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Research Article

A Comparative Quantification in Cellularity of Bone Marrow Aspirated with two New Harvesting Devices, and The Non-equivalent Difference Between A Centrifugated Bone Marrow Concentrate And A Bone Marrow Aspirate As Biological Injectates, Using A Bi-Lateral Patient Model

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ABSTRACT

The first aim of this study was to examine the cellularity and quality of autologous bone marrow aspirates harvested with two novel FDA-cleared devices, namely the Aspire™ bone marrow aspiration system (AS-BMAS) and the Marrow Cellution bone marrow aspiration device (MC-BMAD). Compared to traditional bone marrow harvesting needle systems, both these devices have a closed distal tip, avoiding preferential marrow collection (peripheral blood aspiration) from deeper cavity regions, whereas the side holes facilitate more horizontal marrow extraction. In all patients, a similar harvesting technique was used. The second aim was to demonstrate the effectiveness of mechanical centrifugation of a large volume of extracted bone marrow to produce a bone marrow concentrate (BMC). Finally, we directly compared bone marrow constituents aspirated with MC-BMAD with a BMC, generated by centrifugation of bone marrow harvested using the AS-BMAS. A bi-lateral patient model was used for all comparisons. All cellular analyses included the measurement of Colony-Forming Units-fibroblasts (CFU/f) levels, CD34+cells/ml, Total Nucleated Cells (TNCs)/ml, platelets/ml, and Red Blood Cells (RBCs)/ml in a single, FDA-approved laboratory, compliant with Good Manufacturing Practice regulations. A total of 12 patients consented to the study. In the direct BMA comparison, the AS-BMAS bone marrow yielded significantly higher CFU/f counts and TNC concentrations than the MC-BMAD (1,060/ml, 33.5 × 10^6/ml, and 610/ml and 28.6 × 10^6/ml, respectively), with comparable platelet and RBC concentrations. Data following BMA concentration to produce a BMC revealed highly significant cell yields, fewer RBCs, and lower hematocrit (HCT). A direct cellular comparison between the aspirate of the MC-BMAD and centrifugated BMC following AS-BMAS marrow extraction showed highly significant differences in cellularity. The AS-BMAS produced cell concentrations of CFU/f, CD34+ cells, TNCs, platelets, and RBCs that were comparable, or greater than, the predicate device. We believe that concentrating bone marrow is a consistent and safe method to produce a BMC that has the potential to be clinically effective. Furthermore, data indicate a non-equivalent difference in BMC cellularity, when compared to a non-filtered and non-centrifugated BMA for clinical use.

Keywords: Bone marrow concentrate; bone marrow aspirate; mesenchymal stem cells; preferential aspiration

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INTRODUCTION

Bone marrow aspiration procedures are essential to collect fresh bone marrow (BM) for preparing autologous bone marrow concentrate (BMC) [1] in order to successfully treat patients with a variety of musculoskeletal disorders (MSK-D) [2], as well spinal disorders [3], chronic wounds, and critical limb ischemia [4]. In interventional orthobiologics, BMC injection therapies are primarily performed because BM contains mesenchymal stem (stromal) cells (MSCs), which play pivotal roles in tissue regenerative healing processes [5]. In particular, they are involved in the repair and reconstruction of multiple tissues across a number of interventional procedures, due to the plasticity of multipotent progenitor cells (MPCs), and thus they can differentiate into multiple unique cell lineages, such as bone, cartilage, muscle, nerve, and tendon in vitro [6]. However, the cellular content of BM is more complex and distinct, as it also contains hematopoietic stem cells (HSCs), various nucleated cells, platelets, growth factors, and other cytokines [7]. Unfortunately, the number of MSCs is low, estimated to vary between 0.01-0.02percent of the total BM cell volume [8]. An accepted method for increasing the concentration of MSCs and other BM cells is to harvest a calculated amount of fresh BM volume, in order to employ density gradient centrifugation techniques, to produce BMC at point-of-care. However, in the past the collection of larger volumes of BM contributed to significant peripheral blood aspiration, with lower numbers of MSCs and MPCs [9]. Currently, innovative BM needle harvesting systems are available, which are designed to potentially minimize large peripheral blood aspiration during BM harvesting. Furthermore, the proprietary design characteristics of these novel harvesting devices are aimed towards more efficient marrow aspiration dynamics, contributing to higher cellular yields in collected BMA specimens. However, conditional negative forces occur while pulling the syringe plungers to aspirate BM. These high shear forces are likely to cause cell membrane damage during forceful collection of BM tissue [10]. A distinct difference between the two devices in this study is that the MC-BMAD is intended to be used as an aspirate-collecting device only, with direct application to patients, avoiding filtration and concentration processing steps. The AS-BMAS, however, extracts BM tissue, which is subsequently processed with a dedicated centrifugation and concentration system, to a generate BMC as an injectate. The purpose of this study was to analyze and compare BM cell compositions in three direct comparisons. We compared and analyzed BM aspirates harvested with two novel BMA devices (Aspire™ and Marrow Cellution™), using contralateral posterior superior iliac spine (PSIS) BM extractions from the same patient. The effectivity and cellular yields of mechanical centrifugation of a large volume of extracted BMA was investigated. Most importantly, we quantified the cellular differences between unprocessed BMA injectates (MC-BMAD), with BMA harvested with the AS-BMAS, and subsequently processed to prepare a BMC injectate. We postulate that there are minimal quantitative and viability differences between the BM collected via the two BMA needle systems. However, we hypothesize that BMA cell populations will be significantly concentrated in a viable BMC injectate, with a lower HCT, when compared to the larger BMA volume. Finally, comparison 3 will elucidate the differences between a non-purified BMA injectate (MC-BMAD) and a filtered and concentrated BMC as an injectate.

MATERIALS AND METHODS

Patients

In this multi-center, prospective study, 12 selected patients consented to a bi-lateral BMA harvesting procedure from the PSIS prior to their interventional orthopedic injection procedures to treat a variety of MSK disorders. Patients eligible for inclusion in this study were aged between >40 and <65 years, had not used nonsteroidal anti-inflammatory drugs within 2 weeks of the procedure, had not had a cortisone injection within 6 weeks of their scheduled BMC procedure, did not use oral anticoagulants or immunomodulatory-immunosuppressive medications, and did not have an active systemic infection or active malignancy, with a hemoglobin level <10 g/dL. Patients were only recruited when they were already scheduled for a BMC procedure, i.e. they did not undergo BMA harvesting just for this study.

BMA harvesting devices

In this study, two different BM harvesting devices were evaluated, namely the Aspire™ Bone Marrow Harvesting System (AS-BMAS) (EmCyte Corporation, Fort Myers FL, USA), and the Marrow Cellution Bone Marrow Aspiration Device™ (MC-BMAD) (Ranfac Corporation, Avon MA, USA) (Figure 1). In detail, the AS-BMAS contains minimally invasive instrumentation to collect BM aspirate. The system is designed to gently penetrate the trabecular bone, supporting a quiescent tissue environment during deployment and BMA collection. The actual BM aspiration needle has a completely closed and blunt distal tip, with three specific aspiration orifices to avoid tissue activation, to minimize peripheral blood aspiration, and to induce clotting. The AS-BMAS is primarily intended to collect sufficient BMA volume for centrifugation processing to produce a BMC as an injectate. The intended use for the MC-BMAD device is to collect just 10 ml of BM for direct injection into patients (without processing or filtering). The device has a similarly designed closed-tip as the AS-BMAS, although the tip is not blunt and the aspiration openings have a similar design as the traditional Jamshidi™ needle (Ranfac Corporation, Avon MA, USA).

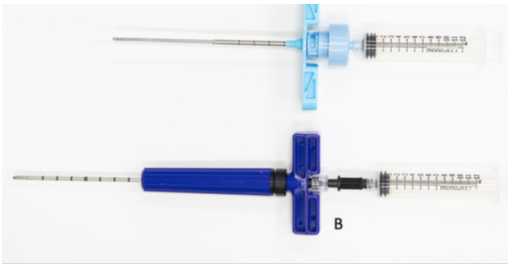


Figure 1: Bone marrow harvesting devices used in this comparative study (A) The Aspire™ Bone Marrow Harvesting System (AS-BMAS) (EmCyte Corporation, Fort Myers FL, USA) (B) The Marrow Cellution Bone Marrow Aspiration Device™ (MC-BMAD) (Ranfac Corporation, Avon MA, USA)..

Study design

In this study, we performed three comparisons to determine the effectiveness of two novel BM harvesting devices, regarding their capacity to yield marrow cellular constituents and provide detailed data on the differences of a BM injectate and a BM concentrate (Figure 2). Prior to harvesting, patients randomly chose which PSIS was used for the MC-BMAD. The contralateral PSIS was then assigned to the AS-BMAS. In the first comparison, a bi-lateral patient model was used to compare the BMA cellular content harvested with the AS-BMAS and the MC-BMAD, following a single cortical puncture. In this comparison, the instructions for use from both companies were followed for introducing the aspiration needle into the PSIS marrow cavity. Thereafter, an identical marrow aspiration

method was performed for both devices using 10 ml syringes and a quick-sharp pull technique. Exactly 10 ml of BM was harvested from each PSIS, using both devices, at the same needle depths, and vials were prepared for laboratory analysis. In the second comparison, after the initial 10 ml of BMA was harvested by the AS-BMAS, another 56 ml of aspirate was collected from the respective PSIS. The collected BM was consolidated into a 60-ml syringe and meticulously passed through a 200-micron BM filter before the filtered aspirate was transferred to the PureBMC® concentration device (EmCyte Corporation, Fort Myers FL, USA) to prepare centrifuged BMC (EC-BMC).The third comparison consisted of comparing the cellular differences between a BMA injectate and a centrifugated BMC injectate.

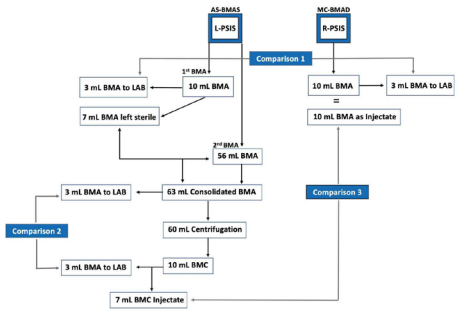


Figure 2: Study design flow chart. Abbreviation: AS-BMA: Aspire bone marrow aspiration system; MC-BMD: Marrow Cellution bone marrow aspiration device; L-PSIS: left posterior superior iliac spine; R-PSIS: right posterior superior iliac spine; BMA: bone marrow aspirate; LAB: laboratory; BMC: bone marrow concentrate.

Bone marrow aspirate collection protocol

Prior to starting the BM harvesting procedures, a thorough heparin wash was performed, using 15 ml of a heparin solution with a concentration of 1,000 IU/ml. All BM device components, aspiration needles, BMA concentration assemblies, collection syringes, and filters were rinsed three times. The 10 ml BM harvesting syringes each contained 1.0 ml of the heparin solution as an anticoagulant. Patients were placed in a prone position. The BM harvesting PSIS sites were confirmed via ultrasound, or fluoroscopy, depending on the clinic. Thereafter, local anesthetics were used for pain management in all patients. The areas of aspiration were sterilely prepared and draped, as per standard protocol. Multiport BM aspiration needles were employed to pass the bone cortex and enter the marrow cavity for both aspirates, and the appropriate volumes for the two comparisons were collected from each posterior iliac crest, using imaging techniques when appropriate. For the first comparison, 10 ml of aspirate was collected with both devices utilizing both PSISs, following the manufacturer's instructions. In all patients, the MC-BMAD was used first, at a PSIS site chosen by the patient. Thereafter, the AS-BMAS was employed in the contralateral PSIS using a 10 ml syringe, applying a quicksharp pull technique to acquire 10 ml of BM, starting subcortically. After aspirating 1.5 ml of BM, the harvesting needle was rotated 90° clockwise, followed by aspirating another 1.5 ml of BM. Thereafter, the needle was advanced 0.5 cm, and the same aspiration steps were repeated until a total of 9 ml was collected to complete the 10 ml total volume, in three steps. This first syringe was clearly labeled and prepared for laboratory sample analysis. The AS-BMAD was carefully kept in place, without exiting the marrow cavity. For all patients, 3.0 ml of this sample was used in a dedicated laboratory vial for analysis, while the remaining 7.0 ml was kept sterile. At this stage, the AS-BMAS device was repositioned, subcortically to start BM collection for the second comparison, obtaining a total of 56 ml of aspirate, using 10 ml syringes, exploiting quick-sharp pulls of the plunger. In the first five syringes (all loaded with 1 ml of heparin) after aspirating 4.5 ml BM, the harvesting needle was turned in a clockwise direction by 90°, and an additional 4.5 ml BM was collected to complete a volume of 10 ml in the syringe. Subsequently, the needle was moved 0.5 cm deeper into the marrow cavity and the same harvesting steps were initiated. In the final syringe, 5.5 ml of BM was collected in 0.5 ml of heparin. Thereafter, all six syringes and the remaining 7 ml BM of the first BM aspiration were connected to the BM filter for passage, and the filtered BM was collected in one consolidation syringe (BMA-60), containing 63 ml for sampling. Prior to sampling, the BMA-60 syringe was adequately agitated by 180° for 10 times, clock to counterclockwise, to assure proper mixing in order to facilitate an even cellular distribution. Furthermore, this provided an adequate state of anticoagulation prior to transferring the BMA-60 to the concentration device for BMC preparation.

BMC preparation

After the sample aliquot was taken, the BMA-60 volume was processed using the PureBMC processing platform (EC-BMC). A two-step centrifugation and preparation protocol concentrated the aspirate cellular content to a BMC. Following a first centrifugation spin, the BMA was sequestered in a BM plasma fraction (BMPF), containing a buffy coat layer and RBCs. The BMPF was aspirated, immediately followed by a separate collection of 2 ml of RBCs, following the manufacturer's instructions of the PureBMC concentration device. Both volumes were then transferred for a second centrifugal spin cycle to the concentration accessory device. During the second spin, the BM cells were concentrated and attached to the bottom of the device. Excess BMPF was manually removed, leaving behind a specific BMC volume for resuspension. The final BMC volume was approximately 10.5 ml, leaving a 3.5 ml sample aliquot for analysis, and 7 ml for patient treatments.

Laboratory analysis

All BMA and BMC preparations were meticulously agitated, following the laboratory instructions; aliquots were then taken and shipped for analysis to an independent, FDA and Good Laboratory Practice accredited laboratory (Bio Sciences Associates, Cambridge MA, USA).

Quantification of platelets and red blood cells

Complete blood counts (CBCs) were performed using a 3-part differential hematology analyzer to quantify the platelets, RBCs, and calculated HCT, contained within the start sample and platelet concentrates. The platelet concentration factor, which is the ratio of the concentration of platelets in the platelet concentrate product to the concentration of platelets in the start sample (adjusted for dilution with anticoagulant), was determined for each device. CBCs were measured according to the BSR TM-076 Coulter Ac-T diff 2 Hematology Analyzer. Total nucleated cell counts were performed using a Beckman Coulter AcT diff2 hematology analyser (Beckman Coulter, Brea, CA) for baseline samples and BM concentrates. Cell counts were performed in open sample mode according to the manufacturer's and laboratory's standard procedures. Prior to sample cell counts, the analyzer passed all system setups, calibration and daily quality control testing.

Flow cytometry

Samples for flow cytometry were prepared and analyzed as recommended by the International Society for Hematotherapy and Graft Engineering [11]. Total Nucleated Cells (TNCs) (1×10^6 cells/sample) were incubated with PE anti-human CD34 and anti-human CD45 Alexa Fluor 647 for 15 min at room temperature. To validate the specificity of the CD34 antibody, a control sample was also prepared with an isotype control. Lysis buffer was added to each sample and incubated for 10 min at room temperature. Cells were washed with PBS, 0.2% BSA before adding Cell Viability Solution and Counting Beads. Stained samples were protected from light and analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) immediately following processing.

The CD34 positive population, implemented as a hematopoietic stem cell (HSC) marker, determined using a single platform methodology, was defined as the CD45 'dim' and CD34 'bright' population. Cell viability was assessed by dye exclusion of 7-AAD solution. The 7-AAD negative population was reported as a percentage of viable cells. Spectral compensation between fluorescent channels was set using beads labeled with the respective fluorophores for corresponding channels. (PE Anti-human CD34, PE IgG1 k Isotype Ctrl, Lysing Buffer, Cell Viability Solution – BD Biosciences, San Jose, CA; Anti-human CD45 Alexa Fluor 647 – BioLegend, San Diego, CA; Counting Beads – Spherotech, Lake Forest, IL).

Colony-forming units-fibroblasts (CFU-f).

Samples were adjusted to a density of 2×10^6 nucleated cells per ml and cultured with supplemented mesenchymal stem cell growth media (StemCell Technologies, Cambridge, MA) at 37°C in 5% CO2. Following 10-14 days of incubation, non-adherent cells were removed by washing with PBS. Adherent cells were stained with Giemsa stain at room temperature (Ricca Chemical Company, Arlington, TX). Excess stain was washed away with distilled water. Colonies containing >50 cells with fibroblast morphology were counted using a Nikon Diaphot 300 microscope and reported as CFU-f per ml of sample. Isolation and expansion of MSCs were quantitatively and qualitatively assessed between testing and control culture conditions using two tailed t-tests.

Statistics

Statistical analyses utilized SAS/STAT software (SAS/STAT version 9.4. Cary, NC: SAS Institute Inc, 2014). Descriptive statistics are reported as mean and standard deviation (±). Statistically significant differences between groups were determined using independent sample or paired t-tests as appropriate, with a 95% confidence level on each principal effect means to account for multiple comparisons. All statistical tests were two-tailed, a p-value < 0.05 was determined to be statistically significant. Regression analyses were performed to quantify the relationship between the pairs of quantitative variables. Pearson product-moment correlation were calculated to ascertain the parametric measure of a linear relationship between pairs of variables. An adjusted R-Squared (Adj-R) value was added, this provides a measure of the amount of variation explained by the independent variable.

RESULTS

BM was harvested from 12 patients (8 men and 4 women), with a mean age of 59 ± 9 years, who all met the inclusion criteria as identified at the initial visit for an interventional orthobiological procedure. All patients underwent BM harvesting without any adverse events or complications.

Comparison 1

In the first comparison, 10 ml of BM was harvested from both PSISs, using single cortical punctures. A comparative quantification of laboratory data between the Aspire system and the Marrow Cellution BMA device is shown in Table 1.

Table 1: BMA cell analysis for comparison 1.

	RBC x 10^9/mL	HCT %	PLT x 10^6/mL	CFU-f/ mL	CD34+ /mL	TNC x 10^6/ mL	Viability
AS-BMAS	3.85 ± 0.64	37.8 ± 5.4	145 ± 70	1,060 ± 1,028	109,066 ± 120,039	33.5 ± 18.5	94.8
MC-BMAD	4.11 ± 0.64	35.8 ± 5.3	153 ± 53	610 ± 691	76,670 ± 48,143	28.6 ± 15.5	94.8
p value	0.06	0.07	0.6	0.041*	0.273	0.013*	0.92

BMA: bone marrow aspirate; AS-BMAS: Aspire bone marrow aspiration system; MC-BMAD: Marrow Cellution bone marrow aspiration device; RBC: red blood cells; HCT: hematocrit; PLT: platelet; CFU-f: Colony forming units-fibroblast; TNC: total nucleated cells

HCT, RBC, and platelet concentrations: The HCT, RBC and platelet count in the BM samples collected with the two different marrow aspiration devices were not significantly different, with a similar SD for both devices.

CD34+ cell concentrations: Overall, the HSC content was higher in the AS-BMAS group (109,066 SD 120,039) than in BM harvested with the MC-BMAD (76,670 SD 48,143), but this difference was not statistically significant.

TNC concentration: Concentration of TNCs were significantly higher in the BM from AS-BMAS patients compared to the MC-BMAD group: 33.5 x 10^6/ml vs. 28.6 x 10^6/ml, respectively (P<0.013).

CFU-f cultures: The mean CFU-f counts after 14 days of cell culture for the AS-BMAS and MC-BMAD was 1,060 (SD 1,028) and 610 (SD 691), respectively. There was significantly greater CFU-f in the BM from AS-BMAS patients compared to the MC-BMAD group (P<0.0412). In both groups, the standard deviation was very large.

Cell viability: Mean cell viability of both BM groups was similar (P>0.92).

CD34+ cells and TNC correlations with CFU/f counts in 10 ml aspirates. The analysis included exploration between CD34+ cell counts, TNCs, and CFU/fs in the 10 ml of bi-laterally harvested BM. Figure 3 shows the plotted CD34+ cell counts from the AS-BMAS against the CFU/f counts. The Pearson's correlation coefficient was 0.5288, with an adjusted R-value of only 0.1894 (P=0.0882).

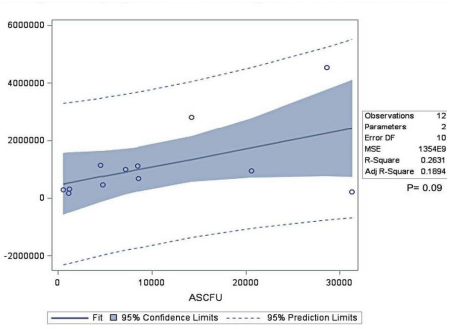


Figure 3: Regression analysis plotted CD34+ cell counts against the CFU/f counts for the AS-BMAS. Abbreviation: ASCD34: Aspire CD 34 cells; ASCFU: Aspire Colony Forming Units.

The TNC cell counts, however, were significantly correlated with CFU/f counts. A strong positive association was seen with an Adj-R value of 0.7939 (P<0.001), with 95% confidence limits. The CFU/f counts of the MC-BMAD aspirates were at an Adj-R value of 0.7072 correlated with TNC counts (P<0.001). The CD34+ cell counts did not correlate with the CFU/f counts (P=0.9445). No data outliers were removed from the correlation analyses.

Comparison 2

The cellularity of the consolidated BMA-60 volume, harvested with the AS-BMAS, was compared with prepared BMC in comparison 2. The BM was always filtered before BMC centrifugation. In Figures 4 and 5, a comparative quantification of laboratory data between the BMA-60 aspirates and EmCyte BMC product is presented. The average BMA volume prior to processing was 61.7 ml (SD 1.5 ml), and the mean centrifugated BMC volume was 11.1 ml (SD 2.9 ml).

HCT, RBC, and platelet concentrations: The HCT percentage and RBC counts were significantly lower in the BMC compared to the collected BMA prior to processing. The HCT was reduced by 26% (Figure 4A), and the RBCs were decreased to 1.23 × 109/ml, P<0.0001 for both parameters (Figure 4B). Conversely, the BMC platelet count was significantly increased to 5.2 times the BMA value to 657,000 × 10^6/ml, P<0.0001 (Figure 4C).

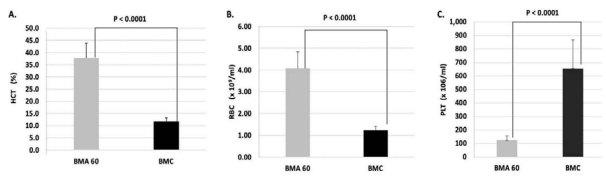


Figure 4: Change in hematocrit, RBC, and platelet concentrations after centrifugation of bone marrow aspirate collected with the AS-BMAD to produce a bone marrow concentrate. Abbreviation: BMA: Bone marrow aspirate; BMC: bone marrow concentrate; HCT: hematocrit; RBC: red blood cell; PLT: platelet

CD34+ cell concentrations: Overall, the CD34+ cell concentration in the BMC group was higher than in the AS-BMAS aspirate group (159,272/ml and 67,602/ml respectively), P<0. 002).

TNC concentration: The mean BMC TNC count increased by 176% compared to the BMA-60 aliquots, 76.4 × 10^6/ml and 33.0 × 10^6/ml, respectively (P<0.001).

CFU/f cultures: After the culturing period for both BMA and BMC specimens, the CFU-f counts increased to 1,182 ± 689 per ml in the BMC group (P<0.0002), as shown in Figure 5B.

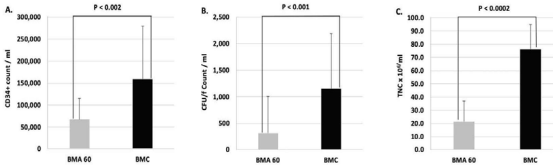


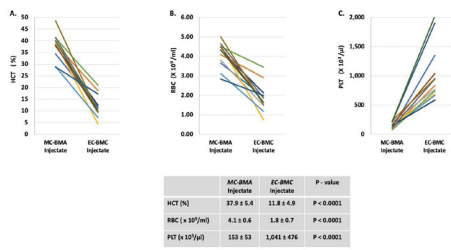
Figure 5: Change in CD34 + cells, CFU/f, and TNC concentrations after centrifugation of bone marrow aspirate collected with the AS-BMAD to produce a bone marrow concentrate. Abbreviation: BMA: Bone marrow aspirate; BMC: bone marrow concentrate; CFU/f: colony forming units for fibroblasts; TNC: total nucleated cells.

Cell viability: The cell viability between the BMA-60 before processing (94.6%) and the final BMC injectate (93.9%) was not statistically different.

Comparison 3

In this comparison, we quantified the cellular differences of a filtered and concentrated BMC specimen (EC-BMC) and a non-filtered and unconcentrated BM tissue sample (MC-BMAD). Both biologics are clinically used in patient treatments. The cellular concentrations in this comparison were corrected for the MC-BMAD and EC-BMC specimens to 10 ml and 7 ml, respectively, to be compliant with the manufacturer's approved instructions and clinical indications for use. In Figures 6 and 7 the laboratory data for all individual patients are vividly presented to visualize the significant dissimilarities between the Marrow Cellution BMA injectate and the Aspire-EmCyte BMC as injectate. Additionally, the graphics provide transparency in the various ranges of the parameters among all individuals.

HCT, RBC, and platelet concentrations: The HCT percentage was reduced by more than 3-fold in the EC-BMC vials to 11.8%, compared to an average HCT of 37.9% in the marrow injectate of the MC-BMAD (P<0.0001). Similarly, the average RBC count was 1.8 × 109/ml, compared to 4.1 × 109/ml in the BMA only sample (P<0.0001). The platelet count in the EC-BMC injectate was 6.8 times higher than in the BMA specimen harvested with the MC-BMAD (P<0.0001) (Figure 6A-C).



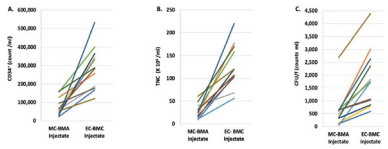
	MC-BMA Injectate	EC-BMC Injectate	P - value
HCT (%)	37.9 ± 5.4	11.8 ± 4.9	P < 0.0001
RBC (x 10^6/ml)	4.1 ± 0.6	1.8 ± 0.7	P < 0.0001
PLT (x 10^3/µl)	153 ± 53	1,041 ± 476	P < 0.0001

Figure 6: Individual cellular differences and mean values and standard deviation for the two groups regarding hematocrit, RBC, and platelet concentrations between injectates prepared with the MC-BMAD and after centrifugation with the EC-BMC procedure. Abbreviation: MC-BMAD: Marrow Cellution bone marrow aspiration device; EC-BMC: EmCyte Corporation bone marrow concentrate; HCT: hematocrit; RBC: red blood cell; PLT: platelet.

CD34+ cell concentrations: Generally, the HSC content (measured as CD34+ cell concentrations) was significantly lower in BM acquired with the MC-BMAD, compared to the BMC product (76,670/ml and 288,168/ml, respectively) (P<0.0001).

TNC concentration: Concentrations of TNCs were significantly higher in centrifugated EC-BMC samples compared to BM

aspirated with the MC-BMAD (EC-BMC: 126,125 × 10^6/ml; MC-BMAD: 28.6 × 10^6/ml, Figure 7B) (P<0.0001).



	MC-BMA Injectate	EC-BMC Injectate	P - value
CD34+ (count/ml)	76,670 ± 48,143	288,168 ± 116,818	P < 0.0001
TNC (x 10⁶/ml)	28.6 ± 15.5	126,125 ± 47,049	P < 0.0001
CFU/f (counts/ml)	610 ± 691	1,835 ± 1,093	P < 0.0001

Figure 7: Individual cellular differences and mean values and standard deviation for the two groups regarding CD34 + cells, CFU/f, and TNC concentrations between injectates prepared with the MC-BMAD and after centrifugation with the EC-BMC procedure. Abbreviation: MC-BMAD: Marrow Cellution bone marrow aspiration device; EC-BMC: EmCyte Corporation bone marrow concentrate; HCT: hematocrit; RBC: red blood cell; PLT: platelet.

CFU/f cultures: The CFU/f counts after culturing are presented in Figure 7C. The EC-BMC prepared vials had a CFU/f count that was 3 times greater than the MC-BMAD vial (P<0.0001). Patient variability was noted by the large standard deviation in both products (610/ml, 1,835/ml, respectively).

Cell viability: The cell viability after BMC processing (93.9%) was not statistically different compared to the MC-BMA BM product (95.0%) (P=0.37).

DISCUSSION

Tissue regeneration and effective remodeling requires the effective recruitment and activation of, for example, BM stem cells, progenitor cells, and other cells, whose progeny are capable of repairing and regenerating tissues. As a result, the rationale of cell-based therapy strategies requires a clear understanding of the functionality of BM harvesting devices, as well as differences between a non-processed BMA injectate and a BMC treatment vial, and the differences in cell concentrations. The purposes of this study, therefore, were to compare the differences in cellular content and viability of BM tissue harvested with two recently introduced BM harvesting devices, following a single cortical stick and a 10 ml marrow aspiration. We used a bilateral PSIS patient model in all patients to strengthen the validity of the results. Furthermore, we presented the increase in cellular yields, while simultaneously reducing RBC content, after concentrating 60 ml of harvested BM aspirate (AS-BMAS) by a commercially available BMC system. Finally, we quantified the cellular differences of an unprocessed BMA injectate (MC-BMAD) with a concentrated and filtered BMC injectate (EC BMC). To our knowledge, no previous studies have evaluated the cellular content of BMAs harvested with two different aspiration systems, both designed with a closed distal tip with multiple side openings. Moreover, we employed similar aspiration volumes, aspiration depths, utilizing a quick-sharp pull technique in comparison 1. BM tissue comprises a heterogeneous mix of cells, including MSCs (measured as CFU/f), HSCs (CD34+ expressing cells), progenitor cells, TNCs, platelets, RBCs, and interleukin-1RA [12]. The role of platelets (megakaryocytes) and RBCs are rarely mentioned in the literature as components of BMderived injectates [7]. Equally important, the roles and deleterious effects of RBCs on autologous-prepared orthobiological treatment vials have scarcely been discussed [10-13]. In a volume of BMA, the concentrations of the cells are similar to the concentration of cells that are present in the BM cavity. However, Scarpone et al. showed predominantly higher CFU/f counts and similar TNC and CD34+ concentrations when the MC-BMAD was used to aspirate 8 to 10 ml of BMA, compared to the BMC prepared using two different centrifugation devices [14]. Noteworthy, in all patients, the BM centrifugation devices processed each 60 ml of BMA, which was harvested with the traditional Jamshidi™ needle (Ranfac, Avon MA, USA). The enumeration and quality of BM harvested with the two different BMA needles suggests the comparison of apples and oranges (i.e. incomparable items), based on the typical design characteristics for both BMA needles. Therefore, we employed two recently introduced BMA systems, both with a completely closed distal tip. The difference in distal tip design between the two aspiration needles is that the AS-BMAS utilizes an entirely blunt tip, while the MC-BMAD has a partially blunt tip, with sharp edges, as shown in Figure 8. A distally closed tip allows lateral BM aspiration only, with a greater horizontal plane to preferentially collect more BM cells, instead of a vertical and deeper plane that facilitates the collection of more peripheral blood [15]. In addition, the lateral ports target the (sub)endosteal BM niche more efficiently, in order to collect more MSC from this area, as previously recognized by Mendez-Ferrer et al. [16]. Another different design characteristic is that the Aspire™ harvesting system has three lateral aspiration orifices, each with a relatively large surface area. Two orifices are located on the same side of the aspiration needle, and a third single port is on the opposite side (at 180°) of the needle. The MC-BMAD has aspiration openings positioned over the entire needle circumference. Theoretically, during 90° needle redirection, the AS-BMAS, at the same harvesting depth, will allow a two-sided horizontal non-harvested and rich cellular and MSC BM tissue plane. Instead, turning the MC-BMD at the same depth, could result in a less rich cellular BM environment, compared to the AS-BMAS, as the aspiration ports are allocated over the entire needle configuration, and a complete non-harvested BM tissue plane by turning 90°, is not feasible. Peer-reviewed articles and textbooks rarely mention the syringe-pulling technique. The rationale for a rapid, so-called quick-sharp BM aspiration technique is to harvest a BM specimen with high cellularity and quality. Gronkjaer et al. concluded that the quality and cell count of BMA using the quick-sharp pull-technique is better than using a slow pulling technique to fill the syringe, although the pain intensity is significantly higher with the rapid pull technique. However, in-depth analysis of the VAS pain scores revealed that the difference in pain intensity is only moderate compared to the general variability of the VAS pain score [17]. During marrow harvesting, rapid and quick-sharp pull techniques force marrow fluid with incorporated cellular content, through the orifices of the harvesting needle, resulting in turbulence, as measured by the Reynolds shear stress (RSS) metric [18]. High RSS results in RBC damage, presented by cell deformability with the release of the inflammatory hemoglobin components heme and iron, measured as a percentage of hemolysis [10]. Therefore, harvesting needles with larger aspiration surfaces, will either produce lower RSS values and thus less cell damage, or allow application of more negative pressure while maintaining the same RSS number, compared to aspiration needles with smaller aspiration openings [19,20]. Interestingly, previously published data indicate that a significant factor in harvesting BM is the size of the harvesting syringe, and the frequency of BM aspirations. Hernigou et al. demonstrated that the first 10 ml of aspirated BM contains the highest number of CFU/fs, compared to larger aspiration syringes, and multiple series of marrow aspiration [21]. In order to control the level of negative pressure in the marrow cavity and to maximize marrow tissue yield during aspiration procedures, 10 ml syringes have been recommended [22]. This is

also in agreement with small-volume rapid and quick-sharp pull aspiration techniques [23]. Furthermore, we believe that 10 ml harvesting syringes contribute to a better anticoagulation regimen, as they fill considerably quicker than larger syringes. Subsequently, smaller syringes can be more rapidly and effectively agitated to mix the anticoagulant with the BM to avoid clotting.

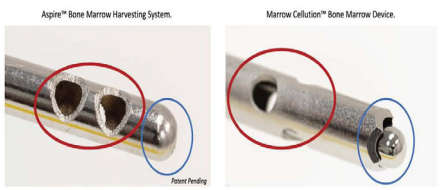


Figure 8: Differences in distal tip design between the two aspiration needles..

In comparison 1, we compared 10 ml of BMA harvested with both needle systems, at the same depths, using one 10 ml syringe while employing quick-sharp pulls to aspirate 10 ml of BM. Data from this comparison revealed that the AS-BMAS produces 10 ml BMA with significantly higher CFU-f and TNC concentrations, compared to the MC-BMAD. In general, the CFU/f cell counts in the BMA harvested with both systems were significantly higher than published BMA data [9,24]. It is well known that BM holds several specific cellular and molecular microenvironments, known as BM niches [6]. More specifically, several authors have indicated that the majority of BM MSC populations originate within the cortical bone, endosteal region, and as perivascular niches [25,26]. These findings are noteworthy, as deeper BMA needle positionings in the PSIS will not facilitate the harvesting of more MSCs, but merely contribute to the collection of large volumes of peripheral blood. This effect is more pronounced when BM harvesting needles are used with an open-ended distal tip, preferentially drawing through the large open tip from the inner marrow space. In contrast, both BMA systems used in this study only draw from the lateral ports and not the center, due to the closed, distal tip, collecting marrow samples rich in CFU/f. In addition, when these harvesting systems are placed below the cortical bone and in the endosteal region, the lateral aspiration ports favor the aspiration of marrow from these regions only. The aspiration ports neighbor these specific areas in a horizontal plane, without aspirating from a vertical, more peripheral plane, as the distal tip is closed. In order to better characterize and compare the marrow tissue cellularity of the 10 ml of marrow aspirates harvested with both devices, we assessed the correlation between CFU/f counts and TNCs by multiple regression analysis (Figure 9).

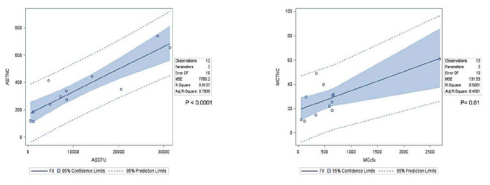


Figure 9: Regression analysis plotted TNCs against the CFU/f counts for the AS-BMAS and MC-BMAD. Abbreviation: ASTNC: Aspire Total nucleated cells; ASCFU: Aspire colony forming units; MCTNC: Marrow Cellution total nucleated cells; MC-CFU: Marrow Cellution colony forming units.

Despite the fact that the BMA procedures were performed by four physicians, the AS-BMAS and MC-BMAD presented a significant correlation, between CFU/f counts and TNC concentrations ($P<0.0001$ and $P=0.01$, respectively). This solid consistency in the marrow cell extraction during the harvesting procedure can be attributed solely to the design characteristics of both needle systems, as the Adj-R value for CFU/f and TNC for both harvesting devices was significant, although the correlation coefficient of the AS-BMAS was higher than for the MC-BMAD, evidenced by high Adj-R values (0.794 vs. 0.450, respectively). No significant correlations, with weak Adj-R values, were observed for CD34+ cells and CFU/f in BMA harvested with both devices. This could be because aspirating 10 ml of BMA from the endosteal and sub-endosteal regions initially contributes to the collection of a higher number of MSCs. The closed distal tip from both aspiration needles minimizes peripheral marrow-blood aspiration from the deeper areas, such as the central and perisinusoidal regions [27]. In the shallow and subcortical area, the aspiration needle side holes expedite a more preferential (horizontal planed) marrow aspiration, with higher yields of MSCs, compared to CD34+ cell concentrations in these regions. Theoretically, this observation indicates that MSCs, and not HSCs, are mainly located in the endosteal and subendosteal marrow regions.

Data from comparison 2 considerably support the beneficial effects of BMA centrifugation to produce a BMC. Our data corroborate other studies showing the effectiveness in concentrating bone marrow with centrifuges to yield higher cell counts and alter the BMC composition [24-28]. RBC content and HCT were significantly reduced in the BMC following the two-step processing method (4.08 to 1.23 × 109/ml and 37.8 to 11.7 % respectively, $P<0.001$). It is worth mentioning that the significance of RBCs in BMC treatment specimens has rarely been discussed, despite the fact that orthobiological injectates containing high RBC concentrations carry the risk of inducing tissue inflammation and cell damage [29]. Equally important is RBC cell membrane damage (hemolysis) as a result of high RSS numbers initiated by high shear forces during a BMA procedure. Destroyed RBCs release several highly inflammatory hemoglobin split products, such as heme, ferric-hemoglobin, and iron [30]. Consequently, these hemoglobin split products induce RBC disintegration, provoking eryptosis, with concomitant development of oxidative stress and potential tissue damage [31]. The platelet concentration in the BMC was increased by more than five times compared to the BMA vial. This significant yield in BMC platelet numbers is a critically important factor in BM regenerative medicine applications. Platelets contain numerous platelet growth factors, lysosomes, and cytokines with specific cellular functions in regenerative tissue healing pathways. Interestingly, several clinical studies have mentioned beneficial outcome effects when platelets were added to BMC treatment vials [32,33]. BM centrifugation significantly increased the CFU/f count in the BMC specimen, compared to the BMA-60 sample (316 vs. 1,157 CFU/f per ml, respectively). Noteworthy was the significant decrease in CFU/fs that was observed in the consolidated BMA-60 volume prior to BMC preparation. During the second harvesting procedure with the AS-BMAS, an additional 56 ml of BM was harvested, following the same method as earlier described.

Many factors may contribute to a wide variety of MSC counts following centrifugation procedures. The design features of BM concentration devices, executed preparation protocols, and various patient variables have been reported in the literature [28,34,35]. The Scarpone study is particularly noteworthy regarding their reported variabilities of CFU/f determinations, as they used three different, internationally located laboratories for CFU/f cell culturing procedures [14]. Pamphilon et al. concluded that when

laboratory data from different laboratories are used to compare MSC counts across BMA and BMC samples, this constitutes a significant confounding factor [36]. In another study, procedural variances were introduced by mandatory HCT settings of the centrifuge in order to yield a concentration of MSCs during the centrifugation process [37]. An accepted standard for BMA and BMC production would eliminate a lot of bias and contribute to more consistent patient results. Presumably, this would reduce the impact of marrow heterogeneity and reduce differences in BM cellular yield composition, positively impacting the biological activity and regenerative potency of the BMC treatment specimen, and thus clinical outcomes [38]. Finally, and importantly in comparison 2, no differences in cell viability were noted, indicating that the mechanical centrifugal forces did not have a negative effect on the cells causing cell damage.

The purpose of the final evaluation (comparison 3) was to assess the distinct characteristics between a non-filtered and unprocessed BMA as a clinical injectate, and a filtered and processed BMC orthobiological injectate with regard to cellular differences and viabilities of both injectates. Figures 6 and 7 represent cellular differences in this bi-lateral study model between the BM harvested with MC-BMAD with its intended use to serve as an unprocessed BM injectate and the centrifugated EC-BMC specimen. While other BMA cellular constituents were increased following BMA centrifugation, the RBC cell count was significantly reduced in the EC-BMC Figure 6A and 6B, respectively). This is a meaningful discrepancy between BMA as an injectate and BMC as an orthobiological treatment specimen. In this study, the RBC concentrations in MC-BMAD samples was 3.5 times higher compared to the centrifugated BMC, with a correspondingly 69% reduction in HCT in the EC-BMC vials. Furthermore, an absolute difference in platelet count in the EC-BMC vials, compared to MC-BMAD preparations, is reflected by 6.8 times increase in platelets of the processed EC-BMC treatment vials. Incongruously, in their comparative analysis, the Scarpone group did not address the RBC content and platelet concentrations of the MC-BMAD and BMC products prepared with two different centrifuges. These cellular differences may negatively affect the treated tissue microenvironment following BM injection therapies. In this regard, a study by the group of Roosendaal demonstrated in a human in-vitro study, that brief exposure of cartilage to blood resulted in lasting cartilage damage [39]. Particularly noteworthy is a recommendation by Mariani and Pulsatelli, to avoid RBC content in autologous biological products to avoid detrimental effects of RBCs to optimize patient outcomes following musculoskeletal therapies [13]. Intriguingly, recent data indicate that platelet growth factors play key roles in several MSC trophic mechanisms, like immunomodulation [40], MSC differentiation [41], and angiogenesis [42]. Hence, higher BM platelet concentrations potentially contribute to better patient outcomes, as the MSC concentrations are accordingly increased when compared to the native MSC counts, enabling additional trophic support mechanisms. In Figure 7A, both stem cell parameters are portrayed. CD34+ cell concentrations in the EC-BMC specimens were on average 159,272/ml, with a range of 75,175 to 333,315/ml, an increase of 2.4 times the pre-processing concentration. This increase is not in accordance with published data from Schafer et al., who noted no increase in CD34+ cells compared to control. This could be due to a higher cell yield during the harvesting procedure with the AS-BMAS in this study, as they were using a traditional BM harvesting needle, possibly inducing preferentially marrow collection from the open distal tip, allowing more peripheral blood aspiration. The CFU/f count in the EC-BMC sample was 93.7% higher than in the MC-BMAD, as shown in Figure 7B. Figure 7C shows the absolute differences of both platelet and TNC concentrations for all EC-BMC subjects. The EC-BMC platelet concentration was significantly higher in all patients compared to the MC-BMA vial, $P<0.0001$. TNC concentrations were almost three times greater in the EC-BMC than in the marrow aspirate only, $P<0.002$.

Some limitations of this study should be noted. Firstly, the two harvesting devices used to harvest BM were different, and it was not possible for the physicians to perform the marrow harvesting procedure in a blind manner using both devices. Secondly, the sample size for comparisons was small, contributing to a wide range of CFU/f values. However, statistical differences were noted in all comparisons. Thirdly, due to the study design and concomitant laboratory analyses, we were not able to use a complete primary BMC sample in comparison 3, comparing the unprocessed BMA injectate with the centrifugated BMC sample. From the initial 10 ml BMA aspirated with the AS-BMAS, 3 ml of this aspirate was sent to the laboratory for analysis. Therefore, a significant number of cells did not contribute to the BMC cellular composition. Based on the data of the BMA cellular analysis harvested with the AS-BMAD, it is reasonable to conclude that countless amounts of MSCs were not calculated for in the BMA vs BMC comparison. Future studies should include a larger sized bi-lateral study, comparing only initial BM drawn volumes to objectively compare a BMA injectate vs. a centrifuged BMC injectate.

The primary purpose of this study was to compare cellular differences between BMA harvested with two novel commercially available BM harvesting devices, as well as to evaluate differences in cellularity between centrifugated BMC and non-processed BMA products. To strengthen the validity of the result, a single donor model was applied to generate both biological products. Both aspiration devices had a completely closed tip, avoiding preferential distal peripheral blood collection, as BM tissue could only be aspirated through the side ports. These ports enable marrow to be withdrawn from across a greater horizontal geography, targeting the endosteal and sub-endosteal niches, regions known to be rich in mesenchymal stem and progenitor cells. From an effectiveness and consistency perspective, the AS-BMAS yielded higher CFU/f cell counts in the 10 ml aspirates, confirmed by the high correlation of CFU/fs and TNCs.

Concentrating 60 ml of BMA to produce a BMC revealed a significantly increased cellularity of all marrow constituents, in particular platelets, TNCs, and CD34+ cells. The CFU/f counts in the BMC were significantly increased compared to the BMA-60. However, the CFU/f counts were not significantly increased when compared to the counts of the first 10 ml of BMA. Our research supports results from the study by Hernigou et al. who showed that large volume aspirates tend to be infiltrated by significant amounts of peripheral blood, which contains fewer MSCs, leading to lower CFU-f counts [43]. In this study, centrifugation caused significantly higher CFU/f, TNC, CD34+ cell, and platelet yields, and considerably fewer RBCs when compared to BMA-60.

Despite enrichment of biologic factors, the question remains as to whether higher MSC content in BMC injectates can lead to improved clinical efficacy, as the role of other BM constituents, such as platelets, is still not established.

CONCLUSION

In conclusion, our study has shown a nonequivalent difference in BMC cellularity when BM is harvested with the AS-BMAS, compared to an aspirate collected with the MC-BMAD, which is intended for injection as a non-filtered and non-centrifugated aspirate.

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