Comparative Bioactivity Analysis for Off-the-Shelf and Culture–Rescued Umbilical Cord-Derived Mesenchymal Stem/Stromal Cells in a Xeno- and Serum-Free Culture System

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Abstract
We recently reported a standardized xeno- and serum-free culture platform to isolate and expand umbilical cord-derived mesenchymal stem/stromal cells (UC-MSCs). Comparing populations from the same passage, cells that were cryopreserved and culture-rescued exhibited characteristics similar to those of their fresh counterparts, continuously cultured cells without interim cryopreservation. The culture rescue after thawing allowed for the cells to be fully recovered. However, since it would be more cost-effective and timesaving if cryopreserved cells can be used as an off-the-shelf product, we set out to compare the bioactivity of freshly thawed UC-MSCs versus culture-rescued UC-MSCs of the same batch that were recultured for an additional passage under our xeno- and serum-free protocol. UC-MSCs showed high viability in both the freshly thawed and the re-cultured group. Both populations displayed a similar proliferation capacity which is indicated by a comparable population doubling time and colony-forming ability. Both freshly thawed and culture-rescued UC-MSCs expressed the characteristic immunophenotype and were capable of differentiating into osteocytes, chondrocytes, and adipocytes. On the other hand, culture-rescued cells appeared to be more potent in immunosuppression than freshly thawed cells. In conclusion, freshly thawed and culture-rescued cell products share comparable bioactivity in cell growth and proliferation, immunophenotype, and differentiation potential. However, the culture-rescued cells that were allowed to grow for an additional passage appear to display a more favorable immunomodulatory potential when compared to their freshly thawed parent cells.

Keywords
umbilical cord, mesenchymal stem cell, mesenchymal stromal cell, cryopreservation, cell therapy, immunomodulation

Abbreviations
MSCs, Mesenchymal Stem/Stromal Cells; UC-MSCs, Umbilical Cord-derived MSCs; BM-MSCs, Bone Marrow-derived MSCs; AD-MSCs, Adipose-derived MSCs; CFU, Colony-Forming Unit

Introduction
Clinical applications of mesenchymal stem/stromal cells (MSCs) have been increasing worldwide, making these cells the most frequently used in cell therapy second only to hematopoietic stem cells. MSCs have been shown to have great potential in treating graft-versus-host disease, anal fistula in Crohn’s disease, neurological diseases, degenerative joint diseases, and cardiovascular diseases, among others, and can also be used in the context of tissue engineering¹,². The cells exhibit unique potential in regulating the immune response.³

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response, promoting angiogenesis, and generating an environment that is expedient for endogenous stem cell repopulation3,4. Their potent in vitro expansion capacity and immune-privileged attributes make them attractive for allogeneic uses.

We have established a standardized culture platform for umbilical cord MSCs (UC-MSCs) and other adult tissue-derived MSC sources that can be used for therapeutic purposes5. This is important for the decision of how the cell product should be prepared for clinical use. Both off-the-shelf products and re-cultured cells are of interest in allogeneic settings. Many advanced phase 3 trials of allogeneic MSCs have employed freshly thawed products such as those of Prochymal (later named Remestemcel-L) (NCT00366145, NCT00482092, NCT02032004, NCT02412735, NCT02336230, NCT04371393) and three other phase 3 trials (NCT04224207, NCT04236739, NCT03042572). However, there are concerns that cryopreservation causes cellular stress due to temperature fluctuation, cryoprotectant-induced cytotoxicity, or mechanical injury6. The infusion of culture-rescued cell products might ensure that the cells have recovered from cryopreservation-induced stress and regained their full physiological bioactivity7. Phase 2 and 3 trials of Darvadstrocel (NCT03042572 and NCT04236739, respectively) used culture-rescued cells and successfully demonstrated the safety and efficacy of the therapy in patients with Crohn’s disease8. However, postcryopreservation re-culture of the cells requires additional laboratory work, including extra quality-control tests and longer processing times. Therefore, the study presented here aimed to compare the qualities of cryopreserved UC-MSC products for clinical applications: freshly thawed and culture-rescued cells that have been re-cultured in our xenogeneic serum-free system for one additional passage.

Methods

Patient Samples

UC-MSCs (n = 5) were obtained from the biobank of the Vinmec Healthcare System. All UC samples were collected from healthy donors at Vinmec International Hospital in 2019 after they signed informed consent forms. Sample collection and data analysis were approved by the Ethics Committee of the Vinmec Healthcare System and were carried out in accordance with the Declaration of Helsinki.

UC-MSC Culture

UC-MSCs were cultured under xeno- and serum-free conditions as described previously5. Briefly, the cells were cultured in StemMACSTM MSC Expansion Media XF (Miltenyi Biotec, Bergisch Gladbach, Germany) in cell culture flasks (Thermo Scientific, Waltham, MA, USA) coated with CellStart™ coating substrate (Gibco, Waltham, MA, USA) and grown until the culture reached 80% confluence. The cells were harvested using CTS™ TrypLE™ Select Enzyme (Gibco, Waltham, MA, USA). MSCs at passage 4 were suspended in cold serum-free, xeno-free, and chemically defined reagent CryoStor® CS10 (Stem Cell Technology, Vancouver, Canada) at a concentration of 1-2 million cells/ml and frozen using a CoolCell® Cell Freezing Container (Biocision, Bath, UK) at −80°C overnight. The cells were cryopreserved in the vapor phase of liquid nitrogen in an automated Brooks system (Brooks Life Science, Chelmsford, MA, USA). The temperature was monitored and maintained at −196°C.

Cryopreserved UC-MSCs were thawed and used for subsequent experiments. Briefly, the cells were thawed rapidly in a water bath at 37°C and then gently diluted to 10 ml warm culture medium per 1 ml of cell suspension. The cells were centrifuged to remove DMSO and resuspended in warm culture medium. An aliquot of the reconstituted cell suspension was treated with Trypan Blue Solution, 0.4% (Gibco, Waltham, MA, USA) to determine the viability of the cells. The samples were then divided into two fractions: one freshly thawed fraction without further treatment, and one fraction to be culture-rescued. The freshly thawed fraction was used immediately for the experiments. The culture-rescued cells were allowed to recover for one more passage before analysis. These cells were plated at a concentration of 5000 cells/cm² in StemMACSTM MSC Expansion Media XF (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured at 37°C, 5% CO₂ for 3-4 days. They were analyzed upon reaching 80% confluence.

Examination of Population Doubling Time

Freshly thawed or culture-rescued UC-MSCs were seeded at a concentration of 5000 cells/cm² in four different 96-well plates (6 wells per sample), each per measuring time point. On day 0, the cells were allowed to attach on the surface for 3 hours after seeding, fixed with paraformaldehyde 4% (w/v) (Sigma-Aldrich, St. Louis, MO, USA), and stained with DAPI Staining Solution (Abcam, Cambridge, UK). Images were captured with the ImageXpress Micro Confocal system and the nuclei were counted using MetaXpress software. The algorithm was calculated with the following parameters: approximate min width: 8 μm, approximate max width: 35 μm, and intensity above the local background of 2000 gray levels. Similarly, cell numbers were counted every day until day 3. Population doublings (PDs) and the population doubling time (PDT) were calculated using the following formulas:

\[
PDs = \frac{N_t}{N_0}
\]

\[
PDT = \frac{\ln(N_t)}{\ln(2)}
\]

Flow Cytometry Analysis

Flow cytometry was performed to test the surface markers and cell viability of both freshly thawed and culture-rescued UC-MSCs. Immunophenotypic analysis was performed...
using a Human MSC Analysis Kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions with antibodies against the following molecules: CD73, CD90, CD105, CD44, CD34, CD45, CD11b, CD19, and HLA-DR. In addition, the cells were stained with a CD34 monoclonal antibody (581) FITC (Invitrogen, Waltham, MA, USA), CD11b monoclonal antibody (ICRF44) PE (Invitrogen, Waltham, MA, USA), CD19 monoclonal antibody (HIB19) PerCP-Cyanine 5.5 (Invitrogen, Waltham, MA, USA), CD45 monoclonal antibody (HI30) APC (Invitrogen, Waltham, MA, USA), and anti-HLA-DR-FITC antibody (Beckman Coulter, Brea, CA, USA). For cell viability analysis, UC-MSCs were stained with Sytox Red dye (Invitrogen, Waltham, MA, USA). The cells were measured by a BD Canto II flow cytometer (Becton Dickinson, Brea, CA, USA) and analyzed using FlowJo software.

**Colony-Forming Unit (CFU) Assay**

Freshly thawed or culture-rescued UC-MSCs were plated in triplicates at concentrations of 4 cells/cm² and cultured in MSC medium that was changed twice per week. After 10 days, cells were fixed with Methanol (Merck, Darmstadt, Germany) and stained with Giemsa (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. They were plated at ratios of 1:1 and 2:1 with UC-MSCs in RPMI supplemented with 10% fetal bovine serum (Life Technologies, Waltham, MA, USA), 100 IU/mL interleukin 2 (Peprotech, Rocky Hill, NJ, USA), and 1% penicillin/streptomycin. Human MSC Suppression Inspector, human (Miltenyi Biotec, Bergisch Gladbach, Germany), which consists of anti-biotin MACSiBead particles that are preloaded with biotinylated CD2, CD3, and CD28 antibodies was added to activate T-cell proliferation. After four days of co-culture, the cells were harvested, labeled with anti-CD3 VioBlue, anti-CD4 APC-Vio770, and anti-CD8 PE-Vio770 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany, clones: BW264/56, VIT4, and REA734, respectively) and 7-AAD (Miltenyi Biotec, Bergisch Gladbach, Germany), and then analyzed with a BD Canto II flow cytometer (Becton Dickinson, Brea, CA, USA). The proliferation state of viable CD3⁺CD4⁻ and CD3⁺CD8⁻ T-cells was analyzed based on the CFSE signal, which was diluted after each time the cells divided. Non-activated PBMCs served as the negative control, and MSC suppression inspector-activated PBMCs without MSCs were the positive control.

**Statistical Analysis**

Data were analyzed by GraphPad Prism 8 software. The comparison between freshly thawed and culture-rescued UC-MSCs was performed using a paired t-test. Statistical significance was defined as P < 0.05.

**Results**

**Cell Morphology and Viability**

Cells were previously expanded until passage 4 and cryopreserved for subsequent analysis. The cells exhibited a fibroblastic spindle-shaped morphology before and after cryopreservation, which is typical for MSCs (Fig. 1A).

Cell viability was analyzed using Trypan Blue and Sytox Red dyes. The average survival rate using Trypan Blue for the freshly thawed sample was 93.90% ± 3.98%, whereas that of the culture-rescued sample was 98.96% ± 0.68% (P = 0.05). The results using Sytox Red were 97.62% ± 0.77% for the freshly thawed sample and 98.71% ± 0.56% for the culture-rescued sample (P = 0.28) (Fig. 1B).

**Cell Growth and Colony-Forming Ability**

The growth ability of the cells reflects their fitness; therefore, we compared the growth rate of the freshly thawed and culture-rescued cells. The cells were stained with DAPI and analyzed with the ImageXpress Microconfocal System every day from day 0 to day 3 (Fig. 2A and Supplemental Fig. S1B). The freshly thawed cells grew exponentially, similarly to the culture-rescued cells (Fig. 2B). The doubling time of freshly thawed UC-MSCs was 19.94 ± 2.78 hours, whereas that of culture-rescued MSCs was 21.46 ± 0.94 hours (P = 0.52) (Fig. 2C). For comparison, the population...
The doubling time of fresh continuously cultured UC-MSCs in our historical cohort was 21.73 ± 3.43 hours (n = 29).

Furthermore, a colony-forming assay was performed to assess the clonal expansion capacity of a single cell. Both groups were able to form colonies, and their numbers were comparable (Fig. 3A). The percentage of cells that formed colonies was 43.06% ± 8.07% in the freshly thawed sample versus 40.35% ± 11.56% in the culture-rescued sample.

Figure 1. Morphology and viability of UC-MSCs under xeno-free and serum-free culture conditions. (A) Isolated UC-MSCs displayed a spindle-shaped morphology and were conserved after cryopreservation. (B) Freshly thawed UC-MSCs showed viability comparable to that of their culture-rescued counterparts (n = 5). Scale bar: 100 μm.

Figure 2. Growth ability of freshly thawed and culture-rescued UC-MSCs. (A) A representative example of cell nuclei stained with DAPI for cell counting. (B) Both freshly thawed and culture-rescued UC-MSCs grew exponentially during the 3-day monitoring period and (C) showed a comparable population doubling time (n = 5).
Expression of Surface Markers

We analyzed the expression of MSC-positive markers (CD90, CD105, CD73, and CD44) and MSC-negative markers (CD19, CD11b, CD34, CD45, and HLA-DR) by flow cytometry (Fig. 4A). There were no significant differences in surface marker expression between the freshly thawed and culture-rescued MSCs after thawing (Fig. 4B, C). Overall, the cells in both conditions of interest highly expressed the positive markers with more than 95% positivity, while the levels of all negative markers remained less than 2%.

Differentiation Ability

To test the ability of UC-MSCs to differentiate into osteogenic, adipogenic, and chondrogenic lineages, cells were placed in Gibco StemPro™ Differentiation Media or culture medium for the control and stained with Alizarin Red S, Oil Red O, and Alcian Blue. Both freshly thawed and culture-rescued UC-MSCs were capable of three-lineage differentiation (Fig. 5).

Immunomodulatory Potential

To analyze the immunomodulatory potency of UC-MSCs under the two conditions of interest, we cocultured UC-MSCs (n = 3) with PBMCs derived from healthy donors (n = 6). CD3^+CD4^+CD8^- helper T-cells and CD3^+CD4^-CD8^+ cytotoxic T-cells proliferated faster upon stimulation with CD2, CD3, and CD28 antibody preloaded beads. Their proliferation was suppressed in the presence of culture-rescued UC-MSCs but not in the freshly thawed conditions at a 1:2 ratio of MSCs: PBMCs (Fig. 6). The freshly thawed fraction showed a moderate immunomodulatory capacity at a 1:1 ratio. However, its effect was weaker on both helper T-cells (P = 0.0092) and cytotoxic T-cells (P = 0.0006) than that of their culture-rescued counterparts (Supplemental Fig. S2).

Discussion

Little is known about the quality of MSCs after cryopreservation in a xeno- and serum-free system. We have demonstrated that our standardized xeno- and serum-free culture platform was able to conserve the characteristics of MSCs from different cell sources that were stored in the master bank. In this study, we addressed the issues of final cell product preparation. To our knowledge, this is the first report comparing freshly thawed versus culture-rescued UC-MSCs under xeno-free and serum-free conditions. Our data revealed that freshly thawed and culture-rescued UC-MSCs had similar cell viability, growth ability, CFU numbers, marker expression, and differentiation characteristics. Al-Saqi et al. indicated that BM- and AD-MSCs cultured in xeno- and serum-free Mesencult-XF (Stem Cell Technologies, Vancouver, Canada) and cryopreserved in chemically defined serum- and xeno-free STEM-CELLBANKER™ (ZENOAQ, Fukushima, Japan) did not show significant changes in the morphology, cell viability, population doubling time or differentiation capacity. MSC Nutristem xeno-free media and MSC freezing media (Biological Industries) also maintained the cell identity and bioactivity of thawed BM-MSCs both in vitro and in polymicrobial septic animals. These data suggest that xeno-free and serum-free culture and cryopreservation systems are appropriate and can...
be used for a more standardized manufacturing of MSCs as a therapeutic product.

Several studies have addressed the effects of cryopreservation on MSCs in serum-containing media. Some reports demonstrated that compared to freshly cultured cells, cryopreserved UC-MSCs maintained their cell viability, MSC marker expression and multilineage differentiation after thawing. In contrast, a study comparing BM-MSCs in fresh, freshly thawed, and 24-hour-culture-rescue post-thaw conditions indicated that compared to the other two groups, the freshly thawed group showed decreased CD44 and CD105 expression, decreased proliferation and clonogenic capacity associated with increased apoptosis. Supporting these results, Bahsoun et al. recently reported that

![Figure 4. Expression of MSC markers in freshly thawed and culture-rescued UM-MSCs.](image)

(A) A representative example of MSC marker expression analysis via flow cytometry and analysis by FlowJo software. (B) Both groups showed a normal phenotype of MSCs as defined by ISCT, in which more than 95% of cells expressed positive markers (CD44, CD73, CD90, and CD105) and (C) less than 2% of cells expressed negative markers (CD34, CD11b, CD19, CD45, and HLA-DR) (n = 5).
cryopreservation altered the cellular and functional potency of BM-MSCs and that a 24 h rescue did not fully recover the properties of the cells. The diverse observations might be due to the different tissue sources and culture conditions used in these studies. Compared to adult tissue-derived partners, UC-MSCs grow faster and exhibited a better colony forming capacity. Compared to adult tissue-derived BM-MSCs, UC-MSCs show slower senescence, indicating that UC-MSCs might be less sensitive to cryopreservation-induced stress than their adult tissue-derived relative BM-MSCs.

Due to their potency in immune cell regulation, MSCs have been extensively tested for graft-versus-host disease and autoimmune diseases. Prochymal (later renamed Remestemcel-L) is the first commercial MSC product tested in phase 3 trials, in which allogeneic, cryopreserved bone marrow-derived MSCs were thawed and infused into patients with severe graft-versus-host disease. There were concerns about the use of freshly thawed cells that might limit the therapeutic efficacy of the intervention. Our data indicate that freshly thawed UC-MSCs were less potent in suppressing T-cell proliferation than their culture-rescued counterparts. On the other hand, MSCs derived from prenatal sources, including UC and cord blood, were reported to retain their immunoregulatory effects.

In the case of adult MSCs, data are controversial so far. Some studies claimed that freshly thawed and fresh bone marrow-derived MSCs showed comparable immunomodulatory potency and therapeutic efficacy in polymicrobial septic mice. However, there is evidence indicating an impaired immune suppression potency of freshly thawed BM-MSCs compared to continuously cultured cells. This could be because these cells suffered from heat shock stress as a result of the cryopreservation and subsequent thawing process. Indeed, the success of the allogeneic adipose-derived stem cells named Darvadstrocel for anal fistula of patients with Crohn’s disease suggested that a culture-rescued step after cryopreservation might maximize the effect of cryopreserved MSCs. Thus, the immunoregulatory functionality of MSCs might vary largely depending on their tissue source, culture conditions and the biological characteristics of the samples. Our study and other research suggest that the use of culture-rescued UC-MSCs can be favorable compared to their freshly thawed counterpart. Research in animal models and clinical trials would be essential to study their bioactivity in vivo.

In conclusion, the xeno- and serum-free manufacturing platform, which employs StemMACS™ MSC Expansion Media XF in combination with CellStart™-coating substrate, TrypLE dissociation enzyme, and CryoStor® CS10 for cryopreservation, demonstrated high-quality of both freshly thawed and culture-rescued UC-MSCs according to the International Society for Cellular Therapy (ISCT)-recommended minimal MSC criteria. However, it is necessary to test the functionality of the off-the-shelf products more stringently. Although the cell phenotypes and bioactivity...
properties such as proliferation, CFU numbers, and differentiation are similar, their therapeutic effects might be reduced due to cryopreservation-induced stress, which can be rescued by the cultivation of the cells for one more passage post thawing.

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**Authorship Contributions**

Conception and design of the study: VTH. Acquisition of data: NQM, HTHB, NTTA, TTHN, DMH, and LNT. Analysis and interpretation of data: NQM, HTHB, and VTH. Drafting or revising the manuscript: VTH, HTHB, NQM, DMH, and LNT. All authors have approved the final article. NQM and HTHB contributed equally to this work.

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**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

The study was approved by the Ethics Committee of Vinmec Healthcare System, Hanoi, Vietnam.

**Statement of Human and Animal Rights**

All procedures in this study were conducted in accordance with the Ethics Committee of Vinmec Healthcare System approved protocols (APPROVAL NUMBER/ID: 122/2019/QD-VMSC).

**Statement of Informed Consent**

Written informed consent was obtained from the donors for their anonymized information to be published in this article.

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**Supplemental Material**

Supplemental material for this article is available online.
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