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# Preservation of stem cells

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**Keywords:** cryopreservation, stem cells, cell therapy, preservation, stabilization

**Abbreviations:** HSC, hematopoietic stem cell; MSC, mesenchymal stem cells; hESC, human embryonic stem cells; DMSO, dimethyl sulfoxide; EG, ethylene glycol; FBS, fetal bovine serum; PBPC, peripheral blood progenitor cells

Adult stem cells (hematopoietic and mesenchymal) have demonstrated tremendous human therapeutic potential. Currently, human embryonic stem cells are used principally for understanding development and disease progression but also hold tremendous therapeutic potential. The ability to preserve stem cells is critical for their use in clinical and research applications. Preservation of cells permits the transportation of cells between sites, as well as completion of safety and quality control testing. Preservation also permits the development of a 'manufacturing paradigm' for cell therapies, thereby maximizing the number of products that can be produced at a given facility. In this article, we will review modes of preservation and the current status of preservation of hematopoietic, mesenchymal and human embryonic stem cells. Current and emerging issues in the area of stem cell preservation will also be described.

## Introduction

Stem cells are being used to treat an expanding number of diseases and disorders. For example, hematopoietic stem cells (HSCs), which have traditionally been used to treat leukemia<sup>1</sup> are now being used to treat heart damage from myocardial infarction,<sup>2</sup> hereditary blood disorders<sup>3</sup> and autoimmune disease.<sup>4</sup> Bone marrow is a complex mixture of cells including cells from hematopoietic, mesenchymal and endothelial origin. The mesenchymal fraction of the cells has been shown to create bone, cartilage and muscle-like cells when cultured under defined conditions.<sup>5</sup> Clinical uses of mesenchymal stem cells (MSCs) include cardiac repair<sup>2</sup> and to improve engraftment of hematopoietic stem cells.<sup>4,6</sup> Human embryonic stem cells (hESCs) hold tremendous promise as not only a tool for understanding disease but also as a basis for cell based therapies.<sup>7</sup> hESCs have specific challenges (controlling differentiation of the cells) but overcome many of the challenges associated with adult stem cells (availability). The Food and Drug Administration has approved the first U.S. clinical trial of hESC-based therapies in humans for the treatment of spinal cord injuries.<sup>8</sup>

Preservation of stem cells is critical for both research and clinical application of stem-cell based therapies. Preservation permits

development of cell banks with different major histocompatibility complex genotypes and genetically modified clones. As collection of stem cells from sources such as umbilical cord blood can be difficult to predict or control, the ability to preserve cells permits the banking of stem cells until later use in the research lab or clinical application. The ability to preserve cells permits completion of quality and safety testing before use as well as transportation of the cells between the sites of collection, processing and clinical administration. Finally, the ability to preserve cells used therapeutically facilitates the development of a manufacturing paradigm for stem cell based therapies. The ability to preserve the cells after production of the therapy facilitates coordination of therapy with a patient care regime and reduces staffing requirements of clinical cell production facilities.

The development of a cryopreservation protocol for a given cell type requires specification of: (1) pre-freeze processing; (2) introduction of a cryopreservation solution; (3) freezing protocol; (4) storage conditions; (5) thawing conditions and (6) post thaw assessment. In this review, we will focus on summarizing the findings of recent preservation studies that have been performed to determine the aforementioned protocol elements for hematopoietic stem cells and mesenchymal stem cells (publications since 2000). A more comprehensive and historical review of hematopoietic stem cell preservation can be found in reviews by Sputtek and colleagues.<sup>9-12</sup> We will also include a discussion of the freezing of human embryonic stem cells and the focus of this review article will be on more recent studies of stem cell freezing (after 2000). In addition, we will discuss emerging issues for cell preservation as new applications for preserved cells are developed and implemented.

## Hematopoietic Stem Cells

Recent studies aimed at improving cryopreservation of hematopoietic stem cells have focused primarily on two areas: (1) modification of the freezing medium; (2) freezing and storage protocols. Dimethyl sulfoxide is used to cryopreserve HSCs, with a DMSO concentration of 10% being the most commonly used concentration.<sup>13,14</sup> DMSO infusion has been associated with negative reactions in patients receiving HSC transplants and there is considerable interest in reducing or eliminating DMSO from preservation solution.<sup>15-21</sup> Abrahamsen compared the effects of 5% versus 10% DMSO on CD34<sup>+</sup> cells and showed that survival improved from 52% to 74% with the lower DMSO

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concentration.<sup>22</sup> In a study by Galmes, no difference in engraftment for patients when using cells preserved using reduced DMSO concentration (5%) while a reduction in DMSO-related toxicity of 60% was shown.<sup>23</sup> Similar results were found by Bakken and colleagues.<sup>24</sup>

Other investigators have studied the use of additives (in addition to DMSO) designed to improve post thaw recovery of HSCs. Low levels of trehalose (25 mg/ml) and catalase (100 µg/ml) were shown to reduce post thaw apoptosis in a murine HSC model<sup>25</sup> and improve recovery of colony forming units for umbilical cord blood and HSCs isolated from fetal liver.<sup>26</sup> Cryopreservation solutions supplemented with membrane stabilizer taurine, ascorbic acid and  $\alpha$ -tocopheryl acetate also demonstrated improved post thaw recovery.<sup>26</sup> These studies suggest that additives beyond what are classically considered cryoprotective agents can be used to protect cells from the stresses of freezing and thawing and improve post thaw recovery.

The second major area of recent research has been on investigating the effects of cooling rate and storage temperature on HSC recovery. Standard practice for preserving HSCs involves a controlled rate freezer and cooling at approximately 1°C/min, followed by storage in liquid nitrogen. A number of studies have investigated the use of -80°C mechanical freezers for cryopreservation of HSCs. Clappisson and colleagues used a 3% hydroxyethyl starch (HES) and 5% DMSO mixture in a -80°C mechanical freezer to achieve cell viabilities and BFU-E and CFU-GM activity slightly better than those obtained using the standard 10% DMSO and controlled rate freezer method.<sup>27</sup> A similar result was observed by Kudo and colleagues who used insulation around the PBPC product to control the cooling rate when using a mechanical freezer.<sup>28</sup>

### Mesenchymal Stem Cells

A limited number of studies have also investigated the freezing behavior of MSCs. A variety of different cryopreservation solutions have been tested. Carvalho and colleagues used culture media supplemented with 10% fetal calf serum (FCS) and 5% dimethyl sulfoxide (DMSO) to freeze MSCs isolated from rat bone marrow.<sup>29</sup> Other investigators used a similar solution composition for the cryopreservation of human MSCs isolated from bone marrow.<sup>30,31</sup> In developing a solution more appropriate for clinical use, Pal, Hanwate and Totey used different parenteral solutions (e.g., saline solution, Plasmalyte A) supplemented with 5% human serum albumin (HSA) and 10% DMSO.<sup>32</sup> Moon and colleagues used a vitrification solution of 40% ethylene glycol (EG) + 18% Ficoll 70 + 0.3 M sucrose to preserve MSCs isolated from amnion.<sup>33</sup> Cryopreservation or post thaw wash solutions are sometimes supplemented with additives intended to modulate cell response to the stresses of freezing and thawing (e.g., apoptosis inhibitors to reduce cell losses associated with post thaw apoptosis<sup>34-37</sup>). Heng and colleagues used Rho-associated kinase (ROCK) inhibitor to improve the long-term post thaw recovery of the cells.<sup>30</sup>

A limited number of studies have examined the influence of freezing conditions on post thaw recovery of MSCs. Some studies have used controlled rate freezing of the samples.<sup>29,32</sup> Carvalho

and colleagues also evaluated other cooling rates (3, 5, 10°C/min) but did not report any variation in the survival of MSCs with cooling rate.<sup>29</sup> Other investigators used mechanical freezers with Heng and colleagues placing the samples directly into a -80°C freezer<sup>30</sup> and Kotobuki and colleagues<sup>31</sup> placing the samples into a -30°C mechanical freezer followed by a -80°C freezer. Vitrification of the MSCs required plunging of the samples in a cryovial into liquid nitrogen.<sup>33</sup>

Studies to date have not examined the role of storage conditions (temperature and duration) or the influence of warming conditions on post thaw recovery. Measurements of post thaw recovery among the studies illustrated several challenges for MSCs. First of all, two studies noted that the post thaw viability measured varied with the timing of the assay/time post thaw.<sup>30,32</sup> Specifically, the viability of the MSCs declined with time post thaw, with frozen-thawed MSCs showing a decrease in viability from >80% at 2 hours to <40% at 8 hours when maintained at 4°C.<sup>16</sup> This was presumably because of post thaw apoptosis but markers of apoptosis were not monitored. Secondly, different measures of viability may yield different outcomes. Carvalho and colleagues noted that the viability measured using trypan blue differed significantly from that measured using 7-AAD (90.6% versus 66.3%). This finding is not unexpected as viability as determined using membrane integrity frequently does not correlate with functional measures of viability.<sup>38</sup> Finally, post thaw assessment of stem cells should include more rigorous assays (surface markers associated with stem cells and differentiation assays<sup>31,33</sup>).

Currently, there is no consensus protocol for preserving MSCs. Further work is needed, clearly to determine the optimal cryopreservation solution composition and cooling rate for MSCs. The use of MSCs for the treatment of cardiac repair and link between DMSO and a variety of cardiovascular-related adverse reactions (hypotension, arrhythmia, etc.,) makes the use of DMSO to preserve MSCs concerning unless the DMSO is removed before infusion/injection.<sup>15-17,19-21,39</sup> The rapid decline in viability with time post thaw observed by other investigators also suggests that post thaw apoptosis is a concern for this cell type. One study suggested that those cells could be 'rescued' through the use of an inhibitor. The growth in the number of disorders and patients treated with MSCs implies that further work is needed to improve preservation of the cells and thereby facilitate their clinical use.

### Human Embryonic Stem Cells

Two basic methods to preserve human embryonic stem cells (hESCs) have been studied and they are based on protocols developed for two different biological systems: (1) embryos and (2) HSCs. Embryos and oocytes from certain species are routinely vitrified.<sup>40</sup> This same basic preservation methodology has been applied to hESCs by a variety of investigators.<sup>41-43</sup> Briefly, colonies of hESCs (100–400 cells) are placed in a vitrification solution composed of 20% DMSO + 20% EG + 0.5 mol/l sucrose after equilibration with a lower concentration DMSO + EG solution. The colonies are loaded into straws and plunged into liquid nitrogen. Post thaw recoveries using this method have demonstrated higher post thaw recoveries (94% colony attachment) when compared to

conventional cryopreservation methods (6% colony attachment).<sup>41</sup> There are several problems with this approach: (1) preservation in straws is open/non sterile; (2) the process is very labor intensive as colonies have to be physically moved from one solution to the next during introduction and removal of the vitrification solution; (3) traditional formulations use animal proteins in the solution. These limitations imply that this technique would be difficult to use in hESC banks as well as individual laboratories. Richards and colleagues developed a closed straw method and used human serum albumin (versus fetal bovine serum) to obtain post thaw recovery of colonies comparable to that obtained using conventional vitrification techniques and have improved the overall technique.<sup>43</sup>

The second method used to preserve hESCs mimics that of HSCs. Briefly, hESC colonies are placed in 10% DMSO solution (the remainder of the solution being typically culture medium or FBS) and placed in an isopropanol bath intended to cool the sample at 1°C/min or a controlled rate freezer.<sup>41,44–46</sup> Ha and colleagues evaluated various compositions of DMSO and EG using a cooling rate of 1°C/min and found that the optimal post thaw recovery (30% colony recovery) was observed using 5% DMSO + 10% EG solution + 50% FBS.<sup>44</sup> It is noteworthy that the solutions evaluated appeared to be introduced using a single step introduction and removal and it is not clear if the post thaw recoveries reflected cell losses from osmotic stresses (versus freezing damage). Kim and colleagues supplemented a 10% DMSO + 90% serum replacement solution with human type IV collagen or laminin. These extracellular matrix molecules were found to improve post thaw recovery and reduce differentiation of the colonies during subsequent culture.<sup>47</sup> Similar results were observed by Zhang and colleagues<sup>48</sup> when a solution containing 10% DMSO + 90% knockout serum replacement was supplemented by 0.2 mol/l trehalose. Adding trehalose to the solution increased the recovery of colonies from 15 to 48%.

In contrast to HSCs and MSCs, hESCs are cryopreserved in small aggregates of a few hundred cells in order to prevent cell loss from apoptosis when detached/dissociated.<sup>49</sup> The cryopreservation of cell aggregates brings with it specific challenges as cell recovery is typically greater for isolated cells that are cryopreserved (versus tissues containing the same cell type<sup>50</sup>). Several investigators have attempted to improve post thaw recovery of hESCs by modifying the configuration of the cells. Martin-Ibanez and colleagues used Rho-kinase inhibitor (ROCK) to improve recovery of hESC colonies after dissociation into single cells and freezing. Post thaw recovery was improved (60%) but only 5–7% of the isolated cells formed colonies when fresh (not frozen) resulting in a lower overall recovery.<sup>51</sup> Ji and colleagues cryopreserved hESC colonies attached to Matrigel-coated plates and observed higher recoveries for colonies that were adherent<sup>52</sup> Nie and colleagues cultured hESCs to microcarriers for culture/expansion and then cryopreserved colonies with and without attachment to the microcarriers.<sup>46</sup> As with previous studies, attachment of the colony to the microcarrier improved post thaw recovery.

The studies described above (independent of method used) also illustrate the difficulty in quantifying outcome of a preservation protocol for hESCs and therefore whether or not the method represents an improvement. As hESCs are typically preserved as

colonies, it is not easy to quantify the fraction of cells that are viable within the colony. Typically the percentage of colonies which are attached is used. It is not simply sufficient to have a colony attach however; the colony must also remain undifferentiated and exposure to DMSO has been associated with differentiation of hESCs.<sup>45</sup> Investigators have attempted to quantify differentiation in colonies and thereby the quality of the colony. Additional studies performed to evaluate the quality of the frozen/thawed colony include: karyotyping, staining for ESC markers, and teratoma formation after implantation into an immunocompromised animal model.<sup>42</sup> In particular for hESCs, it will be important to have several measures of post thaw recovery as the desired post thaw function is very complex.

## Emerging Issues

As the clinical need for stem cells to treat human disease continues to grow, the need for effective and clinically relevant methods for preserving those cell types will also continue to grow. The studies reviewed in this article illustrate ongoing interest in improving and modifying preservation methods. There are several common themes among the three different stem cell types described. In closing, we will discuss those themes briefly:

(1) There is no universal method of preserving stem cells. The inability to simply transfer protocols for HSCs to the preservation of MSCs and the difficulty in effectively preserving hESCs using protocols developed for other biological systems illustrates the unique biology of each cell type and that preservation protocols must reflect that unique biology.

(2) Effective methods of cell preservation that do not involve DMSO must be developed. DMSO is not approved for human infusion; it is associated with adverse effects upon infusion and epigenetic effects on cells. As the clinical use of stem cells continues to grow, the need to develop alternative methods of preserving cells will continue to grow—even for cell types that have been successfully preserved in the past.

(3) We must develop our understanding of the molecular mechanism of damage. Studies of additives designed to stabilize the cell membrane or inhibit post thaw apoptosis represents a glimpse into those molecular mechanisms. Further work is needed to expand that understanding, which will in turn help us preserve all stem cells (those currently amenable to preservation as well as those that respond poorly to conventional preservation protocols).

(4) Existing methods of cell preservation are based on scientific principals, which must drive the development of new protocols or the modification of existing protocols. Each element of the protocol (introduction of the preservation solution, freezing, storage, warming) has the potential to damage the cells if not performed properly. Individuals performing preservation protocols should be trained as to the importance of those elements and the manner by which they should be properly performed.

(5) Post thaw assessment of cells is very difficult and varies significantly from determining the viability of a cell that has not been recently frozen and thawed. Interpretation of cryopreservation studies is frequently hampered by improper, inconsistent and non-validated methods of measuring post thaw viability.

(6) Current methods of preserving cells are very labor intensive and operator dependent and may require equipment that is not easily obtained. New technology will be needed that facilitates high efficiency processing of cells for preservation. Protocols must be developed to permit effective methods of preservation of cells in different contexts.

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