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Transient warming events occurring after freezing impairs umbilical cord–derived mesenchymal stromal cells functionality

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Abstract

Background. Mesenchymal stromal cells (MSCs) have shown promising results for the treatment of refractory acute graft-versus-host disease. While safety of MSC infusion has been demonstrated, the use of cryopreserved MSCs in clinical trials has raised concerns regarding the retention of their functional activity. This has led to the recommendation by experts in the field to use freshly harvested MSCs, even though this approach is much less practical from a logistic point of view. In the present study, we revisited the impact of cryopreservation on MSC functionality and addressed the possibility that warming events on frozen cells rather than cryopreservation *per se* could impact MSC functionality. *Methods.* Following controlled-rate freezing to −130°C, umbilical cord–derived MSCs were left at room temperature (RT) for 2–10 min or on dry ice for 10 min, before being transferred into liquid nitrogen (LqN_2). MSCs of each group were subsequently tested (viability, functionality and cellular damage) and compared with their freshly harvested counterparts. *Results.* We demonstrated that freshly harvested MSCs as well as cryopreserved MSCs that were left on dry ice following step-down freezing have comparable viability, functionality and integrity. In contrast, cryopreserved MSCs that were left at RT before being transferred into LqN_2 were functionally impaired and showed cellular damage upon thawing even though they exhibited high viability. *Discussion.* Warming events after freezing and not cryopreservation *per se* significantly impair MSC functionality, indicating that cryopreserved MSCs can be an advantageous alternative to freshly harvested cells for therapeutic purposes.

Key Words: *cryopreservation, functional activity, immunosuppression, mesenchymal stromal cells, warming events*

Introduction

In the last years, mesenchymal stromal cells (MSCs) have attracted much attention as a possible alternative treatment of steroid-resistant acute graft-versus-host disease (GvHD). Indeed, since Le Blanc et al. reported the case of a 9-year-old boy successfully treated with MSCs [1], these cells have been the subject of several phase 1 and 2 clinical studies that have shown promising results [2–4]. MSC-mediated immune suppression has been widely demonstrated *in vitro* by the inhibition of T-cell proliferation induced by anti-CD3/CD28 antibodies [5,6], and also *in vivo* in a mouse model of GvHD [7–9]. Several mechanisms have been shown to contribute to the anti-inflammatory response of MSCs, including suppression of T-cell proliferation resulting from the interferon (IFN)-γ-induced expression of indoleamine 2,3-dioxygenase (IDO) and secretion of anti-inflammatory cytokines such as tumor necrosis factor–inducible gene 6 protein (TSG-6) [10–12]. Although safety of MSC infusion has been well proven [13], its efficacy in a phase 3 clinical trial did not match the expectations raised by previous clinical and *in vitro* studies [14], although a significant improvement in response rates in the pediatric patient subgroup was observed [15,16].

To have an adequate number of cells for clinical applications, MSCs need to be expanded under tightly controlled conditions. Ideally, expanded cells should be cryopreserved to produce a readily available off-the-shelf therapeutic product. Cell cryopreservation also facilitates quality control and extensive testing prior to use [17]. Importantly, cryopreserved cells must maintain their immunosuppressive properties upon thawing. In this regard, recent reports have suggested that MSC viability and potency were impaired by cryopreservation, which could explain the inconsistencies mentioned above between early phase clinical trials (often done with fresh cells) and large industry-sponsored phase 2/3 clinical trials (done with

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cryopreserved MSCs) [18–20]. However, several recent studies showed that cryopreservation does not alter the immunosuppressive properties of MSCs [21–24], suggesting that steps in the overall banking process and not cryopreservation *per se* might be responsible for these discrepant observations.

Pre-freezing sample preparation and freezing protocols for long-term cryostorage have been optimized over the past years and cell cryopreservation is now a well-controlled process [25–28]. However, it has been recently reported that uncontrolled temperature fluctuations (transient warming events) have negative impacts on cell viability, recovery and functionality of frozen human peripheral blood mononuclear cells (PBMCs), suggesting that similar effects could also occur on frozen MSCs [28]. The objective of the present study was thus to determine the potential impact of both cryopreservation and transient warming events after cell freezing on the integrity and immunosuppressive properties of umbilical cord (UC)–derived MSCs.

Methods

MSC isolation and culture

This study has been approved by Héma-Québec's Research Ethics Committee and all participants in this study have signed an informed consent. MSCs were extracted from human UC Wharton's jelly using a proprietary explant culture method developed by Tissue Regeneration Therapeutics Inc. and bone marrow MSCs (BM-MSCs) were purchased from Lonza. For expansion, cells were seeded at 1000 cells/cm^2 (UC-MSC) or at 3500 cells/cm^2 (BM-MSC) in multilayer flasks (Corning Falcon Cell Culture Multi Flasks, Corning) and cultured in a chemically defined MSC culture medium (TheraPEAK Mesenchymal Stem Cell Medium- Chemically Defined [UC-MSCs] and in Minimum Essential Medium Eagle alpha medium supplemented with L-glutamine and 10% fetal bovine serum [FBS; BM-MSCs], Lonza). Medium was changed after 4 days and lactate concentration (used as an index of cell proliferation) was measured using the YSI 2300 Stat Plus Glucose Lactate Analyzer (Marshall Scientific). After 7 days of culture, MSCs were washed with Dulbecco's Phosphate-Buffered Saline (DPBS; Life Technologies) and harvested using (TrypLE Express; Life Technologies) for 10 min at room temperature (RT). Trypsin dilution was done by adding an equal volume of Plasma-Lyte A (Baxter Canada) containing 10% Human Serum Albumin (HSA) (Alburex 25, CSL Behring). Cells were centrifuged (600*g*, 10 min), suspended in Plasma-Lyte A containing 5% HSA and the cell suspension was analyzed (count and viability) on a Nucleocounter NC-250 (ChemoMetec). MSCs were then re-seeded as described above for one or two additional passages. All assays were performed using MSCs between passage two and four. Cells were tested using flow cytometry (BD Accuri C6, BD Bioscience) for expression of CD44, CD73, CD90 and CD105 and for the absence of CD45 and CD31 expression using the BD Stemflow hMSC Analysis Kit (BD Bioscience).

Cryopreservation and warming event assay

The strategy used in the present study is depicted in Figure 1. Following harvest, a portion of freshly harvested MSCs (FR-MSCs) was immediately tested and used as a positive control in our experiments. The remaining portion of MSCs was suspended at 2×10^6 cells/mL in a cryoprotectant solution containing 10% dimethyl sulfoxide (DMSO), 0.9% dextran 40 (CryoSure-DEX40, WAK-Chemie Medical GmbH) and 5% HSA. The cell suspension was distributed in cryovials (2×10^6 cells/cryovial) and placed in a CryoMed controlled-rate freezer (Thermo Fisher Scientific) until sample temperature reached –130°C. Immediately after step-down freezing, cell vials were separated into two groups. For the first group, vials were left at RT (RT-MSCs) for 2, 4, 6, 8 or 10 min, whereas, for the second group, vials were kept on dry ice (DI-MSCs) for 10 min before being transferred into liquid nitrogen where they were stored until further use (Figure 1). Temperature was monitored in one sample of each group by inserting thermocouple leads within the vial. Data were recorded using the portable datalogging system OM-3000 (Omega).

T-cell proliferation assay

Human PBMCs were isolated using density centrifugation over Ficoll-Paque (GE Healthcare Bio-Sciences) from whole blood collected from healthy volunteers who signed an informed consent. MSC vials (DI-MSC and RT-MSC) were removed from liquid nitrogen and kept on dry ice before being placed in a ThawSTAR instrument (Biocision). The thawed cells were then slowly diluted (1:3) in pre-warmed growth medium and the suspension was analyzed (count and viability) on a Nucleocounter NC-250. PBMCs (5×10^5 cells/well in a 24-well plate) were activated using 20 ng/mL of anti-human CD3 and CD28 antibodies (clone OKT3 and CD28.2, respectively, from eBiosciences) and incubated with FR-MSCs or freshly thawed DI-MSCs or RT-MSCs at a ratio of 1 MSC for 10 PBMCs (1:10 ratio) in RPMI 1640 supplemented with 10% FBS and L-glutamine (all from Life Technologies). Four days later, all cells (adherent and nonadherent) in each well were harvested after a 10-min treatment with TrypLE Express and incubated for 30 min with Fc Receptor blocking reagent (Miltenyi Biotec), 7-Aminoactinomycin D; BD Bioscience), anti-CD3 (clone SK7, allophycocyanin [APC]; eBioscience)