize the current centrifugation method used in most clinics for nucleated cell concentration in terms of hVSEL cells yield, and
2. To develop a method for the enrichment of hVSEL cells derived from human bone marrow that is amenable to full implementation in a clinical setting.

HYPOTHESIS

Red blood cell flocculation will yield a concentrate of nucleated cells from human bone marrow with a higher content of hVSEL cells when compared to the common two-step centrifugation process.

IRB

This study was approved for by the Texas State University IRB.

KEYWORDS

Regenerative medicine, VSEL, hVSEL, hMSC, stem cells, multipotency, pluripotency

PROJECT DESCRIPTION

In the study we characterized *the presence of hVSEL cells in marrow fractions produced by centrifugation,* and developed a *fractionation protocol for marrow based on an initial Hespan-induced RBC flocculation step* to yield a leukocyte plus platelet fraction enriched in hVSEL cells. Samples of bone marrow aspirate (BMA), and bone marrow concentrates (BMC) were collected and prepared at Precision Regenerative and Functional Medicine (PRFM) and sent to Texas State for processing and analysis (FIG 1).

RESULTS

FLOCCULATION PARAMETERS

The hetastarch Hespan (BBraun, Bethlehem, PA) increases the sedimentation rate of red blood cells in human whole blood (FIG 2) and it does so in a concentration and time dependent manner. The effect of Hespan on RBCs is not due to dilution (data not shown), and it is concentration and time dependent (FIG 3). It reaches a maximum between 30-40% and between 30-60minutes, respectively. The flocculation effect is primarily on RBC and it does not significantly affect the sedimentation rate of leukocytes or platelets, as indicated by the increase in TNC, platelets, and MNC with time and Hespan concentration (data not shown). Flocculation was more pronounced during the first 10-20 minutes and it tends to even out towards the end of the incubation of incubation period (FIG 3B).

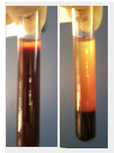

Figure 2. Whole blood after incubation with 40% (v/v) Hespan (right) for 30 minutes versus no Hespan added (left).

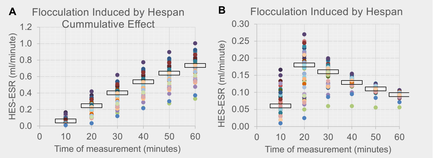

Figure 3. RBC flocculation induced by Hespan. A total of 55 whole blood samples from 55 different donors were treated with 30% Hespan and incubated for 10-30 minutes. At each time interval the volume of supernatant was measured. The net increase in supernatant volume at each time point is shown in Panel B. Panel A shows the cumulative supernatant volume at each time point. The supernatant is defined as the portion of blood devoid of red blood cells. HES-ESR is the Hespan-induced erythrocyte sedimentation rate.

Erythrocyte Sedimentation Rate (ESR), also known as sed rate, is a non-specific clinical measure of inflammation. We wanted to know if the effect of Hespan on RBC flocculation correlated with ESR. Hespan has a *normalizing effect* on ESR, i.e Hespan-induced flocculation was inversely related to the ESR so that *the higher the ESR, the lower the effect of Hespan on red blood cell flocculation*. (FIG 4).

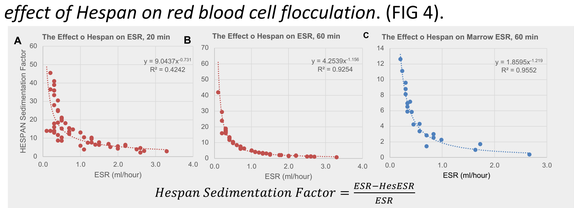

Figure 4. Hespan-induced flocculation, indicated by Hespan sedimentation factor, versus ESR. Whole blood was incubated with 30% Hespan for 20 and 60 minutes, Panels A and B, respectively. Whole marrow was incubation with 30% Hespan for 60 minutes (Panel C).

This normalizing effect is of great importance to assay and protocol development. We can rely on the conditions set forth by this new method to work well for the majority, if not all, of marrow (and blood) samples we will see in the clinic.

Another measure of robustness is the non-dependence of this method on hematocrit, a measure of RBC concentration. (FIG 5).

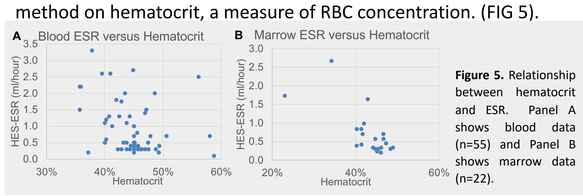

Figure 5. Relationship between hematocrit and ESR. Panel A shows blood data (n=55) and Panel B shows marrow data (n=22).

CELL YIELD

Flocculation increased the recovery of nucleated cells by about 4.5-fold over centrifugation alone. The yield of VSEL cells and hMSC was also improved by flocculation over centrifugation alone (FIG 6 & 8).

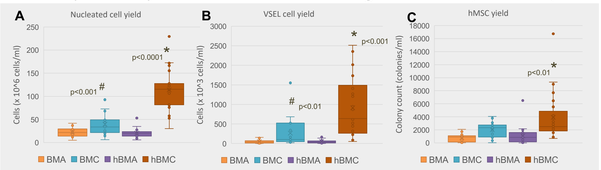

Figure 6. Bone marrow cell yields. Panel A: total nucleated cell counts, Panel B: VSEL cells counts (CD45-/Lin-, Small, CD34+, CD184+ and CD133+). *indicates statistically significant difference between BMC and hBMC and # between BMA and BMC, p values as indicated, paired student t-test, n=24 in all groups.

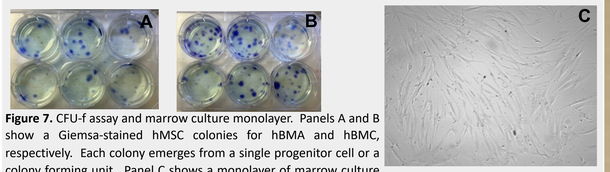

Figure 7. CFU-f assay and marrow culture monolayer. Panels A and B show a Giemsa-stained hMSC colonies for hBMA and hBMC, respectively. Each colony emerges from a single progenitor cell or a colony forming unit. Panel C shows a monolayer of marrow culture expanded for cytometry or ICC. Conditioned media (CM) were collected from this monolayer similar to the one shown here.

One of the most remarkable findings of this work is that VSEL cells ($CD45^-/Lin^-/Small/CD34^+/CD184^+/CD133^+$) underwent growth and expansion in tissue culture in every sample cultured so far. Viable cells were found associated with the monolayer of adhered cells by cytometry (FIG 8-TC) and ICC (data not shown), and also free-floating or "released" into the conditioned media of cultured cells (FIG 8).

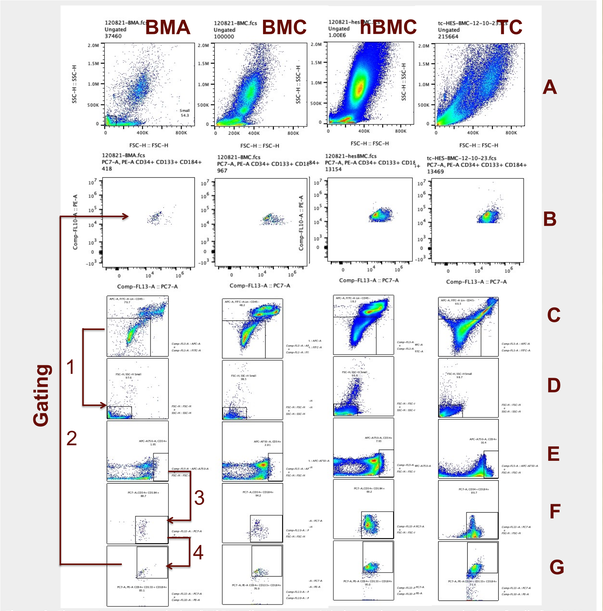

Figure 8. Flow cytometry analysis of VSEL cells. Top row (A) shows the entire population of cells for BMA, BMC, hBMC and *in vitro* expanded cells. The second row (B) shows the population of VSEL cells corresponding to the samples on the top row. Rows C-G show the gating used to identify the VSEL cell population. CD45 and Lineage negative cells were first selected (C), from which cells <7μm were selected (D), followed by the sequential selection of CD34 (E), CD184 (F), and finally CD133 (G) positive cells.

When probed for the markers of pluripotency, Nanog and Oct 3/4, a subset of the population expresses one or both markers along with Stage Specific Embryonic Antigen 4 (SSEA4), all of which are markers of pluripotency in embryonic stem cells (Figure 8).

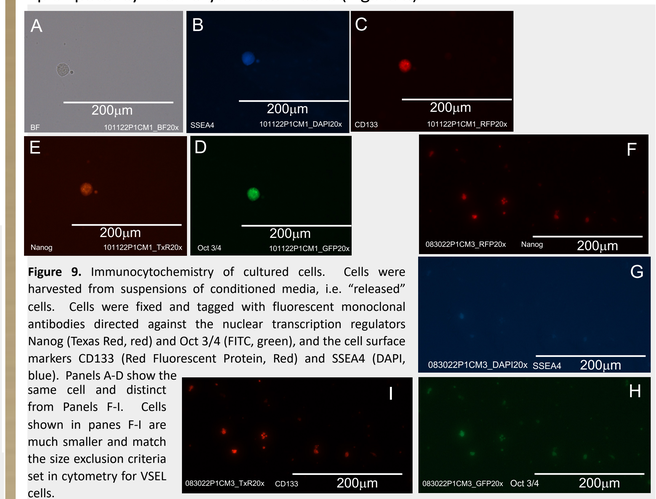

Figure 9. Immunocytochemistry of cultured cells. Cells were harvested from suspensions of conditioned media, i.e. "released" cells. Cells were fixed and tagged with fluorescent monoclonal antibodies directed against the nuclear transcription regulators Nanog (Texas Red, red) and Oct 3/4 (FITC, green), and the cell surface markers CD133 (Red Fluorescent Protein, Red) and SSEA4 (DAPI, blue). Panels A-D show the same cell and distinct from Panels F-I. Cells shown in panes F-I are much smaller and match the size exclusion criteria set in cytometry for VSEL cells.

Among the positive cells we found at least two populations that appear to be distinct based on their size (FIG 9). Additional work is necessary to fully characterize these cells at a molecularly and functional level, and to develop a culture system specific for their expansion.

SUMMARY

- *RBC flocculation by Hespan is donor independent* as it is not a function of hematocrit nor ESR. Importantly, it has a normalizing effect on ESR.
- *An initial flocculation step removed greater than 95% of RBC* enabling the production of a substantially RBC-depleted marrow concentrate with nucleated cell concentration as high as 300mill/ml possible in volumes of 0.5 to 1ml (the highest we historically get with BMC is about 30-40mill/ml).
- *hBMC contained a greater number of hMSC and VSEL cells* than centrifugation alone; however, it appears that flocculation may not be specific to RBCs, and also affect a subpopulation of nucleated cells, as indicated by a disproportionate increase in $CD45^+$cells in hBMC (data not shown).
- *hVSEL cells grow and undergo expansion in tissue culture* under the conditions used here. Viable cells are found in greater numbers than seeded in the monolayer as well as free-floating in the media, suggesting a physiological release mechanism.
- *Free (i.e. released) cells express nuclear transcription factors associated with pluripotency*.

PROJECT IMPACT

The results of this study enable the following:

- Implementation of point-of-use **protocol** for autologous supra-concentrate for percutaneous injections in combination with scaffolds (e.g. autogenous Fibrin) approved morphogens (e.g. rhBMP2/Infuse™) and vascular modulators (e.g. Avastin™) for pilot clinical studies.
- Molecular and functional **cell characterization** of isolated marrow-derived hVSEL cell, expanded and released.
- Development of **a culture system** for the expansion of hVSEL cells, alone or in coculture, to investigate the cellular niche that regulates hVSEL physiology and microanatomy.
- Creation of a new Biologics technology: a **single use disposable bioreactor** for the **decentralized expansion** of autologous hVSEL cells in the clinic.

GRAPHIC METHODOLOGY

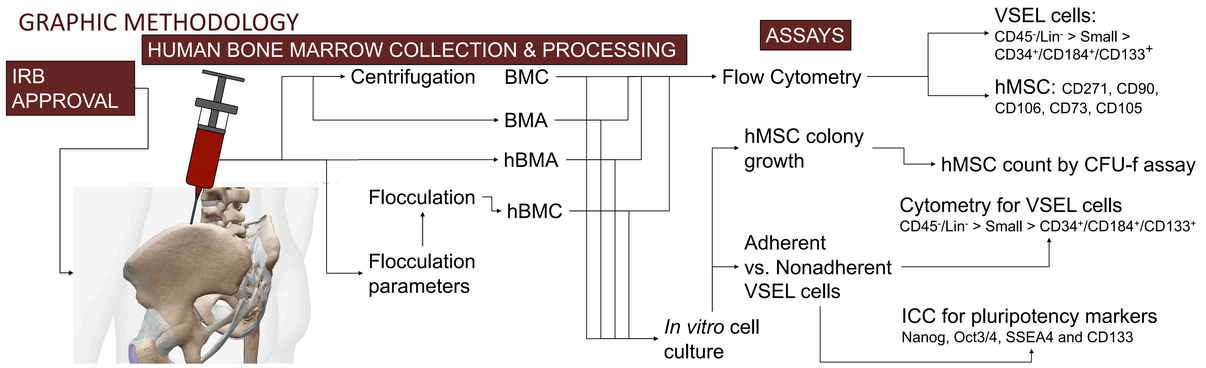

Figure 1. Methods. Exemption was granted by the Texas State University Institutional Review Board. Samples were aspirated over anticoagulant (ACDA). Purchased human blood was used to develop flocculation parameters (Lonza, Morristown, NJ). Samples from the same donor were used both methods side by side. Cells were quantified by flow cytometry and colony formation (CFU-F, for hMSC only). Marrow concentrates were expanded *in vitro* and the presence of hMSC and VSEL cells were measured in adhered cells. Non adherent cells were probed for the presence of VSEL cells by cytometry and immunocytochemistry.