

Comparative study to assess the differences between fresh versus frozen Bone Marrow Aspirate Concentrate

Jacob Singer¹, Haruki Nishimura¹, Shelbi Green¹, Aiping Lu¹, Johnny Huard¹

¹Steadman Philippon Research Institute, Vail, CO.

Email of Presenting Author: jsinger@sprivail.org

Disclosures: Johnny Huard (ORS Board of Directors, royalties Cook Myosite, Inc.)

INTRODUCTION: Bone marrow aspirate concentrate (BMAC) is a popular OrthoBiologic with exciting results in reducing inflammation and pain in patients with osteoarthritis (OA) and people suffering from musculoskeletal issues. BMAC is derived from the patient's bone marrow in the form of bone marrow aspirate (BMA) which is then concentrated to an injectable form to reduce the number of erythrocytes and leukocytes, while increasing the concentration of mononuclear and mesenchymal stem cells (MSCs). BMAC is unique as it is an autologous product that can be retrieved through a minimally invasive procedure at a reasonable price considering the market for stem cell therapy. As the market for BMAC and OrthoBiologic products increases so will the need for a system of quality assurance, universal freezing method, and approval from government agencies. The goal of this study is to develop a BMAC harvesting, banking and delivery service that facilitates multiple injections from a single bone marrow harvest without expansion or significant manipulation of the cells. We hypothesize that there will be no difference in comparing fresh and frozen BMAC in assessing its monocyte and platelet count, fibroblast colony-forming unit (CFU-F) potential and multi-lineage differentiation.

METHODS: Patients and BMAC collection: Study participants were accrued between March 2023 and August 2023 (n= 11) at the Steadman Clinic (TSC) in Vail, Colorado (IRB#2020-050). BMAC was obtained through TSC and processed by the affiliate biologics company ProofPoint Biologics (PPB) from BMA to BMAC, residuals of the BMAC were then obtained by Steadman Philippon Research Institute (SPRI) to be further analyzed. **Platelet and monocyte count** was obtained through PPB's Ruby hematology analyzer, platelet and monocytes were counted at day 0 (fresh), day 7, day 14, and day 30 (n=3). **BMAC freezing:** BMAC was frozen down with 10% Dimethyl Dioxide (DMSO) and 90% human plasma using Mr. Frosty freezing container in -80°C freezer. One month later, the cells were thawed, and experiments were performed to compare to fresh BMAC. **Mesenchymal stem cell (MSCs) isolation:** The BMAC was centrifuged at 1500g for 10 minutes followed by carefully extracting the prevalent monolytic layer (buffy coat), the buffy coat was washed with PBS 3 times. The mononuclear cells were plated in growth media (alpha minimum essential medium eagle, aMEM with 20% FBS, 1% Penicillin/Streptomycin, and 50ng/mL FGF) and grown for 1 week. **CFU-F Assay:** CFU-F assay was conducted in passage 3. The cells were seeded in six-well plate in concentration of 200 cells per well. CFUs were cultivated for 14 days, and then fixed with PFA 4% and dyed for 30 minutes with 1% crystal violet solution. The colony number was counted to determine the percentage of clones per 100 seeded cells. **Osteogenic and Adipogenic differentiation in vitro:** MSCs at passage 3 were then plated at a density of 35,000 in 24-well plate for osteogenic and adipogenic differentiation following the protocols from Lonza. MSCs were grown in Lonza Osteogenic differentiation media with addition of BMP-2 for 14 days, and Alizarin-Red staining was performed for analyzing the osteogenesis (quantification calculated by absorbance measurement on Alizarin Red staining). Oil red O stain was performed after 18 days for analyzing the adipogenesis (quantification calculated by absorbance measurement on Oil Red staining). **Statistical analysis:** All results are presented as mean ± standard deviation (SD). Means from fresh and frozen BMAC were compared using One-way ANOVA with a significance value of p < 0.05.

RESULTS: The platelet count of frozen BMAC significantly decreased over a 4-week period whereas monocyte count stayed consistent. We found that the platelet counts significantly decreased after the BMACs were frozen for 2 and 4 weeks (Fig. 1A, p<0.027), however the result of monocyte counts showed there is not significant changes after BMAC at different time points (Fig. 1B, p>0.05), suggesting that the number of MSCs stayed consistent after frozen. Low platelet count is most likely due to the freezing method of BMAC, further evaluation on freezing BMAC is needed. **MSCs from frozen BMAC formed greater number of CFU-F than the MSCs from fresh BMAC.** To determine relative CFU-F activity for each MSC preparation from fresh and frozen BMACs, we performed CFU-F assay. By counting the number of colonies per 100 seeded cells, we observed that fresh BMAC formed fewer colonies that were bigger in size, whereas frozen BMAC had more colonies that were smaller (Fig. 2A, 2B). The difference in colony formation regarding number and size of colonies needs to be further investigated to determine if it is affecting proliferation potential. **There are no significant differences seen between MSCs from fresh and frozen BMACs for Osteogenic differentiation.** To determine if the osteogenic differentiation potential changes after frozen, we compared osteogenic differentiation potential of MSCs from fresh and frozen BMACs, Alizarin Red Stain was conducted then quantified via absorbance levels and compared to fresh and frozen groups. The result revealed that fresh and frozen MSCs from BMAC both were able to undergo osteogenesis with no significant difference between fresh and frozen groups (Fig. 3A, 3B, p>0.05). A larger cohort of patients will be needed to validate frozen BMAC osteogenic differentiation potential. **There are no significant differences seen between MSCs from fresh and frozen BMACs on adipogenic differentiation.** It was necessary to confirm adipogenic differentiation potential between MSCs from fresh and frozen BMAC, Oil Red O Stain was conducted then quantified via absorbance levels and compared to control group. The results revealed that fresh and frozen BMAC were able to differentiate to adipogenesis with no significant difference between fresh and frozen groups (Fig. 3C, 3D, p> 0.05). Results indicate that the function of MSCs remain similar after being frozen for one month, a larger cohort of patients of frozen BMAC group will be needed to conform these results.

DISCUSSION: BMAC, a specialized OrthoBiologic product obtained through a minimally invasive procedure, faces inherent limitations on the frequency of its application. Given this constraint, it becomes imperative to consider preservation methods, such as freezing, to extend its utility over time. Consequently, the evaluation of BMAC quality in a comparative context between fresh and frozen states gains paramount importance. This study advocates an investigation into critical parameters including platelet and mononuclear cell viability, CFU-F potential, and multi-lineage differentiation capacities. By comprehensively examining these aspects, we aim to ascertain the efficacy of frozen BMAC, offering insights that can potentially shape strategies for enhancing its therapeutic effectiveness. It is important to note that we were able to determine that there is no significant difference between CFU-F formation and multi-lineage differentiation, which leads us to believe that freezing has no effect on the potential of MSC's. It is noted that a significant reduction in platelet count was seen after 2 and 4 weeks of BMAC being frozen. This is most likely due to the specific freezing technique of the BMAC. BMAC is unique in that it is comprised of many different cell types that play a large role in the therapeutic effect of BMAC. To further understand how to preserve BMAC and its different compartments together, experiments to optimize freezing methods will be needed to validate the most effective banking system. **SIGNIFICANCE:** Assessing the differences between fresh and frozen BMAC holds significant implications for the field of OrthoBiologics. Understanding how the freezing process affects the cellular and molecular composition of BMAC can ensure that clinicians offer patients the utmost therapeutic potential.

