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# Functional impairment of MSC induced by transient warming events: Correlation with loss of adhesion and altered cell size

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**Abstract**

*Background:* We recently showed that transient warming effects decreased the functional and adhesion properties of mesenchymal stromal cells (MSC) while post-thaw viability remained high. In an attempt to better predict functional impairment of cryopreserved MSC, we further analysed the correlation between viability, immunosuppressive activity and adhesion of cells exposed or not to warming events. *Methods:* MSC prepared from six umbilical cords were frozen to −130°C and immediately transferred in a dry ice container or exposed to room temperature for 2 to 10 min (warming events) prior to storage in liquid nitrogen. Viability, functionality (inhibition of T-cell proliferation), adhesion and expression of various integrins were evaluated. *Results:* The monotonic loss of functional activity with time was proportional to the length of warming events to which MSC were subjected and correlated with the monotonic loss of adhesion capacity. In contrast, post-thaw viability assessment did not predict functional impairment. Interestingly, flow cytometry analyses revealed the emergence of a $FSC^{low}$ population present in the viable cell fraction of freshly thawed MSC, which displayed poor adhesion capacity and expressed low levels of integrin $\beta 5$. The prevalence of this $FSC^{low}$ population increased with the length of warming events and correlated with impaired functional and adhesion properties. *Discussion:* Our results reveal that loss of functional activity (4-day test) induced by transient warming events could be predicted by evaluating adhesion (2-hr test) or FSC profile (10-min test) of MSC immediately post-thaw. These observations could lead to the development of surrogate tests for rapidly assessing the functional quality of cryopreserved MSC.

**Key Words:** *adhesion, cryopreservation, functionality, mesenchymal stromal cells, size, surrogate test*

## Introduction

The efficacy of mesenchymal stromal cells (MSC) for the treatment of graft vs host disease and other immune-related disorders is currently being assessed in several clinical trials [1]. In the last few years, the impact of cryopreservation on phenotype, proliferation, viability and functionality of MSC has been investigated following reports showing that MSC functionality was impaired by cryopreservation, raising doubt on the possibility of using these cells as an off-the-shelf therapy [2-7]. We recently shed light on this issue by demonstrating that warming events occurring after MSC freezing, rather than cryopreservation *per se*, are responsible for the reduction in their immunomodulatory potency [8]. While properly cryopreserved MSC were shown to inhibit T-cell proliferation as efficiently as freshly harvested MSC, cells that were exposed to room temperature (RT) for a few minutes following freezing at -130°C were dramatically impaired while their post-thaw viability remained high. This latter observation indicates that post-thaw viability is a poor predictor of potency. In addition to the loss of immunosuppressive properties, their ability to bind to plastic or ECM-coated surfaces was also compromised [8], suggesting that expression of integrins could be affected by warming events, given the role of these molecules in adhesion to extracellular matrix [9]. The present study was thus undertaken to extend our previous observations on the impact of transient warming events on post-thaw viability, functional activity and adhesion properties of umbilical cord-derived MSC. More precisely, we investigated

*Abbreviations:* MSC, mesenchymal stromal cell; DI, dry ice; RT, room temperature; DI-MSC, MSC kept on dry ice after freezing; RT-MSC, frozen MSC exposed to warming events; PBMC, peripheral blood mononuclear cells

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whether some methods for assessing post-thaw viability correlated better than others with the immunosuppressive properties of MSC subjected to warming events. The concordance between functional activity and adhesion as a function of the length of warming events was also investigated as well as the expression of alpha and beta integrins on the MSC surface. The emergence of a viable MSC population with altered cell size was uncovered during flow cytometry analyses. The correlation between this cell population and functional impairment was examined. Overall, this study permits to better characterize the impact of transient warming events on MSC properties and characteristics.

## Methods

### *MSC isolation and culture*

This study has been approved by Héma-Québec's Research Ethics Committee and all participants have signed an informed consent. MSC from the Wharton's jelly of 6 human umbilical cords were extracted using a proprietary explant culture method developed by Tissue Regeneration Therapeutics Inc. (TRT, Toronto, ON, Canada). Briefly, the amniotic membrane was removed and the umbilical vessels were separated. The perivascular Wharton's Jelly was dissected from the tunica media of the individual vessels, diced, and used as a tissue source for explant culture [10]. Cell expansion was done as previously described [8]. Briefly, cells were seeded at 1000 cells/cm$^2$ in multi-layer flasks (Corning$^{TM}$ Falcon$^{TM}$ Cell Culture Multi Flasks, Corning, NY, USA) and cultured in a chemically defined MSC culture medium (TheraPEAK$^{TM}$ MSCGM-CD$^{TM}$, Lonza, Rochester, NY, USA). Medium was changed after 4 days and cells were harvested after seven days of culture. MSC were washed with Dulbecco's Phosphate Buffered Saline (Life Technologies, Burlington, ON, Canada) and harvested using TrypLE$^{TM}$ Express (Life Technologies). MSC were then reseeded as described above for one or two additional passages. All assays were performed using MSC between passage 2 and 4. Cells were tested by flow cytometry (BD Accuri$^{TM}$ C6, BD Bioscience, Mississauga, ON, Canada) for the expression of CD44, CD73, CD90 and CD105 and for the absence of hematopoietic marker expression using the BD Stemflow$^{TM}$ hMSC Analysis Kit, as well as for the absence of the endothelial cell marker CD31 (all reagents from BD Bioscience). Briefly, $5 \times 10^5$ cells were incubated with the various antibodies or isotype controls for 30 min at RT in the dark, washed twice and analyzed. The MSC preparations used in this work met the ISCT phenotypic criteria (>95% positive CD44, CD73, CD90, CD105 and <2% positive for the hematopoietic markers) and were < 2% positive for CD31. Three-lineage differentiation was confirmed using the STEMPRO® Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kits (Life Technologies). MSC characterization data are presented in Supplementary Figure 1 and Supplementary Table 1.

### *Cryopreservation and warming events*

Following harvest, MSC were suspended at $2 \times 10^6$ cells/ml in a cryoprotectant solution containing 10% dimethyl sulfoxide, 0.9% dextran 40 (CryoSure-DEX40, WAK-Chemie Medical GmbH, Taunus, Germany) and 5% Human Serum Albumin (Alburex® 25, CSL Behring, Ottawa, ON, Canada) at RT. The cell suspension was distributed in cryovials ($3 \times 10^6$ cells/cryovial) and placed in a CryoMed$^{TM}$ controlled-rate freezer (Thermo Fisher Scientific, Waltham, MA, USA) until sample temperature reached -130°C (2°C/min from RT to 0°C; 1°C/min until -40°C; 10°C/min until -130°C; hold -130°C for 5 min). Immediately after controlled-rate freezing, cell vials were separated into two groups. For the first group, vials were left at RT (RT-MSC) for 2, 4, 6, 8 or 10 min, whereas vials from the second group were kept on dry ice (DI-MSC) for 10 min before being all transferred into liquid nitrogen where they were stored for at least 7 days before testing.

### *Analysis of post-thaw viability and integrin expression*

Vials containing MSC (DI-MSC and RT-MSC) were recovered from the liquid nitrogen container and kept on dry ice before being placed in a ThawSTAR$^{TM}$ instrument (Biocision, San Rafael, CA, USA). The thawed cells were then slowly diluted (1:3) in pre-warmed growth medium. Cell viability was determined 30 min after thawing using four different methods. First, cells were stained with a solution containing DAPI and acridine orange (solution 18) and analyzed on a Nucleocounter® NC-250$^{TM}$ (both from ChemoMetec, Lincoln, NE, USA) according to the manufacturer's instructions. Second, for trypan blue viability analysis, cells were stained with the Trypan Blue solution (Thermo Fisher Scientific) and the number of total and blue-stained cells was immediately determined using a hemacytometer (Hausser Scientific, Horsham, PA, USA) and a light microscope (Olympus CK30, Olympus Canada Inc., Toronto, ON, Canada). Finally, for 7AAD- (BD Bioscience) and SYTOX Blue- (Thermo Fisher Scientific) based analyses, cells were stained with the fluorescent dye according to the manufacturer's instructions. 7AAD and

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