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Clinical mesenchymal stromal cell products undergo functional changes in response to freezing

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Abstract

Background aims. Current methods of mesenchymal stromal cell (MSC) cryopreservation result in variable post-thaw recovery and phenotypic changes caused by freezing. The objective of this investigation was to determine the influence of *ex vivo* cell expansion on phenotype of MSCs and the response of resulting phenotypes to freezing and thawing. *Methods.* Human bone marrow aspirate was used. MSCs were isolated and cells were assessed for total count, viability, apoptosis and senescence over 6 passages (8–10 doublings/passage) in *ex vivo* culture. One half of cells harvested at each passage were re-plated for continued culture and the other half were frozen at 1°C/min in a controlled-rate freezer. Frozen samples were stored in liquid nitrogen, thawed and reassessed for total cell count, viability and senescence immediately and 48 h after thaw. *Results.* Viability did not differ significantly between samples before freeze or after thaw. Senescence increased over time in pre-freeze culture and was significantly higher in one sample that had growth arrest both before freeze and after thaw. Freezing resulted in similar initial post-thaw recovery in all samples, but 48-h post-thaw growth arrest was observed in the sample with high senescence only. *Conclusions.* High pre-freeze senescence appears to correlate with poor post-thaw function in MSC samples, but additional studies are necessary to obtain a sample sizes large enough to quantify results.

Key Words: *apoptosis, freezing, function, mesenchymal stromal cells, senescence, viability*

Introduction

Mesenchymal stem/stromal cells (MSCs) are currently being investigated for a variety of clinical treatment applications. To date, more than 300 clinical trials involve the use of MSCs, with more than 2000 patients safely treated with MSCs [1]. MSCs are being investigated for the treatment of cardiovascular disorders (stroke, myocardial infarction), diabetes, connective tissue disorders (cartilage defects, osteonecrosis and limb ischemia), chronic obstructive pulmonary disease, nervous system disorders (multiple sclerosis, Parkinson's disease and spinal cord injury), kidney diseases and more [2,3].

Effective preservation of MSCs is critical, in particular, for their use as a highly functional off-the-shelf therapy for patients. The ability to store the cells allows for completion of safety and quality control testing before use of the cells, permits transportation from the site of processing to the site of administration and streamlines coordination of the cell therapy with patient care regimes [4]. Development of MSC-based therapies requires standardization of methods of culture and cryopreservation. MSCs are typically cultured *ex vivo* and expanded to a sufficient cell number before patient administration. Uniform, optimized methods of cell expansion have not been developed, and media composition (basal media, serum and additional supplements), seeding density, expansion vessel and *in vitro* population doublings can vary considerably among investigators.

Ex vivo culture of cells has been associated with changes in cell phenotype [5,6]. One such change observed in MSCs is the development of a senescent phenotype [7]. Senescent cells exhibit an inflammatory secretome [8], and, as such, may cause undesirable results in immunomodulatory therapies. *Ex vivo* culture of cells can also influence freezing response. Both hematopoietic progenitors and lymphocytes exhibited changes in subzero water transport and intracellular ice formation tendencies after *ex vivo* culture [9,10], which in turn can influence freezing response. Francois *et al.* [11] quantified diminished response for indoleamine 2,3-dioxygenase (critical to immunomodulatory cell function)

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for frozen and thawed MSCs when compared with fresh non-frozen cells. A recent study by Moll *et al.* [12] also showed that cryopreserved MSCs had reduced immunomodulatory and blood regulatory properties immediately after thaw.

These temporal and freezing-induced changes in cell behavior can lead to confounding outcomes for clinical studies with the use of cryopreserved MSCs. One investigator hypothesized that poor post-thaw MSC function may have been responsible for the failure of a recent clinical trial [13]. The objective of this investigation was to determine the influence of *ex vivo* cell expansion on phenotype of MSCs at harvest and the response of resulting phenotypes to freezing and thawing. This information will help to clarify the influence of culture conditions on the biological characteristics of MSC products and potential shifts in composition or behavior resulting from the freezing process.

Methods

Cell culture and processing

The MSCs used for this study were isolated from bone marrow purchased from Lonza (Walkersville, MD, USA) and were shipped overnight on ice. Volume, cell count and viability of samples were recorded on arrival. Mononuclear cells (MNCs) were isolated from the bone marrow by use of Ficoll Paque Premium (GE Healthcare, Pittsburgh, PA, USA) density gradient centrifugation and separation. On initial receipt, the 10-mL bone marrow sample was diluted with 10 mL of 0.9% saline. In a 50-mL conical tube, this dilute marrow cell suspension was carefully layered over 15 mL of GE Ficoll Paque Premium. The resulting layered suspension was centrifuged at 300*g* for 25 min at room temperature with no brake. The cell layer was collected and was then washed with 50 mL of Hank's balanced salt solution (no phenol red, calcium or magnesium; Lonza) and centrifuged at 300*g* for 5 min. A second wash was performed with the use of the same procedure described above. The supernatant was discarded after both washes.

The MNCs isolated with the use of this method were resuspended in MSC complete culture medium (MSC CCM) composed of α-Modified Eagle Medium (MEM) base (Invitrogen, Grand Island, NY, USA), 16.5% fetal bovine serum (Hyclone, Thermo Scientific, Waltham, MA, USA) and 1% Glutamax (200 mmol/L, Invitrogen). Characteristics of the cell population including cell count and viability were measured again at this stage, along with flow cytometry testing for the negative marker CD45 and positive marker CD90. Cells were seeded at a density of $1.0-1.5 \times 10^5/cm^2$ in appropriately-sized tissue culture–treated t-flasks (Corning, Corning, NY, USA) at a media depth of 1.6 mm.

Growth conditions and passaging

Cells were cultured in a 5% CO_2, 37°C incubator in MSC CCM on appropriately-sized t-flasks. At 24 h and 48 h after seeding, non-adherent cells were removed by means of media change. In these culture conditions, only MSCs from the mononuclear cell population adhered to the surface. Cell enumeration and characteristics (doubling time, viability, senescence and apoptosis) taken on and after the first harvest reflect only MSC characteristics.

Media changes were performed every 2–4 days until cells reached the desired 70–80% confluence between days 8–12. When cells reached the desired 70–80% confluence, they were passaged. MSCs were washed with Dulbecco's phosphate-buffered saline (Invitrogen), removed from the culture surface with TrypLE select (Invitrogen), diluted with MSC CCM to quench the action of TrypLE select and centrifuged. Cells were resuspended in MSC CCM and analyzed for viability, senescence and apoptosis. Half of the remaining cells that were not used for assays were seeded in new flasks at densities of 40–50 cells/cm^2, and the rest were frozen down at each passage. A total of 6 passages were performed, which corresponds to approximately 35–40 population doublings in healthy cells.

Freezing/thawing

At each passage harvest, $1-10 \times 10^6$ cells were frozen down in a total volume of 1 mL per cryovial (Nunc/Nalge Thermo Scientific, Waltham, MA, USA). Cells were harvested, washed in DPBS and resuspended in 5% human serum albumin to a concentration of $2-20 \times 10^6$ cells/mL (2× the desired freezing concentration). The cells were combined stepwise with an equal volume of 2× freezing media composed of 60% Plasmalyte A (Baxter, Deerfield, IL, USA), 20% of 25% HSA (Baxter) and 20% dimethylsulfoxide (Bioniche Pharma, Belleville, Ontario, Canada). The final concentration of dimethylsulfoxide was 10% by volume. Cells were frozen in a controlled rate freezer with the use of the following protocol: (i) Wait at 0.0°C (place sample in freezer at this step); (ii) Wait at chamber = 0.0°C until sample = 1.0°C; (iii) Ramp −1°C/min until sample = −12°C; (iv) Ramp −20°C/min until chamber = −60°C; (v) Ramp +15°C/min until chamber = −18°C; (vi) Ramp −1°C/min until sample = −60°C; (vii) Ramp −3°C/min until sample = −100°C; (viii) End.

After completion of the freezing protocol, cell vials were removed from the controlled-rate freezer (with the use of insulated gloves) and transferred quickly (<30 seconds) to liquid nitrogen storage to