Biologic Orthopedic Journal

Original Article
DOI: 10.22374/boj.v6i1.61

ISOLATION OF BONE MARROW MESENCHYMAL STEM CELLS EMBEDDED IN NATIVE TISSUE STROMA YIELDS ENRICHED HARVEST, IMPROVED ADHERENCE AND PROLIFERATION, AND UNIQUE SECRETOME

Ryan Dregalla PhD[1], Jessica Herrera BS[1], Lucanus Koldewyn BS[1,2], W. Sealy Hambright PhD[2], Chris Donner BA[1,2], Jacob Singer BS[2], E. Jeff Donner MD[1,2]*, Johnny Huard, PhD[3]

[1]Colorado Spine Institute, Johnstown, CO
[2]Elite Regenerative Stem Cell Specialists, Johnstown, CO
[3]Steadman Philippon Research Institute, Vail, CO

Author for correspondence: Jeff Donner: ejdonner@gmail.com

Submitted: 4 November 2023. Accepted: 21 February 2024. Published: 24 April 2024

Abstract

Background: The field of orthobiologics traditionally utilizes cellular products, including bone-marrow aspirate concentrate (BMAC), micronized adipose tissue, and platelet preparations to address pain from degenerative processes, orthopedic injuries and medical conditions characterized by chronic inflammation and tissue degradation. For BMAC, maximizing the concentration of mesenchymal stem cells (MSCs) in a reduced volume is thought to allow for the therapeutic delivery of the cellular concentrate, secretome, and extracellular vesicles to a site of orthopedic injury or surgical repair. The extracellular matrix (ECM) within the bone marrow stroma contains collagens and proteoglycans known to regulate cell proliferation, migration, differentiation, and cell-cell communication among resident bone marrow cells. This study aimed to evaluate the cellular effects on MSC health and function when harvested to retain their native tissue stroma.

Methods: We evaluated a novel and unique processing method and device (BMAX™) to mechanically generate a purified MSC product derived from bone marrow in a nonenzymatic manner. BMAX™ products, including cells and stroma, were plated in MSC culture media and incubated for 3–14 days (P0-P1) before evaluation with flow cytometry for cell phenotyping and immunoassays for secretome profiling.

Results: The orthobiologic product containing three-dimensional stromal components can be produced in minutes using an automated bedside device requiring minimal benchtop space. We found increased MSC adherence, improved proliferative density in culture, and significantly elevated enrichment of stromal-derived MSCs versus traditional BMAC centrifugation-based preparations. Further, we demonstrate a unique secretome profile in BMAX™ versus traditional BMAC centrifugation-based preparations.

Conclusions: These qualities provide a novel and unique platform for autologous and allogeneic bone-marrow-derived therapy to better address inflammatory and destructive processes that may improve bone-marrow-derived cell therapies' efficacy.

Keywords: *MSCs, bone marrow, bone-marrow aspirate concentrate, orthobiologics, osteoarthritis*

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

BACKGROUND

Mesenchymal stem cells (MSCs) are multipotent precursor cells with adipogenic, osteogenic, and chondrogenic potential. Through the secretion of various cytokines and growth factors, MSCs have also exhibited paracrine anti-inflammatory and trophic effects that promote tissue repair.[1–5] Bone-marrow aspirate concentrate (BMAC) has become a popular autologous source of MSCs for therapeutic use in many regenerative medicine applications.[5–10] The bone marrow microenvironment contains an array of cellular and structural components that mediate the health and function of resident MSCs and hematopoietic stem cells (HSCs). The cellular and acellular components of bone marrow have been found to regulate MSC proliferation, self-renewal, and differentiation and promote a pro-regenerative MSC secretome.[11] This includes the proteoglycan, glycosaminoglycan (GAG), and collagen-rich extracellular matrix (ECM) that promote cell adhesion and communication, which are important for hematopoiesis and bone formation.[12,13] The bone-marrow microenvironment is thus paramount for MSC health and function thus, the retention of native niche components may significantly improve autologous and allogeneic MSC-based therapies.

BMAC is obtained following step-wise centrifugation and bone-marrow aspirate (BMA) concentration to reduce red blood cells and granulocytes while enriching progenitor cells and associated cytokine/growth factors.[8] The procedure done minimally manipulatively is currently compliant with the US Food and Drug Administration guidelines for autologous use under the same surgical procedure exemption for homologous uses. In the case of BMAC, the objective has long been to maximize MSC yield in a reduced volume to allow delivery of the cellular concentrate to a site of injury. The percentage of MSCs in the bone-marrow compartment is relatively low, making up 0.001 to 0.01% of the nucleated cell population (i.e., does not account for mature red blood cells or platelets).[14,15] Nonetheless, aspiration and density gradient concentration of bone marrow into BMAC has been demonstrated to increase the concentration of MSCs from marrow by 400 to 600%.[16,17] BMAC is also enriched with soluble factors such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF-AA/BB/AB), and vascular endothelial growth factor which promote tissue repair.[18–20]

BMA can be harvested from different sites, including the tibia, calcaneus, or posterior iliac crest (pelvis/ilium). BMA is most often harvested from the iliac crest due to procedural ease and accessibility. It has yielded a greater number of MSCs and desirable amounts of cytokine/growth factor concentrations.[21] Multiple studies have found diminishing returns for volumes over 120 mL due to overt blood dilution and deviation from subcortical regions known to contain the highest amount of MSCs and other target cells.[22–24] Here, we report a human bone-marrow harvesting and processing technique that captures subcortical core samples that retain the three-dimensional properties of native stroma with minimal manipulation using a handheld device in an intraoperative window of time. As a result, adherent MSCs remain in contact with ECM components in culture with distinct morphological and secretory phenotypes compared to traditionally processed BMAC. The harvesting technique also yields a smaller volume negating diluting blood effects and results in a significantly lower hematocrit which has been shown to have deleterious effects on MSC function and tissue repair mechanisms.[25,26] Overall, this technique may improve efficacy for bone-marrow-derived therapies through retention of MSC functionality while also decreasing procedure time and patient/physician burden.

BMAC is a known orthobiologic product for treating many orthopedic-related injuries and musculoskeletal disorders, such as osteoarthritis (OA) and cartilage damage. Patient reports have shown BMAC's potential to attenuate pain associated with the specific injury while initiating a faster healing response. One issue is that BMAC still only contains a small percentage of MSCs, which might be why BMAC has limited cartilage regrowth. We propose that obtaining MSCs from the subcortical bone from the bone marrow maintains more of the "stemness" of the MSC's bone-marrow niche which might lead to better cartilage regeneration, making it a more promising therapeutic. Current ongoing *in vitro* and

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

in vivo experiments have confirmed that BMAX™ has more potential as an orthobiologic product than traditional BMAC.

METHODS

Human donors

BMA samples for a pilot series of patients (n=3) were obtained from consented human donors at the clinic site in conjunction with an ongoing procedure. BMAX™ processed samples were compared to patient-matched BMAC samples prepared through two-step centrifugation as described.[8]

Bone-marrow aspirate concentrate and BMAX™ preparation

Bone marrow was harvested from a single side from the iliac crest as described,[27] using the heparin 1,000 U/mL protocol. For the BMAX™ system, bone cores (BC) were harvested from the iliac crest using an 11-gauge trocar (Supplemental Figure 1). The BC was loaded into the sterilized BMAX™ prototype with 2.0 mL of total media to submerge the BC (as shown in Figure 1C). The BC enters a compartment that includes a rotating and actuating upper grinding plate that is designed to both grind the BC and create a microfluidic turbulence that pulls the GAG and cells from the bone and further micronizes the GAG. Subsequently, having been separated from the bone and in fluid suspension, the BMAX™ product containing micronized GAG and MSCs is removed from the device with a syringe (leaving the bone particles behind). It would be ready for immediate administration back to the patient via injection or further centrifuged into a pellet to treat defects. The prototype was mounted onto a test stand equipped with a variable speed motor to control RPM and a distance meter to control travel of the grinding plate (see Supplemental Video 1). The mobile (upper) grinding plate was set to 1.5 mm above the immobile grinding plate and then set at 180 RPM. Every 60 seconds, the grinding plate advanced in 0.25–0.05 mm increments for 2 min. At this point, supernatant was collected via aspiration including the glycosaminoglycans-rich component of the BC (BC-GAG) (Figure 1C.3) while leaving behind the denser residual calcified bone from the BC (Calcified-BC) which had settled at the bottom. Lastly, the Calcified-BC was collected separately from the BC-GAG for separate analysis.

Total Nucleated Cell and Viability Quantification

For donor-matched BMAX™ and BMAC-processed specimens (n=12), 10 µL from each sample was diluted before quantification using Neubauer hemocytometer/counting and viability assessment via Calcein-AM as described.[25]

In vitro Cell Culture Conditions

BMAX™ and BMAC products (including cells and stroma) were plated as described.[25] For TNF-α challenge, BMAX™ and BMAC-products from 100 mg equivalent of starting trabecular BC material were plated in 6-well plates with 2.5 ng/mL of TNF-α (BioLegend, Cat.# 570102) and carried out for 8-days.

Flow cytometry

MSCs from BMAX™ and BMAC cultures were identified via flow cytometry following International Society for Cell & Gene Therapy (ISCT) minimal criteria for multipotency[28] (minimum 10,000 cells analyzed).

Cytokine Chemokine Secretome Analysis from BMAX™ and BMAC Products

Cell culture medium from BMAX™ and BMAC preparations challenges with TNF-α described above were sampled at Day 0 (immediately after plating), Day 1, Day 4 and Day 8. These samples were stored at −20°C until analyzed via bead-based multiplex (BioLegend 13-plex LEGENDplex™, Cat.# 740502).

Statistical analysis

Data was analyzed in GraphPad Prism version 8.4.3. For total nucleated cell counts (TNC), viability and adherent cell counts, and densities from P0 and MSC populations (by percent) as determined by flow cytometry, an unpaired two-tailed t-test was used to compare the groups. In cytokine/chemokine concentration comparisons in TNF-α challenged

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

cultures, BMAX™ and BMAC groups were analyzed via an unpaired one-tailed t-test based on our hypothesis that the cellular constituency and cell-tissue construct of BMAX™-products will result in less inflammatory factors compared to BMAC-products. For all tests, normality testing of the data set determined if parametric or non-parametric parameters were used.

RESULTS

BMAX™ processing generates a customizable homogenized bone marrow product that contains viable MSCs within their native stroma

Subcortical core marrow samples are removed and ejected from the trocar with a typical weight range between 80–120 mg and 1.0–1.5 cm long, which we call the BC. Interested in the composition and cellular distribution of cells in the bone-marrow stroma, we stained the BC with Alizarin Red (red) and Alican Blue (blue) and positively detected calcified bone and glycosaminoglycan (GAG)-rich compartments, respectively (Figure 1A). In addition, freshly harvested BCs were briefly stained with calcein-AM and we identified spherical cells in the GAG component of the BC (Figure 1B). We designed many iterations of an enzyme-free custom prototype, BMAX™, to grind/mill the small BC samples and mechanically separate the calcified bone from the GAG component, yielding viable cells associated with the soft stroma (Figure 1C). The noncalcified GAG-rich material remains suspended immediately after processing

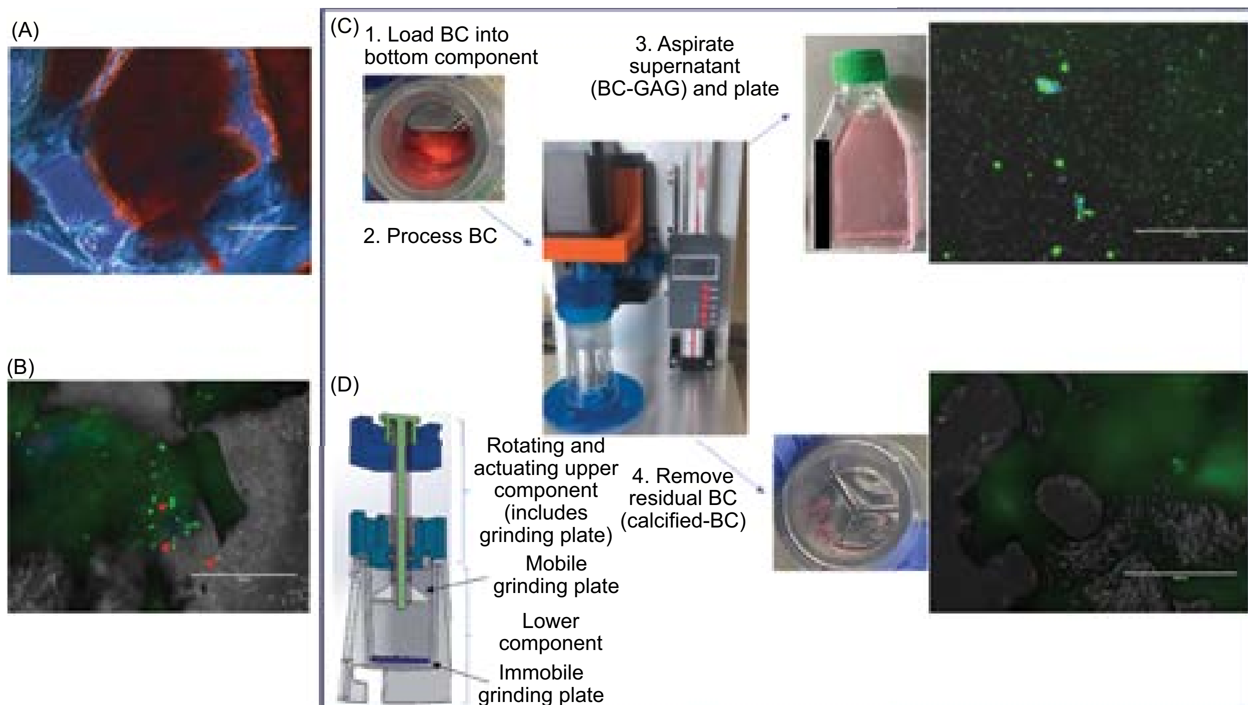


Figure 1. BMAX™ processing methodology. (A) A core derived from the iliac crest contains a calcified compartment (red positive staining via Alizarin Red) and a GAG-rich compartment (blue positive staining via Alican Blue). (B) Viable cells with a spherical morphology are observable within the loose GAG-rich compartment via staining with calcein-AM (red arrows). (C) The step-wise use of the BMAX™ processing system to mechanically strip the GAG-region (BC-GAG) from the calcified bone (calcified-BC) of the BC. The GAG-rich component remains in suspension, allowing it to be easily separated from the residual calcified BC. The GAG-rich component contains viable cells which were plated for further analysis. (D) Key components of the BMAX™ prototype.

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

and is easily collected apart from the dense calcified residual BC.

BMAX™ yields cells with clinically relevant viability, with lower nucleated cell counts compared to BMAC with unique in vitro growth characteristics

Following the processing of BC with BMAX, we performed post-processing assays that are typical of BMAC, using donor-matched BMAC as a benchmark. Immediately following the production of BMAX™ and BMAC-processed BCs, TNC and viability were determined. Because BMAC is a liquid volume, originating from a liquid starting material 50 mL BMA, the counts are reflected as count/microliter (µL) in the final product. In contrast, the BC is solid and based on weight (milligrams) and so, the TNC is reported based on the cells yielded from BMAX™ processing per mg of BC processed. Of clinical importance, this means that a physician can deliver the final BMAX™ product in any volume desired. On average, BMAC yielded 76,188 (±8358) cells per µL, whereas BMAX™ resulted in 46,050 (±6338) cells per mg of BC (P = 0.0088) (Figure 2A). Similarly, the viability of BMAC cells were significantly higher compared to BMAX™-processed BC cells, 95.83% (±0.6822) and 89.38% (±1.497), respectively (P = 0.0007) (Figure 2B).

We next plated and cultured a subset of BMAC and BC-paired donors to determine if the cells embedded within the GAG-rich stroma can establish culture. While BMAC formed typical colony forming units with a fibroblast morphology (CFU-f) by Day 12 (Figure 3A), BMAX™-derived cells (in donor-matched cultures) emerged from the GAG-particles which immobilized to the cell culture flask surface (Figure 3D). As is common in BMAC cultures, we observed that CFU-fs varied in cell density and size (Figure 3A[i] and 3A[ii]) and remained distinguishable from one another. In contrast, BMAX™-derived adherent cells grew consistently throughout the culture vessel, with several cells remaining attached to the GAG-stroma and no evidence of CFU-f formation (Figure 3 D[i]. and 3D[i]). We also allowed a subset of BMAX™ and BMAC-derived cell cultures to reach >90% confluence, as we observed that the BMAX™-derived cells appeared to grow in smaller confines before submitting to cell-contact inhibition. Cell quantitation at >90% confluence at P0 revealed that in donor-matched BMAC/BMAX™ cultures, BMAX™-derived cell cultures resulted in 1.7× more cells than BMAC cultures (Figure 2C), growing at a density of $34.90*10^3$ (±4.154) and $20.03*10^3$ (±1.846) per cm^2 (Figure 2D) (P = 0.0170).

BMAX™-derived adherent cells possess MSC characteristics

To verify that cellular content obtained from BMAX™ processed samples, adherent cells were analyzed using flow cytometry targeting ISCT-validated epitope signatures for MSC populations in cells from Passages 0, 1, 2 and 3 concurrent with donor-matched BMAC adherent cells. After gating to remove white blood cells, it was found that BMAX™-derived cells directly matched the percentage donor-matched BMAC adherent cells for CD90+/CD73+/CD105+ cells at each passage, resulting in 86.86% (±6.663) and 90.01% (±3.313) of the cells being positive for ISCT MSC surface marker criteria by Passage 3 (Figure 4A).

BMAX™-derived adherent cells have shown characteristics of an MSC based on plastic adherence, proliferation potential in standard culture conditions, and surface marker phenotypes. To verify that the actively proliferating BMAX™-derived cells contain a substantial MSC population, as our flow cytometry analysis suggested, we initiated a trilineage differentiation protocol on these cells from Passage 2. Treatment and staining protocols for adipogenic, osteogenic, and chondrogenic differentiation revealed positive results for BMAX™-derived adherent cells (Figure 5 A–C, respectively) compared to untreated BMAX™-derived cell controls for each stain (Figure 5 D–F).

Challenge with TNF-α reveals unique secretory responses that differentiate uncultured BMAX™ and BMAC products

BMAC is not a purified nor refined mesenchymal stem cell therapy, where ~99% of the nucleated cells are hematopoietic. The BMAX™ processing

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

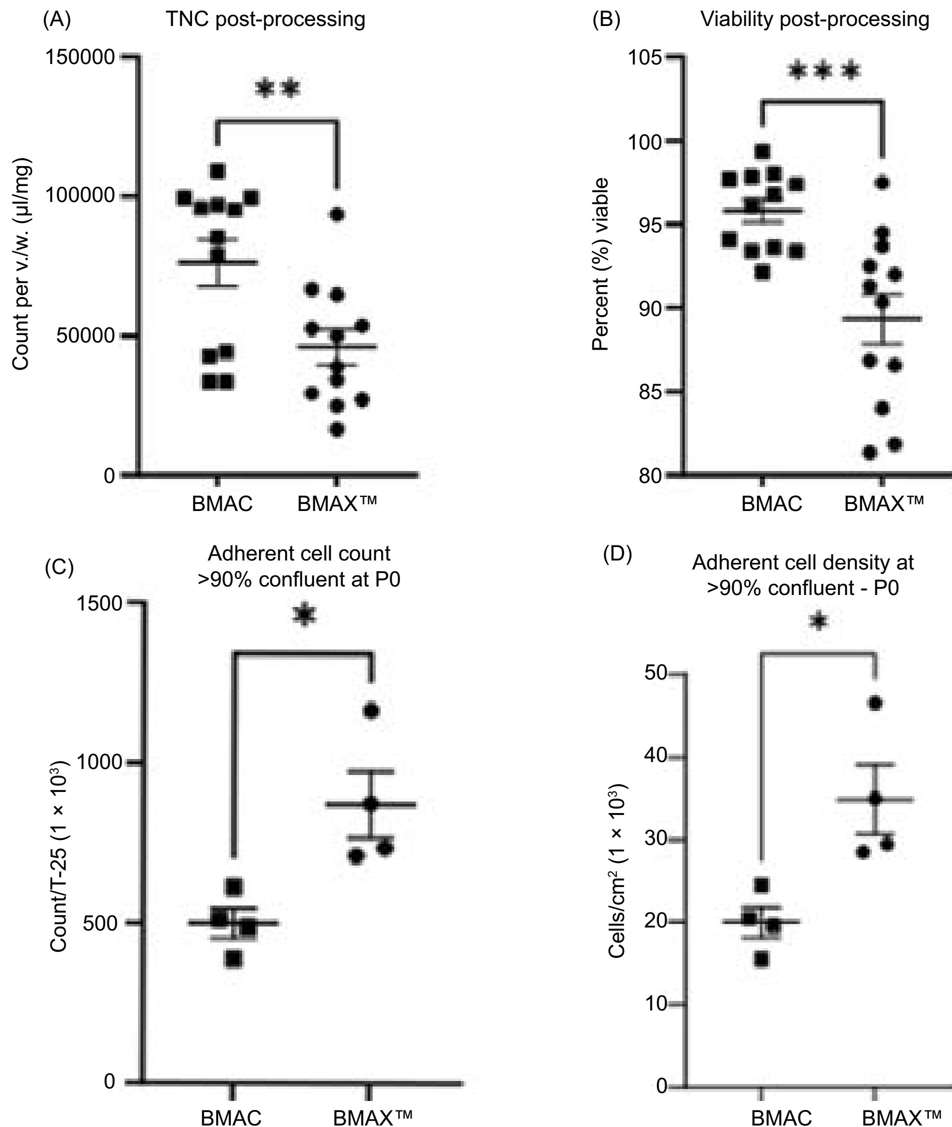


Figure 2. Contrasts in total nucleated cells, viability and cell culture densities in BMAX™ and BMAC-derived cells. (A) TNC quantification and (B) viability for BMAX™ and BMAC samples assessed using fluorescent microscopy. Cultures were carried out until each respective condition reached >90% confluence and adherent cells were quantitated and report ed as (C) total cells per culture vessel and (D) the respective cell density per cm^2.

system results in a tissue-cell construct that is lower in cellularity compared to BMAC, which we suspect may be a pivotal differentiating factor with therapeutic value based on the delivery of fewer leukocytes and MSCs embedded in their native ECM. Under inflammatory conditions, this difference may be revealed via changes in immunomodulatory factors. To assess this possibility, BMAX™-derived cell-tissue construct and donor-match final BMAC products were seeded immediately after processing and challenged with 2.5 ng/mL TNF-α. Cell culture supernatants were sampled immediately after plating (Day 0) and at Days 1, 4 and 8 and assayed for various secreted factors. We only observed differences

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

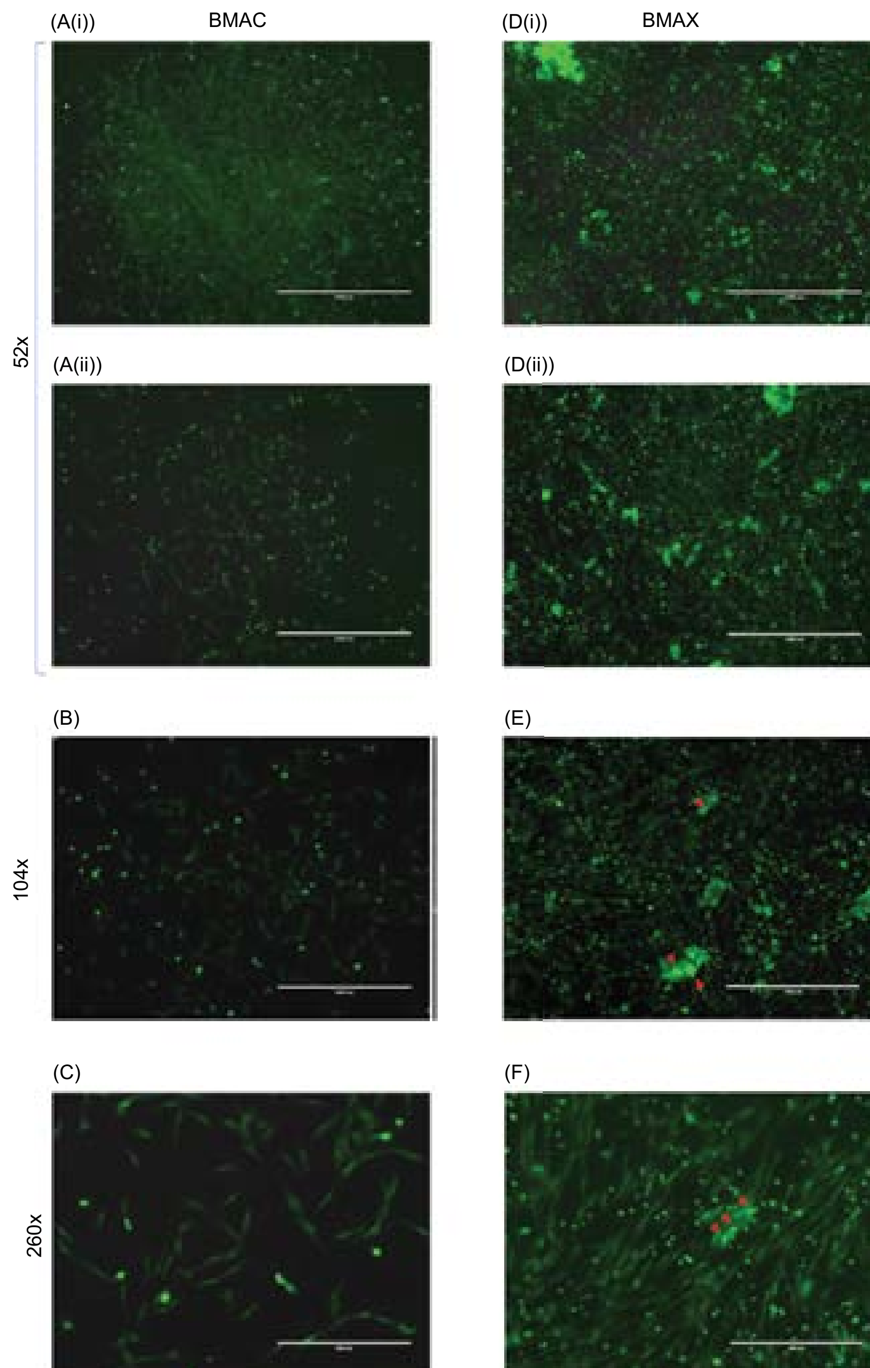


Figure 3. Differences in vitro characteristic between BMAX™ and BMAC-derived MSCs. Calcein-AM stating of donor-matched cultures (cells are stained green) reveal that A BMAC-derived adherent cells form CFU-fs at (i). high and (ii). low densities whereas D(i).(ii). cells derived from the BMAX™ system results in cells embedded in native stroma (large green foci, highlighted by red arrows in E and F). Compared to BMAC-derived adherent cell cultures, BMAX™-derived cells grow rapidly at a higher density throughout the culture vessel and do not form CFU-fs. B. Increased magnification highlights differences in cell density between cells in culture derived from BMAX™ system (right column) and BMAC (left column).

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

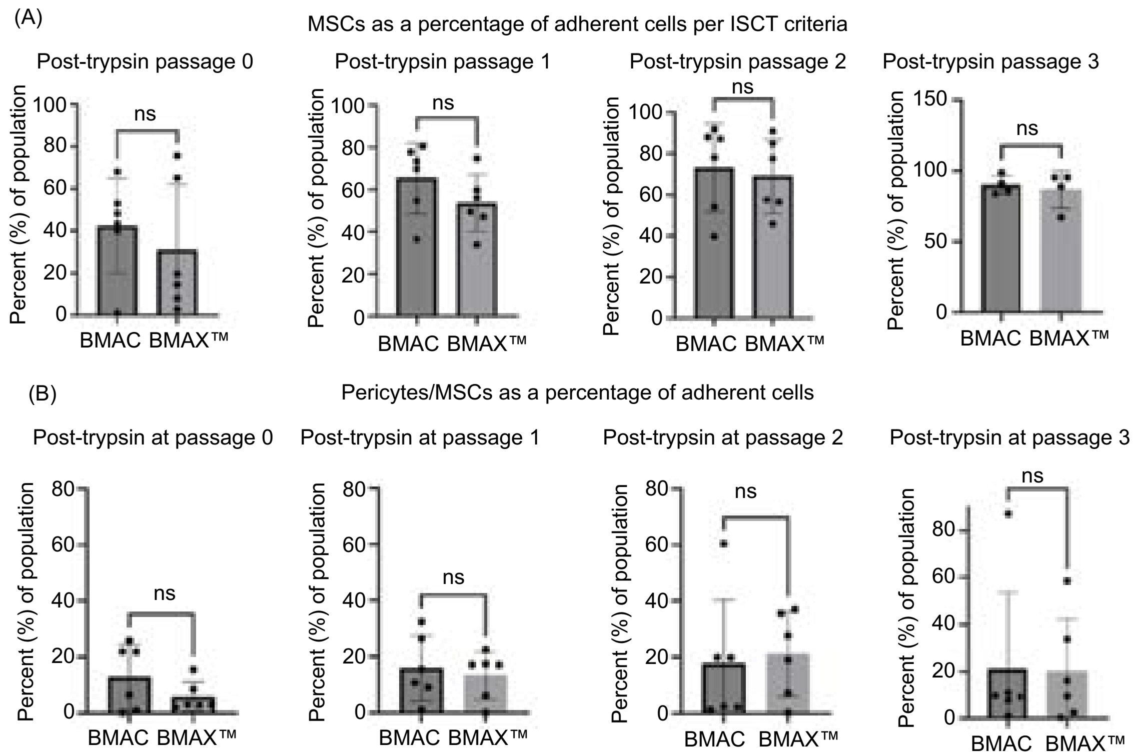


Figure 4. Donor-matched BMAX™- and BMAC-derived adherent cells match MSC surface marker phenotypes. (A) Labeling and assessing ISCT standards for MSC surface markers in BMAX™ and BMAC-derived ex vivo expanded adherent cells reveals no difference in the MSC population (by percentage) through passage 3. (B) Adherent cell cultures for each adherent cell culture show no difference in the frequency of pericyte-like MSC phenotypes.

between BMAX™- and BMAC-products in IL-6 and IP-10 levels within the 8-day time course. In IL-6, although levels remained relatively low in both TNF-α challenged BMAX™- and BMAC-products at Day 1, averaging 2.268 pg/mL (±0.922) and 5.306 pg/mL (±0.8592) respectively, the difference was statistically significant, elevated in BMAC cultures (*P* = 0.0368). At Day 4, IL-6 levels in BMAX™-cultures were rapidly rising compared to BMAC cultures and reached significance by Day 8 where BMAX™-cultures averaged 6849 pg/mL (±3644) and BMAC cultures averaged 46.47 pg/mL (±22.64) (P = 0.050) (Figure 6A). Regarding IP-10, TNF-α challenged BMAC cultures resulted in significantly higher levels compared to BMAX™-cultures at Day 1 (9.89 pg/mL (±1.05) and 2.737 pg/mL (±1.02 respectively), Day 4 (38.46 pg/mL (±15.30) and 0.72 pg/mL (±0.36 respectively)) and Day 8 (80.56 pg/mL (±30.98) and 0.72 pg/mL (±0.35 respectively) (P = 0.0041, P = 0.0346 and 0.0500 respectively) (Figure 6B). We also show that IL-1 receptor antagonist protein (IRAP) levels in both BMAC and BMAX™-product cultures stimulated with TNF-α trend upwards over the time course and overlap at Day 8 (Figure 6C).

CONCLUSIONS

MSC delivery via BMAC is widely used in orthopedic settings with positive clinical outcome data for orthopedic ailments such as OA,[7,9,29,30] lower back pain,[31,32] and as an adjunct to improve healing in rotator cuff repair[33] and foot and ankle repair procedures.[34,35] The immunomodulatory signaling attributes of MSCs that augment regeneration are now considered a more critical therapeutic property of

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

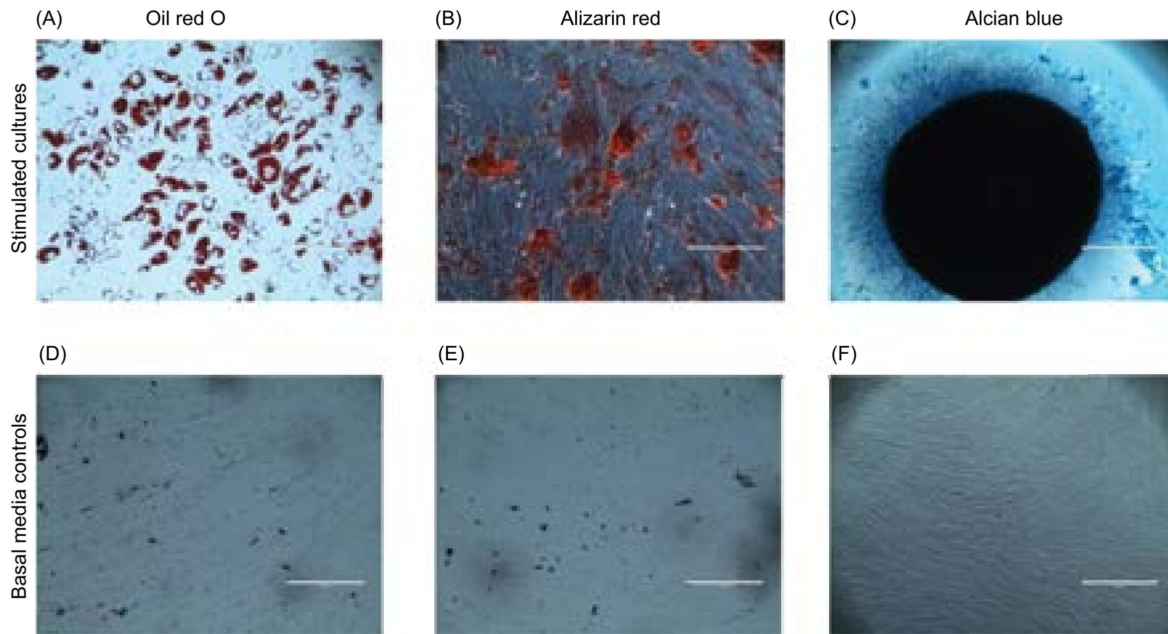


Figure 5: Tri-lineage differentiation potential of BMAX™-derived adherent cells. Exposure of BMAX™-derived cells to commercially available differentiation kits confirm BMAX™-derived adherent cells (A) adipogenic (oil red O staining), (B) osteogenic (Alizarin Red staining) and (C) chondrogenic (microsphere and Alcian blue staining) properties. Basal media controls for each respective stain are shown for (D) oil red o, (E) alizarin red and (F) alcian blue. Scale bars in images (A, B, D, E) = 200 µm; (C, F) = 400 µm.

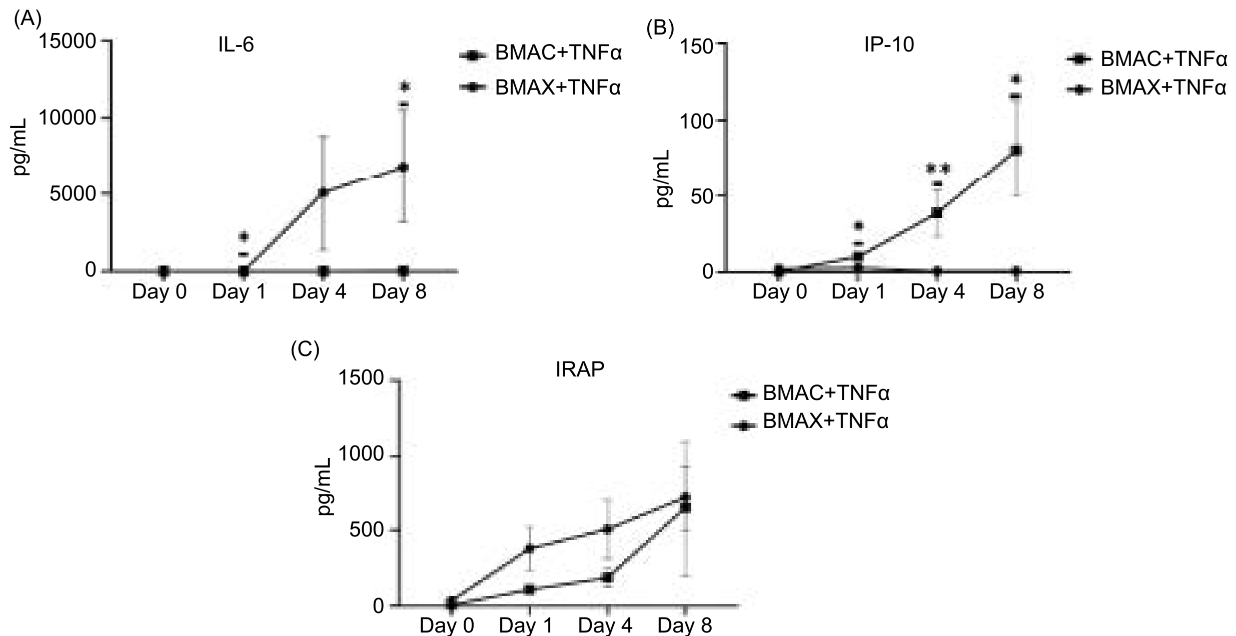


Figure 6. Secretomes from Donor-Matched BMAX™ and BMAC Primary Cultures Differ with TNF-α Stimulation while Retaining Active IRAP Expression. Relative concentrations of (A) IL-6, (B) IP-10/CXCL10 and (C) IRAP in primary cultures of BMAX™ and BMAC-products over 8 days. Concentrations are normalized per 1 million cells plated.

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

the cells than replicative cellular replacements for damaged tissue.[36] However, evidence suggests that the lack of MSC persistence and proliferation at injury sites *in vivo* may be due to disrupted focal adhesion-associated pathways and homing signaling caused by the dissociation of MSCs from their native niche.[36,37] Although MSCs are a rare population in the marrow niche, they interact directly with HSCs, providing physical support and secreting various factors that modulate HSC-driven hematopoiesis.[38] Outside of progenitor cells, nonhematopoietic cells (pericytes, osteocytes, adipocytes, endothelial cells) and hematopoietic cells (neutrophils, lymphocytes, monocytes, and megakaryocytes) are present that aid in the maintenance of the bone-marrow niche.[3,39,40] Another critical component is the physical three-dimensional architecture comprising the extracellular matrix (ECM). The ECM contains proteoglycans, collagens, elastins, glycosaminoglycans, heparin, and various matricellular proteins.[12,13] The ECM also acts as a reservoir for growth factors and proteases[41] and as a junction for cell-cell communication and receptor engagement that regulates hematopoietic cell localization and migration.[13,42]

The retention of the physical/chemical properties of the marrow microenvironment may significantly improve the functionality of MSCs in regenerative processes that are lost when expanded in monoculture or disrupted during BMAC processing. Current BMAC processing standards neglect the critical niche elements of the marrow compartment by removing ECM structural components through centrifugation and aspirating large marrow volumes significantly diluted with blood.[2,8] previous studies evaluating various BMAC harvesting protocols using different devices, anatomical locations, or techniques have yielded noticeably heterogenous products.[23,43] There is also significant variation in accepted standardized BMA harvest volumes which often surpass 120 mL (either unilateral or bilateral) thereby introducing large amounts of diluting blood.[22,23]

Here, we demonstrate a human bone-marrow processing technique using a newly developed device (BMAX™) that retains the native properties of the marrow stroma important for MSC health and function. The method produces a smaller concentrated volume of injectate, significantly reducing mitigating effects of diluting blood and associated erythrocytes which can negatively impact MSC properties and tissue repair ability.[25,26] Using the BMAX™ device we isolated viable adherent MSCs with unique growth characteristics including the close association with marrow ECM that were distinct from BMAC-derived cells in culture. BMAX™ derived cultures also had enhanced proliferative ability when compared to standard processed BMAC isolates. We posit that the retention of marrow niche elements in BMAX™ may preserve native MSC signaling and function that is lost when attached in purified monoculture.

We further found BMAX™ derived MSCs to exhibit an enhanced responsive pro-regenerative and anti-inflammatory secretory profile following stimulation with TNF-α, a potent pro-inflammatory cytokine upregulated at injury sites that regulates paracrine signaling in MSCs.[36,44] TNF-α and IL-1β positively feedback on one another during OA promoting inflammation, cartilage degeneration, and pain.[45–47] IL-1 receptor antagonist (IRAP) is a protein that downregulates pro-inflammatory IL-1β as a competitive antagonist and is an emerging small molecule treatment strategy for OA.[4,46,47] It was found that when challenged with TNF-α, both BMAX™ and BMAC-*products* (i.e., not culture expanded) actively expressed IRAP *in vitro*. Interestingly, compared to donor-matched BMAC, the BMAX™ derived cell-tissue construct also responded to TNF-α insult with significantly higher levels of IL-6, a cytokine known to have both anti-inflammatory and pro-inflammatory properties and a key factor in retaining MSC "stemness' or "plasticity" and for maintaining the bone-marrow niche.[48,49] These differences only demonstrate that innate differences of the secretome between therapeutic-grade BMAX™ and BMAC products exist and are not inclusive of all factors that may be clinically relevant.

BMAC is a known orthobiologic product used for various orthopedic-related indications or disorders such as osteoarthritis, osteonecrosis, tendon/ligament repair, fracture repair, and cartilage defects. BMAX™ is a similar product that may be used for similar indications as an injectable form

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

alone or as an adjuvant to other procedures, devices, or biologics. Using the novel approach described herein, BMAX™ processing of bone marrow offers potential benefits over traditional BMAC preparations in its potential ability to (1) Retain MSCs at the injection/delivery/engraftment site (2) Enhance *in vivo* survival, (3) Provide adequate means for proliferation and signaling and (4) Maintain plasticity/ "stemness" due to the undisrupted contact of MSCs with their native scaffold. We posit that these properties within the BMAX™ product will improve efficacy and clinical outcomes versus traditional BMAC preparations. Thus, for degenerative joint conditions, particularly facet and knee joints, BMAX™ may offer significant improvements in pain relief for these common conditions given the enhanced immunomodulatory effects highlighted herein. Future studies will evaluate BMAX™ processing methods *in vivo,* including ongoing preclinical studies underway by our group using a murine OA model to test the improved ability to mitigate OA symptomology in BMAX™ versus BMAC.

AUTHORS' CONTRIBUTIONS

RD, JH, and LK designed and performed all experiments described including analysis and figure preparation. LK recruited and consented all study participants, assisted in the sample harvest, conceived and designed the study. WSH performed data interpretation and initial manuscript draft. JD conceived, designed and sponsored the study and harvested samples. All authors have reviewed and approved the final manuscript.

CONFLICTS

Dr. Jeff Donner invented and patented the BMAX process US Patent 11,655,454. Lucanus Koldewyn Shareholder & Officer BMAX Medical LLC & Assistant Director Elite Regenerative Institute.

ACKNOWLEDGEMENTS

This study was sponsored by The Colorado Spine Institute by Jeff Donner MD.

REFERENCES

1. Anil U, Markus DH, Hurley ET, et al. The efficacy of intra-articular injections in the treatment of knee osteoarthritis: A network meta-analysis of randomized controlled trials. Knee. 2021;32:173–182. https://doi.org/10.1016/j.knee.2021.08.008
2. Auw Yang KG, Raijmakers NJH, van Arkel ERA, et al. Autologous interleukin-1 receptor antagonist improves function and symptoms in osteoarthritis when compared to placebo in a prospective randomized controlled trial. Osteoarthritis Cartilage. 2008;16(4):498–505. https://doi.org/10.1016/j.joca.2007.07.008
3. Birbrair A, Frenette PS. Niche heterogeneity in the bone marrow. Ann N Y Acad Sci. 2016;1370(1):82–96. https://doi.org/10.1111/nyas.13016
4. Bogers SH. Cell-Based Therapies for joint disease in veterinary medicine: what we have learned and what we need to know. Front Vet Sci. 2018;5:70. https://doi.org/10.3389/fvets.2018.00070
5. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol. 2007;213(2):341–347. https://doi.org/10.1002/jcp.21200
6. Cavallo C, Boffa A, Andriolo L, et al. Bone marrow concentrate injections for the treatment of osteoarthritis: evidence from preclinical findings to the clinical application. Int Orthop. 2021;45(2):525–538. https://doi.org/10.1007/s00264-020-04703-w
7. Chahla J, Dean CS, Moatshe G, Pascual-Garrido C, Serra Cruz R, LaPrade RF. Concentrated bone marrow aspirate for the treatment of chondral injuries and osteoarthritis of the knee: a systematic review of outcomes. Orthop J Sports Med. 2016;4(1):2325967115625481. https://doi.org/10.1177/2325967115625481
8. Chahla J, Mannava S, Cinque ME, Geeslin AG, Codina D, LaPrade RF. Bone Marrow Aspirate concentrate harvesting and processing technique. Arthrosc Tech. 2017;6(2):e441–e445. https://doi.org/10.1016/j.eats.2016.10.024
9. Keeling LE, Belk JW, Kraeutler MJ, et al. Bone marrow aspirate concentrate for the treatment of knee osteoarthritis: a systematic review. Am J Sports Med. 2022;50(8):2315–2323. https://doi.org/10.1177/03635465211018837
10. Zhang R, Ma J, Han J, Zhang W, Ma J. Mesenchymal stem cell related therapies for cartilage lesions and osteoarthritis. Am J Transl Res. 2019;11(10):6275–6289.

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

11. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. Front Pharmacol. 2020;11. https://doi.org/10.3389/fphar.2020.00757
12. Coutu DL, Kokkaliaris KD, Kunz L, Schroeder T. Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. Nat Biotechnol. 2017;35(12):1202–1210. https://doi.org/10.1038/nbt.4006
13. Klamer S, Voermans C. The role of novel and known extracellular matrix and adhesion molecules in the homeostatic and regenerative bone marrow microenvironment. Cell Adh Migr. 2014;8(6):563–577. https://doi.org/10.4161/19336918.2014.968501
14. Chu W, Zhuang Y, Gan Y, Wang X, Tang T, Dai K. Comparison and characterization of enriched mesenchymal stem cells obtained by the repeated filtration of autologous bone marrow through porous biomaterials. J Transl Med. 2019;17(1):377. https://doi.org/10.1186/s12967-019-02131-y
15. Drela K, Stanaszek L, Snioch K, et al. Bone marrow-derived from the human femoral shaft as a new source of mesenchymal stem/stromal cells: an alternative cell material for banking and clinical transplantation. Stem Cell Res Ther. 2020;11(1):262. https://doi.org/10.1186/s13287-020-01697-5
16. Gobbi A, Karnatzikos G, Scotti C, Mahajan V, Mazzucco L, Grigolo B. One-step cartilage repair with bone marrow aspirate concentrated cells and collagen matrix in full-thickness knee cartilage lesions: results at 2-year follow-up. Cartilage. 2011;2(3):286–299. https://doi.org/10.1177/1947603510392023
17. Schäfer R, DeBaun MR, Fleck E, et al. Quantitation of progenitor cell populations and growth factors after bone marrow aspirate concentration. J Transl Med. 2019;17(1):115. https://doi.org/10.1186/s12967-019-1866-7
18. Veronesi F, Giavaresi G, Tschon M, Borsari V, Nicoli Aldini N, Fini M. Clinical use of bone marrow, bone marrow concentrate, and expanded bone marrow mesenchymal stem cells in cartilage disease. Stem Cells Dev. 2013;22(2):181–192. https://doi.org/10.1089/scd.2012.0373
19. Wang S, Wei X, Zhou J, et al. Identification of α2-macroglobulin as a master inhibitor of cartilage-degrading factors that attenuates the progression of posttraumatic osteoarthritis. Arthritis Rheumatol. 2014;66(7):1843–1853. https://doi.org/10.1002/art.38576
20. Woodell-May J, Steckbeck K, King W. Potential mechanism of action of current point-of-care autologous therapy treatments for osteoarthritis of the knee-a narrative review. Int J Mol Sci. 2021;22(5). https://doi.org/10.3390/ijms22052726
21. Li S, Cao P, Chen T, Ding C. Latest insights in disease-modifying osteoarthritis drugs development. Ther Adv Musculoskelet Dis. 2023;15:1759720X231169839. https://doi.org/10.1177/1759720X231169839
22. Hernigou J, Picard L, Alves A, Silvera J, Homma Y, Hernigou P. Understanding bone safety zones during bone marrow aspiration from the iliac crest: the sector rule. Int Orthop. 2014;38(11):2377–2384. https://doi.org/10.1007/s00264-014-2343-9
23. Hernigou P, Homma Y, Flouzat Lachaniette CH, et al. Benefits of small volume and small syringe for bone marrow aspirations of mesenchymal stem cells. Int Orthop. 2013;37(11):2279–2287. https://doi.org/10.1007/s00264-013-2017-z
24. Santos Duarte Lana JF, Furtado da Fonseca L, Mosaner T, et al. Bone marrow aspirate clot: A feasible orthobiologic. J Clin Orthop Trauma. 2020;11(Suppl 5):S789–S794. https://doi.org/10.1016/j.jcot.2020.07.003
25. Dregalla RC, Herrera JA, Donner EJ. Red blood cells and their releasates compromise bone marrow-derived human mesenchymal stem/stromal cell survival in vitro. Stem Cell Res Ther. 2021;12(1):547. https://doi.org/10.1186/s13287-021-02610-4
26. Everts PA, Malanga GA, Paul R V., Rothenberg JB, Stephens N, Mautner KR. Assessing clinical implications and perspectives of the pathophysiological effects of erythrocytes and plasma free hemoglobin in autologous biologics for use in musculoskeletal regenerative medicine therapies. A review. Regen Ther. 2019;11:56–64. https://doi.org/10.1016/j.reth.2019.03.009
27. Dregalla RC, Herrera JA, Koldewyn LS, Donner EJ. The choice of anticoagulant influences the characteristics of bone marrow aspirate concentrate and mesenchymal stem cell bioactivity in vitro. Stem Cells Int. 2022;2022:8259888. https://doi.org/10.1155/2022/8259888
28. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315–317. https://doi.org/10.1080/14653240600855905
29. Kraeutler MJ, Chahla J, LaPrade RF, Pascual-Garrido C. Biologic options for articular cartilage wear (platelet-rich plasma, stem cells, bone marrow aspirate concentrate). Clin Sports Med. 2017;36(3):457–468. https://doi.org/10.1016/j.csm.2017.02.004
30. Di Matteo B, Vandenbulcke F, Vitale ND, et al. Minimally manipulated mesenchymal stem cells for the

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

treatment of knee osteoarthritis: a systematic review of clinical evidence. Stem Cells Int. 2019;2019:1735242. https://doi.org/10.1155/2019/1735242
31. El-Kadiry AEH, Lumbao C, Rafei M, Shammaa R. Autologous BMAC Therapy improves spinal degenerative joint disease in lower back pain patients. Front Med (Lausanne). 2021;8:622573. https://doi.org/10.3389/fmed.2021.622573
32. Haines CM, Bhatt FR, Orosz LD, et al. Low back pain, disability, and quality of life one year following intradiscal injection of autologous bone marrow aspirate concentrate. Stem Cells Int. 2022;2022:9617511. https://doi.org/10.1155/2022/9617511
33. Schoch BS, Werner BC, Shapiro SA, Camp CL, Chalmers PN, Cancienne JM. Effect of bone marrow aspirate concentrate and platelet-rich plasma augmentation on the rate of revision rotator cuff repair. Orthop J Sports Med. 2022;10(11):232596712211270. https://doi.org/10.1177/23259671221127004
34. Glenn R, Johns W, Walley K, Jackson JB, Gonzalez T. Topical review: bone marrow aspirate concentrate and its clinical use in foot and ankle surgery. Foot Ankle Int. 2021;42(9):1205-1211. https://doi.org/10.1177/10711007211021017
35. Harford JS, Dekker TJ, Adams SB. Bone Marrow Aspirate concentrate for bone healing in foot and ankle surgery. Foot Ankle Clin. 2016;21(4):839-845. https://doi.org/10.1016/j.fcl.2016.07.005
36. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM, Caplan AI. Mesenchymal stem cell perspective: cell biology to clinical progress. NPJ Regen Med. 2019;4(1):22. https://doi.org/10.1038/s41536-019-0083-6
37. Levy O, Mortensen LJ, Boquet G, et al. A small-molecule screen for enhanced homing of systemically infused cells. Cell Rep. 2015;10(8):1261–1268. https://doi.org/10.1016/j.celrep.2015.01.057
38. Zanetti C, Krause DS. “Caught in the net”: the extracellular matrix of the bone marrow in normal hematopoiesis and leukemia. Exp Hematol. 2020;89:13–25. https://doi.org/10.1016/j.exphem.2020.07.010
39. Kumar R, Godavarthy PS, Krause DS. The bone marrow microenvironment in health and disease at a glance. J Cell Sci. 2018;131(4). https://doi.org/10.1242/jcs.201707
40. Lana JF, da Fonseca LF, Azzini G, et al. Bone marrow aspirate matrix: a convenient ally in regenerative medicine. Int J Mol Sci. 2021;22(5). https://doi.org/10.3390/ijms22052762
41. Hynes RO. The extracellular matrix: not just pretty fibrils. Science (1979). 2009;326(5957):1216–1219. https://doi.org/10.1126/science.1176009
42. Domingues MJ, Cao H, Heazlewood SY, Cao B, Nilsson SK. Niche extracellular matrix components and their influence on HSC. J Cell Biochem. 2017;118(8):1984–1993. https://doi.org/10.1002/jcb.2590
43. Hegde V, Shonuga O, Ellis S, et al. A prospective comparison of 3 approved systems for autologous bone marrow concentration demonstrated nonequivalency in progenitor cell number and concentration. J Orthop Trauma. 2014;28(10):591–598. https://doi.org/10.1097/BOT.0000000000000113
44. Choi H, Lee RH, Bazhanov N, Oh JY, Prockop DJ. Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. Blood. 2011;118(2):330–338. https://doi.org/10.1182/blood-2010-12-327353
45. Malemud CJ. Anticytokine therapy for osteoarthritis: evidence to date. Drugs Aging. 2010;27(2):95–115. https://doi.org/10.2165/11319950-000000000-00000
46. Meszaros E, Malemud CJ. Prospects for treating osteoarthritis: enzyme-protein interactions regulating matrix metalloproteinase activity. Ther Adv Chronic Dis. 2012;3(5):219–229. https://doi.org/10.1177/2040622312454157
47. Pricola KL, Kuhn NZ, Haleem-Smith H, Song Y, Tuan RS. Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. J Cell Biochem. 2009;108(3):577–588. https://doi.org/10.1002/jcb.22289
48. Xing Z, Gauldie J, Cox G, et al. IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest. 1998;101(2):311–320. https://doi.org/10.1172/JCI1368
49. Grunke M, Schulze-Koops H. Successful treatment of inflammatory knee osteoarthritis with tumour necrosis factor blockade. Ann Rheum Dis. 2006;65(4):555–556. https://doi.org/10.1136/ard.2006.053272
50. Hyer CF, Berlet GC, Bussewitz BW, Hankins T, Ziegler HL, Philbin TM. Quantitative assessment of the yield of osteoblastic connective tissue progenitors in bone marrow aspirate from the iliac crest, tibia, and calcaneus. J Bone Joint Surg Am. 2013;95(14):1312–1316. https://doi.org/10.2106/JBJS.L.01529
51. Ziegler CG, Van Sloun R, Gonzalez S, Whitney KE, DePhillipo NN, Kennedy MI, Dornan GJ, Evans TA, Huard J, LaPrade RF. Characterization of growth factors, cytokines, and chemokines in bone marrow concentrate and platelet-rich plasma: a prospective analysis. Am J Sports Med. 2019;47(9):2174–2187. https://doi.org/10.1177/0363546519832003

This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.

SUPPLEMENTARY

(A)

(B)

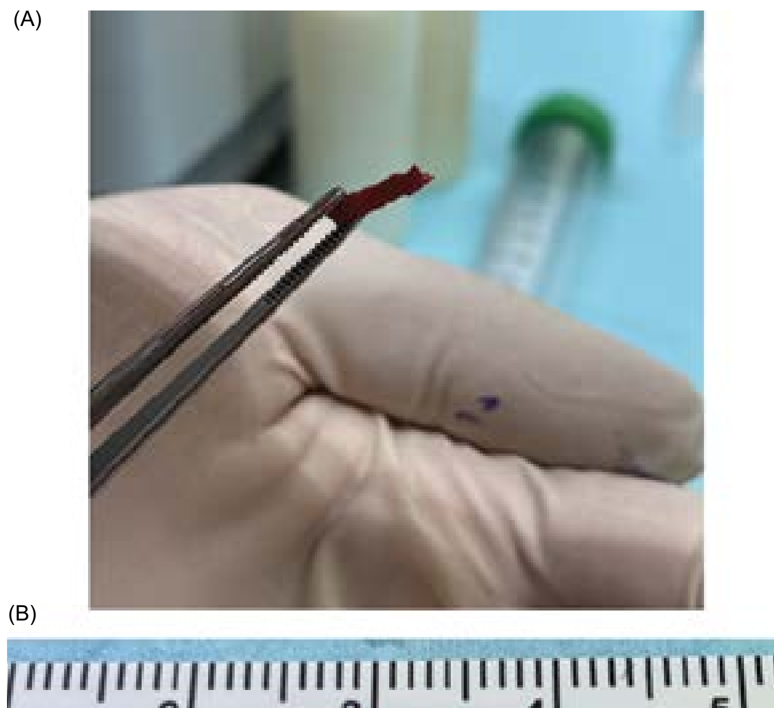
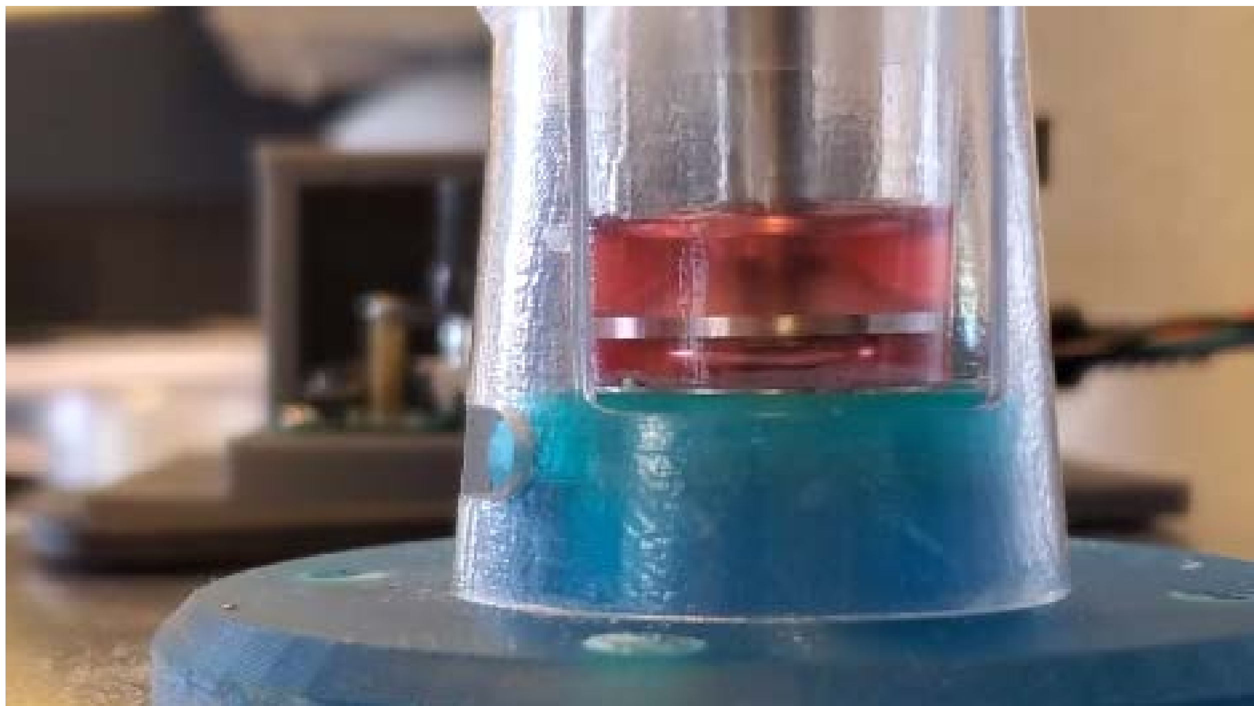


Figure S1. (A) Bone core (BC) collected before BMAX™ processing demonstrating general rigidity and pliability. (B) Size of average BC collected for use in BMAX™ device.



Video S1. Video demonstrating BMAX™ processing device.

This open access article is licensed under Creative Commons Attribution 4.0 International
(CC BY 4.0). http://creativecommons.org/licenses/by/4.0 © Dregalla R, et al.