Cryopreservation increases cellular senescence in Human Bone Marrow Aspirate Concentrate (BMAC).

Noah Knezic, Xueqin Gao, Ping Guo, Aiping Lu*, Johnny Huard*

Steadman Philippon Research Institute, Vail, CO, *Corresponding authors.

INTRODUCTION: Bone Marrow Aspirate Concentrate (BMAC) is a commonly used orthobiologic therapy for musculoskeletal repair and regeneration. However, BMAC contains as few as 0.001% functional mesenchymal stem cells (MSCs) (1,2), often necessitating multiple injections and repeat harvests that can lead to scarring at the extraction site and patient discomfort. BMAC banking through cryopreservation could enable various treatments from a single harvest; however, the impact of freezing on MSC function and senescence remains poorly understood. Cellular senescence, a hallmark of aging and tissue dysfunction, impairs regenerative capacity and has been implicated in osteoarthritis (3-5). This study investigated whether cryopreservation alters the senescence phenotype of the mononuclear cells derived from BMAC.

METHODS: **BMAC samples:** Human Bone Marrow Aspirate (BMA) was purchased in 50mL aliquots from STEMCELL Technologies (n = 3 males, age 29, 52, and 55 years) and processed to BMAC as previously described (2,6). **Cell isolation:** Mononuclear cells were isolated via Ficoll gradient centrifugation. Samples were then analyzed fresh or cryopreserved at –80°C using 10% DMSO and 90% autologous plasma for 30 days. **Q-PCR:** Expression of senescence-associated genes p16 and p21 was measured by qPCR. The RNA was extracted from fresh and frozen BMAC using a Qiagen RNA isolation kit. Primers were designed by our group members. **Flow cytometry:** The percentage of senescence-associated β-galactosidase (SA-β-gal) + cells was assessed using flow cytometry**.** Cells were stained with 1:1000 10 µM baflomycin for 1 hr, followed by 1:1000 SPiDER-βGal (Dojindo) for 30 minutes. The cell suspension was filtered through a 40 µm mesh (BD Sciences) and treated with 1:2000 SYTOX Blue dead cell stain (Invitrogen). Samples were analyzed on a Cytek Northern Lights (16v, 14b, 8r) cytometer. **Statistical analysis:** All results are presented as mean ± standard deviation (SD). Means from fresh BMAC were compared with frozen BMAC using a paired T-test with a significance value of $p < 0.05$.

RESULTS: **Cryopreservation increased P16 and P21 expression.** Q-PCR result demonstrated a significant increase in p16 (**Figure 1**) and p21 (**Figure 2**) expression when comparing frozen to fresh BMAC-derived mononuclear cells (**$p < 0.001$). **Cryopreservation increased SA-β-gal + cells:** As visualized in **Figure 3A,** cells analyzed for SA-β-gal by flow cytometry showed a positive shift in the "Bright" stained population in the frozen BMAC-derived mononuclear cells group when compared to fresh. Flow cytometry analysis revealed a significant increase in SA-β-gal bright cells in frozen samples compared to fresh samples (**$p < 0.01$) (**Figure 3B**), indicating a rise in senescent cells after cryopreservation.

DISCUSSION: Cryopreservation is a crucial step in preserving cells for biological research and medical purposes. There is a lack of data on the effect of cryopreservation on cellular senescence, particularly under certain conditions. Our previous data showed that the multilineage differentiation of MSCs isolated from BMAC remained similar after being frozen for 4 weeks at -80°C. In the present work, we found that cryopreservation significantly increases markers of cellular senescence in BMAC-derived mononuclear cells, as shown by elevated expression of p16 and p21 and increased SA-β-gal activity. These findings suggest that freezing and thawing may induce stress responses in cells from BMAC, leading to a shift in their phenotypic state, which may increase the number of senescent-like cells in the frozen sample. However, further work is needed to verify and determine which cell population is undergoing cellular senescence. While the long-term implications of this shift remain unclear, increased senescence could influence MSC behavior in several ways, including altered secretory profiles (SASP), reduced proliferation dynamics, or changes in immunomodulatory signaling. Such changes may affect how these cells interact with their microenvironment following transplantation. These results underscore the importance of further characterizing how cryopreservation affects the quality and behavior of MSCs, and they support continued investigation into optimized storage protocols or senescence-modulating interventions. Cryopreservation is a vital tool; it's essential to be aware of its potential to induce senescence and to optimize protocols to minimize this risk. Treating the patients with senolytic agents at the time of injection may eliminate the senescent cells in the transplanted BMAC and consequently improve their regenerative potential.

SIGNIFICANCE: These findings suggest that cryopreservation may increase functional senescence in BMAC-derived MSCs and alter the expression of senescence-related genes, underscoring the need for further research into the effects of banking orthobiologic products through cryopreservation.

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Fig.1

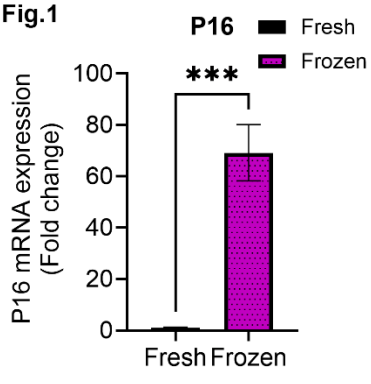

Fig. 2

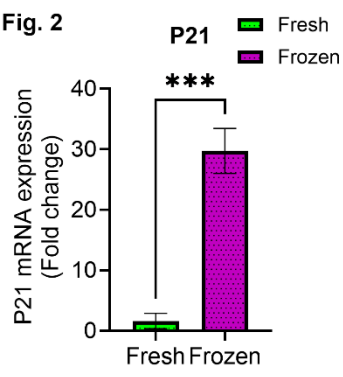

Fig.3