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# Progress in Cryopreservation of Stem Cells and Immune Cells for Cytotherapy

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/60620>

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## Abstract

Cellular therapy with stem and immune cells has demonstrated significant success both in clinical treatments and the industrial market. Cryopreservation is a necessary and essential component of cellular therapy. In this chapter, first of all, some basic theories of cryoinjury and techniques in cryopreservation are reviewed. Then it focuses on the progress of cryopreservation of stem cells and immune cells, including new protocols and techniques, alternative cryoprotective agents (CPA), side effects after transplantation, and advances in reducing adverse reactions. Strategies to minimize adverse effects include medication before and after transplantation, optimizing the infusion procedure, reducing the CPA concentration or using alternative CPAs for cryopreservation, and removing CPA prior to infusion. Traditional and newly developed approaches including methods and devices for CPA removal are discussed. Future work is recommended including further optimization of cryopreservation protocols especially for lymphocytes; standardization of the optimized protocols with temperature monitoring and quality control; exploration of DMSO-free, serum-free, and even xeno-free media for cryopreservation; development of simple, reliable, and cost-effective devices for cryopreservation; and more fundamental cryobiological studies to avoid cellular injury. Keywords: cryopreservation, stem cell, immune cell, cytotherapy

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## 1. Introduction

Cell therapy products, including stem cells and the stem cell-derived immune cells, with a more than \$600 million market size, have been approved for use in the USA and EU[1]. With the anticipated approval of additional products, the cell therapy market is expected to grow at an annualized rate of greater than 40% through the remainder of the decade, reaching greater than \$5 billion by 2020. Stem cell therapy in the USA cleared a record \$250 million in 2013. All these data prove the significance of cell therapy both in clinical practice and industry.

Importantly, for most types of transplants, cryopreservation is a necessary and essential component of the clinical protocol. Long-term storage provides a solution to various logistical aspects such as the obligatory time interval between collection of the cell product, treatment with high-dose therapy, and subsequent infusion of the product in the case of autologous transplantation, or in the case of transplantation with mismatch between supply (when the baby is born) and demand (when the patient is ready to receive the unit). Cryopreservation also supports better cell therapy product characterization and quality control, improved donor screening for human leukocyte antigen (HLA) or other markers that can impact successful outcomes, and optimal transportation from the point of collection to the site of infusion.

Meanwhile, cryopreservation can be a critical issue determining the success or failure of cell transplantation. Typical cell therapeutic products must be [1] harvested from culture vessels and concentrated, followed by [2] cryopreservation permitting the cells to be stored or shipped. Cryopreservation process generally includes the addition of cryoprotective agents (CPA), cooling of samples with a specific optimal cooling rate, which is cell-type dependent, and then storage at a low temperature (such as  $-80^{\circ}\text{C}$  or  $-196$  to  $-150^{\circ}\text{C}$ , temperature of liquid nitrogen or the vapor phase of liquid nitrogen). [3] Finally, the CPA must be removed post-thaw in preparation for patient administration. Herein, each step may cause injuries to cells, and therefore influence the graft function. Optimization of cryopreservation of a cell product requires understanding the mechanisms of injury caused in cryopreservation (cryoinjury), knowledge of the cryobiological characteristics of the cells, and the development of techniques and instruments to perform the optimal protocol.

In this chapter, first of all, some basic theories of cryoinjury and techniques in cryopreservation are reviewed. Then it focuses on the progress of cryopreservation in cellular therapy of two kinds of cells: stem cells and stem cell-derived immune cells.

Transplantation of stem cells, especially hematopoietic stem cells (HSC) has been successfully developed as a part of treatment protocols for a large number of clinical indications. In this part, we briefly review the progress in stem cell cryopreservation and transplantation, the side effects after transplantation along with strategies for reducing adverse reactions. Direct infusion of cryopreserved cell products into patients has been associated with the development of adverse reactions, ranging from relatively mild symptoms to much more serious, life-threatening complications. Strategies to minimize the adverse effects are reviewed. In many cases, the CPA — typically dimethyl sulfoxide (DMSO) — is believed to cause these adverse reactions and thus many studies recommend depletion of DMSO before cell infusion. Tradi-

tional and newly developed approaches including methods and devices for CPA removal are discussed.

Immune cells function to protect our body from diseases, including detection of a wide variety of pathogens, distinguishing them from the body's own healthy cells, killing infected cells, and others. They exit the bone marrow and may stay in circulation, or recirculate between blood and tissues, or go to tissues and stay there until they die. Transplantation of immune cells has been applied for the treatments of many diseases. However, despite many reports of success in immune cell cryopreservation, some conflicting and negative results are also shown in the literature. In this chapter, progresses in cryopreservation protocols and techniques for immune cells are also reviewed.

## 2. Fundamental cryobiology and cryopreservation processes

Generally, cryopreservation consists of several steps: preprocessing, CPA addition, cooling, storage, thawing, CPA removal, and postprocessing. Each step can affect the viability of cells after cryopreservation. Optimizing each step is necessary to maximize the retention of cell viability and functionalities. Obviously, the better understanding of the cellular injury mechanisms in cryopreservation can help minimize cellular injury. In this part, some fundamental knowledge in cryobiology is summarized. Table 1 shows the general process, cellular injury mechanisms, and proposed optimization work for cell cryopreservation [2].

Procedures	Injury mechanisms	Keys to success
Preprocessing		Cell isolation, dilution or concentration, etc.
CPA addition	Osmotic injury, CPA cytotoxicity	CPA selection (with high cryoprotective function and low cytotoxicity), CPA concentration, CPA addition (gradual addition at lower temperature).
Cooling	Mazur's "Two-Factors": ice injury and solution injury	Cooling rate and cooling history profile, reliable and simple instruments for controlled cooling, monitoring of sample temperatures during cooling.
Storage		Monitoring of sample temperatures during storage. Storage at -80°C for weeks to months and storage in liquid nitrogen or vapor phase of liquid nitrogen for months to years.
Thawing	Ice injury (recrystallization)	Fast and uniform thawing is recommended.
CPA removal	Osmotic injury, CPA cytotoxicity	Stepwise removal of CPA is recommended.
Postprocessing		Sometimes, resting the thawed cells is beneficial for the recovery of cell functions.

**Table 1.** Cryopreservation procedures, injury mechanisms, and optimization

## 2.1. Freezing of cells

Optimization of isolated cell cryopreservation requires a quantitative understanding of the biophysical response of cells during the freezing process [3-6]. As cells are cooled to between about  $-5^{\circ}\text{C}$  and  $-15^{\circ}\text{C}$ , ice forms in the external medium but the cellular contents remain unfrozen and supercooled, presumably because the plasma membrane blocks the growth of ice crystals into the cytoplasm. The supercooled water inside the cells has a higher chemical potential than that of water in the partially frozen extracellular solution, so water flows out of the cell and freezes externally.

The subsequent physical phenomena in the cells depend on the cooling rate. These cell responses to freezing were first expressed quantitatively by Mazur [6] and directly linked with cell cryoinjury by Mazur's "Two-Factors" hypothesis: (a) at slow cooling rates, cryoinjury occurs due to a "solution effect", that is, the intracellular solute/electrolyte concentration increases as water leaves the cell, to a point where severe cell dehydration occurs, and (b) at high cooling rates, water is not lost fast enough and cryoinjury occurs due to intracellular ice formation (IIF), which ruptures the cell membrane. The optimal cooling rate for cell survival should be slow enough to reduce IIF but fast enough to minimize the solution effects. The freezing behavior of the cells can be modified by the addition of cryoprotective agents (CPAs), which affect the rates of water transport, ice nucleation, and ice crystal growth.

More detailed information about theoretical cryobiology can be found in the paper published in 1970 by Mazur in *Science* [5]. Important milestones in cryobiology since then include the development of cryomicroscopy allowing the observation of cell behavior during freezing and thawing [7, 8], devices to model and measure cell membrane permeabilities [9-13], and mathematical modeling to describe the probability of IIF as a function of cooling rate, temperature, and cell type [14, 15]. Karlsson and Toner incorporated the effect of CPA addition on IIF formation into these models and successfully predicted IIF formation as a function of cooling rate, temperature, and CPA concentration, leading to optimal cooling protocols preventing IIF [16].

## 2.2. Thawing of cells

Cells that have survived cooling to low temperatures still face the challenges of thawing, which can exert effects on survival comparable to those of cooling [5, 17]. The effects depend on whether the prior rate of cooling has induced intracellular freezing or cell dehydration. In the former case, rapid thawing can rescue many cells, possibly because it can prevent the harmful growth of small intracellular ice crystals into larger crystals by recrystallization. After severe cell dehydration, no rescue from cell death is possible.

## 2.3. Addition and removal of CPAs

Cells require equilibration with molar concentrations of CPAs to survive freezing. Previous research has shown a high concentration of CPA to be beneficial for preventing IIF and severe cell dehydration, thus leading to increased cell survival [18-24]. However, these CPAs have dramatic osmotic effects on cells. Cells exposed to molar concentrations of permeating CPAs

undergo extensive initial dehydration followed by rehydration and potential gross swelling when the CPAs are removed. Unless precautions are taken, this shrinkage and/or swelling can be extensive enough to cause cell damage and death. Knowledge of cell membrane permeability to water and CPAs allows the prediction of the minimal and maximal cell volume excursions during addition and removal of CPAs, thus providing for a quantitative optimization approach (e.g. stepwise increase or decrease of CPA concentration in cell suspensions) to avoid osmotic damage [19-21].

## 2.4. Storage

Biological specimens can be stored at low temperatures for extended periods because the chemical reactions and metabolism will be slowed down or even stopped at those low temperatures. The “life clock” of these materials (RNA/DNA, cells, tissues, etc.) will be slowed or halted during cryo-storage, and then resumed after thawing to normal temperatures. The most widely applied methods for long-term storage are preserving in -80°C freezers or liquid nitrogen tanks (in liquid or vapor phases of liquid nitrogen). However, in order to totally stop any reactions, storage temperature should be below the glass transition temperature ( $T_g$ ) of the aqueous solution (e.g.,  $T_g = -123$  °C for concentrated aqueous DMSO solutions [25]). Therefore, storage in liquid nitrogen tanks is recommended for preservation for months and years. Some reports have suggested that storage in the vapor phase rather than the liquid phase of liquid nitrogen would be better to avoid potential contamination between samples.

Procedures	Recommended protocol	Comments
CPA selection	<ul style="list-style-type: none"> <li>· Mostly, 10% DMSO in basal medium supplemented with proteins, sometimes DMSO with lower concentrations (5–10%)</li> </ul>	<ul style="list-style-type: none"> <li>· Lower DMSO concentration leads to lower cytotoxicity.</li> <li>· DMSO-free, serum-free, and even xeno-free cryomeia are desired.</li> </ul>
CPA addition	<ul style="list-style-type: none"> <li>· Add CPA stock solution (precooled at 4°C, 2x concentration) to cell suspension stepwise at 4°C or room temperature (with final volume ratio 1:1). Cell concentration can be <math>10^7</math>–<math>10^8</math> cells/mL.</li> <li>· Equilibration (e.g., for 10min)</li> </ul>	<ul style="list-style-type: none"> <li>· Slow CPA addition can reduce cell volume excursion and osmotic injury.</li> <li>· Lower temperature may reduce CPA cytotoxicity.</li> </ul>
Cooling	<ul style="list-style-type: none"> <li>· Controlled slow cooling to a temperature (e.g., -40°C) with 1-2.5°C/min, then cooling down to a lower temperature (e.g., -80°C) at a little faster rate (about 3-5°C/min)</li> <li>· Put into -80°C freezer or liquid nitrogen tank.</li> </ul>	<ul style="list-style-type: none"> <li>· Slow cooling is desired to reduce ice injury to cells.</li> <li>· Temperature recording in whole process is recommended.</li> <li>· Intervention and compensation for crystallization heat by transient chilling during freezing procedure is beneficial for cell viability.</li> </ul>
Storage	<ul style="list-style-type: none"> <li>· Storage in -80°C freezer, vapor phase of liquid nitrogen, or liquid nitrogen.</li> </ul>	<ul style="list-style-type: none"> <li>· -80°C for storage for weeks to months.</li> <li>· Liquid nitrogen for storage for months to years.</li> </ul>

Procedures	Recommended protocol	Comments
		<ul style="list-style-type: none"> <li>· Storage in vapor phase rather than liquid phase of liquid nitrogen could decrease the contamination risk.</li> <li>· Temperature monitoring in whole process is recommended.</li> </ul>
Thawing	<ul style="list-style-type: none"> <li>· Fast thawing by stirring in 37°C water bath until thoroughly melted.</li> </ul>	<ul style="list-style-type: none"> <li>· Fast thawing is beneficial to reduce recrystallization and ice injury.</li> </ul>
CPA removal	<ul style="list-style-type: none"> <li>· Add washing medium slowly to the thawed cell suspension followed by equilibration and washing.</li> <li>· Repeated dilution/washing may be needed.</li> </ul>	<ul style="list-style-type: none"> <li>· Slow CPA removal can reduce cell volume excursion and osmotic injury.</li> <li>· DMSO may be associated with adverse reactions after transplantation without CPA removal; however, so far, no specific consensus exists regarding removal of DMSO prior to infusion, instead leaving the decision to physicians and clinical institutions.</li> </ul>

**Table 2.** Cryopreservation protocol for HSC

### 3. Progress in HSC cryopreservation

#### 3.1. HSC cryopreservation protocol

Stem cell transplantation has been performed using hematopoietic stem cells (HSC) from allogeneic, autologous, and syngeneic donors. In addition to bone marrow, HSC collected from mobilized peripheral blood or umbilical cord blood are currently in widespread clinical use, with the potential for transplantation of HSC derived from embryonic stem cell or induced pluripotent stem cell sources in the not-too-distant future [21,22]. Since the first studies of HSC freezing by Barnes and Loutit in 1955 [23], many experiments have been performed to optimize cryopreservation protocols to enhance the overall recovery and functional capacity of HSC after freezing–thawing and transfusion. Numerous excellent reviews of stem cell cryopreservation have been published, ranging from basic scientific principles to clinical cell processing protocols [24–28]. The most widely applied cryopreservation protocols for HSC have the following general features: after collection, cells are washed and resuspended in a basal saline solution supplemented with some proteins, which also contains one or more CPA. Dimethyl sulfoxide (DMSO) is the most commonly used CPA, typically at a final concentration of 5–10% (v/v). The cell suspension is frozen using a rate-controlled freezer or mechanical passive cooling methods with an optimal cooling rate of -1 to -2.5°C/min [27,28] to a subzero temperature such as -40°C [25,27,29,30], then cooled down to a lower temperature (e.g., -80°C) at a little faster rate (about 3–5°C/min), followed by transferring to a liquid nitrogen tank for long-term storage at temperatures below -150°C. Just prior to transplantation, the cryopreserved cell products are thawed quickly in a 37°C water bath and infused immediately into the patient. Sometimes, DMSO is removed before transplantation.

Table 2 shows the generally applied protocol for HSC cryopreservation. In order to maximize the quality of the frozen samples, following the optimal protocol in each step is recommended, such as gradual addition and removal of CPA, controlled freezing of samples by reliable cooling rate controlled device, fast thawing, and temperature recording during the whole process of cooling, storage, and transportation.

### **3.2. Adverse reactions after HSC transplantation**

Infusion of thawed products has been associated with several types of adverse reactions (ARs), ranging from mild events like nausea/vomiting, hypotension or hypertension, abdominal cramps, diarrhea, flushing and chills to more severe life-threatening events like cardiac arrhythmia, encephalopathy, acute renal failure, and respiratory depression. In some cases, these adverse reactions have been directly attributed to DMSO [26-29], while others have suggested additional factors such as red cell lysate [30, 31], or infusion of high numbers of damaged granulocytes that do not survive cryopreservation [32] are the main causal trigger of these adverse reactions. To minimize such ARs after infusion, many institutions have chosen to limit the total amount of DMSO that can be infused at any one time, while others have evaluated washing protocols to first remove the DMSO and other damaged cell products prior to infusion [33-36]. In addition, patient-specific factors such as age, weight, gender, specific disease, or the type of prior treatments given and chemotherapeutic agents received can also contribute to the development of ARs, as can the infusion procedure itself (speed of injection, pausing for short periods, and the time gap between thawing of frozen cells and infusion). These topics have been intensively reviewed by Shu et al. [37], including ARs after HSC transplantation with cryopreserved products, the physiological role of DMSO in these adverse events, strategies to reduce these ARs, and new options for removal of DMSO before transfusion.

### **3.3. Reducing the ARs after infusion of cryopreserved HSC grafts**

Many approaches have been applied to reduce the adverse effects after transplantation of cryopreserved HSC, such as:

1. systematic premedication before infusion [38],
2. hydration and allopurinol administration after infusion [38],
3. slowing the infusion speed and prolonging the infusion time [36, 38],
4. dividing the infusion into multiple aliquots given several hours or days apart [38, 39],
5. further concentrating HSC grafts to reduce the cryopreservation volumes and corresponding DMSO content [36],
6. reducing DMSO concentration for cryopreservation to lower than 10%, or using alternative CPA to mix with or replace DMSO [36, 40-42]; and
7. removing DMSO before infusion [33-36].

Since the side effects are idiosyncratic and unpredictable so far to our knowledge, all these approaches are suggested to be combined together to reduce the reaction incidence as low as possible. Several studies examining the use of lower concentrations of DMSO or alternative CPAs have been conducted, such as 7.5% DMSO+ 3% hydroxyethyl starch (HES), 5% DMSO + 6% HES, 5% DMSO+6% pentastarch [40, 42, 43]. Simply reducing DMSO concentration may decrease the toxicity and improve the kinetics of engraftment; however, it is also likely to reduce the recovery rate of HSC after cryopreservation and thawing as well. Therefore, other cryoprotective agents, such as HES or trehalose, are recommended to combine with any proposed reduction in DMSO concentration.

### 3.4. Removal of DMSO

A summary of options of methods and devices used for removal of DMSO from cryopreserved products is presented in Table 3 [37]. Conventional manual methods of removing DMSO from cell suspensions based on centrifugation have not changed much since the 1970s. The most widely used procedure was proposed in 1995 [44]. This process can result in cell clumping and HSC loss, cell activation, and carries a risk of product contamination. This procedure is also time-consuming and labor intensive. Several commercially developed devices have been evaluated for CPA removal, such as the CytoMate™, Sepax S-100, and Cobe-2991 instruments. Using user-definable programs DMSO can be efficiently reduced by these automated systems, resulting in reduced labor and reduced risk of contamination due to the closed fluid path. However, these devices are expensive, and again can cause cell clumping, osmotic injury, and loss of cells since they are all still based on centrifugation as their primary mode of operation.

Several new methods/technology for DMSO removal without using centrifugation have recently been developed. Fleming et al. investigated an elegant microfluidic method for small samples based on diffusion [45, 46]. However, this method has not yet been scaled up for the preparation of HSC units for transplantation. Ding et al. proposed a dialysis method for DMSO removal using hollow fiber modules with semipermeable membranes [47, 48]. Zhou and Shu et al. have developed a novel dilution-filtration method and system [49, 50], which can be used to precisely control the removal process to effectively reduce CPA concentration and prevent cell osmotic injury. Research data suggest this method promises to be a fast, safe, easy-to-operate, automated, and cost-effective approach with low cell loss and low contamination risk.

Methods or devices	Working mechanism	Comments
Centrifugation [44]	Centrifugation	<p>Most widely applied procedure for CPA removal.</p> <ul style="list-style-type: none"> <li>· Pros: conventional devices available widely</li> <li>· Cons: high time and labor consumption, cell loss, high risk of contamination</li> </ul>
CytoMate™ [51, 52]	Filtration by spinning membrane	<ul style="list-style-type: none"> <li>· Pros: automated, effective and allowing a step-by-step user definable programming, low risk of contamination</li> <li>· Cons: high cost and cell loss due to clumping</li> </ul>

Methods or devices	Working mechanism	Comments
Sepax S-100 [53, 54]	Consisting steps of dilution and centrifugation using a rotating syringe	<ul style="list-style-type: none"> <li>• Pros: fast, automated processing, low risk of contamination</li> <li>• Cons: high cost and cell loss due to clumping</li> </ul>
Cobe 2991 [35, 55]	Centrifugation	<ul style="list-style-type: none"> <li>• Pros: fast, automated processing, low risk of contamination</li> <li>• Cons: high cost and cell loss due to clumping</li> </ul>
Microfluidic method [45, 46]	Diffusion-based extraction in microfluidic channels	<ul style="list-style-type: none"> <li>• Pros: automated processing, elegant, effective for CPA removal for samples with small volumes</li> <li>• Cons: hard to scale up to large volumes</li> </ul>
Dialysis through hollow-fiber dialyzer [47, 48]	Dialysis across semipermeable hollow fiber membranes	<ul style="list-style-type: none"> <li>• Pros: automated processing, effective CPA removal, low risk of contamination</li> <li>• Cons: high cell loss, optimization needed for samples with small volume</li> </ul>
Dilution-filtration through hollow-fiber dialyzer [49, 50]	Controlled dilution and controlled filtration through semipermeable hollow fiber membranes	<ul style="list-style-type: none"> <li>• Pros: fast, automated processing, low risk of contamination, low-cost, controllable, effective CPA removal</li> <li>• Cons: optimization needed for samples with small volume</li> </ul>

**Table 3.** Methods and devices for CPA removal

To go along with these approaches, DMSO-washing solutions are needed. Generally, washing solutions consist of saline or cell culture medium together with nonpermeable macromolecules (dextran, albumin and/or acid citrate dextrose (ACD)), which are nontoxic, infusible, and provide a mild hyperosmotic environment to help extract the DMSO from cells. This is also why slow addition of such solutions (e.g., dripping) is preferable, as it allows the cells to slowly equilibrate to the changing osmotic environment, and minimizes the rapid uptake of water that can damage the cell membranes.

In conclusion, many progresses on effective devices and methods for removal of DMSO from cryopreserved HSC grafts have been achieved in the last decade, but challenges still remain: further studies are needed to develop the optimal (fast, safe, simple, automated, controllable, effective, and low-cost) methods and devices for CPA removal with minimum cell loss and damage.

### 3.5. Alternative CPAs for stem cell cryopreservation

Although DMSO has been widely accepted and utilized for stem cell cryopreservation, in some situations it may be desirable to employ other CPAs, in combination with or even replacing DMSO. The criteria for optimal CPAs include: (1) protecting cells during cryopreservation; (2) being nontoxic and able to be metabolized or digested by the body with minimal effects, which eliminates the necessity for CPA removal prior to infusion; and (3) cost and availability. Some agents, such as ethylene glycol, hydroxycellulose, sucrose, maltose, trehalose, and some macromolecules (dextran, hydroxyethyl starch, Polyvinylpyrrolidone, etc.) could be poten-

tially used as alternative CPAs. In the last two decades, trehalose has drawn a lot of interest in this field due to its unique properties. It has very high glass transition temperature, and is extremely effective in forming a fragile glass state to protect cells during freezing/thawing and drying, maintaining the thermodynamic stability of cell membranes, and inhibiting lipid-phase transition and separation during freezing and drying [56-58]. However, for HSC, DMSO is still the most widely used CPA. In the future, searching for alternative CPAs could be another strategy to reduce the adverse reactions after HSC transplantation with DMSO.

Another possible significant development in alternative CPAs would be the development of novel, DMSO-free, serum-free, or even xeno-free cryoprotection media for the cryopreservation of stem cells. This effort will address the problems of DMSO toxicity and immunological depression posed by the presence of DMSO, animal serum, and xeno-component to the recipient after transplantation. Thirumala et al. demonstrated the feasibility of a serum-free media with reduced DMSO concentration for cryopreservation of adipose-derived adult stem cells [59]. Schulz et al. showed important advancements toward a xeno-free, chemically defined cryopreservation medium for peripheral blood mononuclear cells (PBMCs) [60,61]. These studies demonstrate the possibility of finding novel cryopreservation media for stem cells.

## 4. Progress in immune cell cryopreservation

### 4.1. Immune cell cryopreservation protocol

Cryopreservation of immune cells has been continuously studied since the 1960s. Improvement of the cryopreservation protocol was conducted based on intensive research, including fundamental cryobiological studies and experimental trials. Successful cryopreservation of lymphocytes (e.g., PBMC) has been reported and applied in clinical practice; however, negative or conflicting results also exist in the literature, especially if assessments of lymphocyte functionality rather than cell viability were applied to evaluate the impacts of cryopreservation.

Procedures	Recommended protocol	Comments
CPA selection	<ul style="list-style-type: none"> <li>Basal medium supplemented with proteins (e.g., RPMI 1640 + fetal calf serum or human serum or plasma) and DMSO (6-10% v/v)</li> </ul>	<ul style="list-style-type: none"> <li>Lower DMSO concentration leads to lower cytotoxicity.</li> <li>DMSO-free, serum-free, and even xeno-free cryomedia are desired.</li> </ul>
CPA addition	<ul style="list-style-type: none"> <li>Add CPA stock solution (precooled at 4°C, 2x concentration) to cell suspension stepwise at 4°C with final volume ratio 1:1 and Cell concentration <math>10^6</math>–<math>10^7</math> cells/mL.</li> <li>Equilibration (e.g., for 10 min)</li> </ul>	<ul style="list-style-type: none"> <li>Slow CPA addition can reduce cell volume excursion and osmotic injury.</li> <li>Lower temperature may reduce CPA cytotoxicity.</li> </ul>

Procedures	Recommended protocol	Comments
Cooling	<ul style="list-style-type: none"> <li>· Cooling to a temperature (e.g., -40°C) with 1°C/min by a cooling rate controlled freezer, then to a lower temperature (e.g., -80°C) at faster rate.</li> <li>· Put into -80°C freezer or liquid nitrogen.</li> </ul>	<ul style="list-style-type: none"> <li>· Slow cooling is desired to reduce ice injury to cells.</li> <li>· Temperature recording in whole process is recommended.</li> <li>· Intervention and compensation for crystallization heat by transient chilling during freezing (-6 to -8°C) is beneficial for cell viability.</li> </ul>
Storage	<ul style="list-style-type: none"> <li>· Storage in -80°C freezer, vapor phase of liquid nitrogen, or liquid nitrogen.</li> </ul>	<ul style="list-style-type: none"> <li>· -80°C for storage for weeks to months.</li> <li>· Liquid nitrogen for storage for months to years.</li> <li>· Storage in vapor phase, instead of in liquid phase of nitrogen, could decrease the risk of contamination/infection.</li> <li>· Temperature monitoring in whole process is recommended.</li> </ul>
Thawing	<ul style="list-style-type: none"> <li>· Fast thawing by stirring in 37°C water bath.</li> </ul>	<ul style="list-style-type: none"> <li>· Fast thawing is beneficial to reduce recrystallization and ice injury.</li> </ul>
CPA removal	<ul style="list-style-type: none"> <li>· Add washing medium (e.g., RPMI 1640 + fetal calf serum) stepwise at 4°C or room temperature to cell suspension followed by equilibration and centrifugation.</li> <li>· Repeated dilution/washing may be needed.</li> </ul>	<ul style="list-style-type: none"> <li>· Slow CPA removal can reduce cell volume excursion and osmotic injury.</li> </ul>
Resting	<ul style="list-style-type: none"> <li>· Incubation at 37°C with 5% CO<sub>2</sub> for a minimum of 120 min.</li> </ul>	<ul style="list-style-type: none"> <li>· Some results showed resting of cryopreserved lymphocyte can promote cell viability and functionality [62, 63].</li> <li>· Incubation with cytokine cocktail may increase the retention of T cell function [64].</li> </ul>

**Table 4.** Cryopreservation protocol for immune cells

Similar to the facts described above, each step in cryopreservation can affect the final status of the immune cells after freezing and thawing. Optimizing each step is necessary to minimize the potential injuries to the cells and maximize the retention of the cell functionality after processing. Table 4 shows the currently recommended protocol for immune cell cryopreservation, which is very close to that for HSC.

#### 4.2. Impacts of cryopreservation on immune cell functions

Viability of immune cells after freezing/thawing varies from 60 to 90%. Some investigators have reported that lymphocyte functionality can be well-retained after cryopreservation. Wang et al. found that although freezing damage could result in about 10% loss of human T lymphocytes isolated from peripheral blood, cytokine producing capability was well-retained, and no apparent change in cell cycle pattern could be detected in T lymphocytes after cryo-

storage for 3-50 days compared to fresh samples [62]. Human peripheral lymphocytes maintained the capacity to respond to antigenic and mitogenic stimulation and to produce micronuclei in cytokinesis-blocked cells [65-67]. Cryopreservation does not induce alterations in lymphocyte surface markers and karyotypes [63, 68, 69]. Riccio et al. showed that with 24 h resting after thawing, the frequency of spontaneous apoptosis in cryopreserved lymphocyte cells was not significantly modified [70]. Hori et al. suggested that freeze-thawing did not affect the viability, phenotype, subsequent maturation, or function of dendritic cells at any stage of maturation [71].

However, alternations of lymphocyte functionality caused by cryopreservation have also been reported in the literature. Mononuclear cells (MNC) are more sensitive than stem cells. Some reports showed that cryopreservation can cause detrimental damage to lymphocyte functions [72-76]. Costantini et al. and Owen et al. found that cryopreservation could induce a consistent set of changes in PBMC from both healthy and HIV-infected patients, including a profound decrease of surface marker expression, significant changes of proportions of some cell types, loss of proliferative responses to some HIV antigens [77, 78]. Results suggested that cryopreservation induced higher levels of apoptosis in PBMC [78-80]. DNA repair capacity (DRC) has a profound influence on DNA stability and ultimately cancer incidence. Fresh human lymphocytes could repair hydrogen-peroxide-induced DNA strand breakage, while cryopreserved lymphocytes did not possess this capability [81]. Similarly, Chang found that the DRC of the cryopreserved peripheral lymphocytes was on average 14% lower than that of the fresh samples, possibility due to the destruction of DNA repair enzymes during cryopreservation [82]. A small delay in the activation of cryopreserved PBMC was also found, which implies the importance of knowing at which time points the desired cellular analyses should be carried out [83]. These results imply that special precautions should be taken in the selection of fresh or frozen lymphocyte samples for analysis and the interpretation of immune studies performed on cryopreserved lymphocytes with different functionality assays.

It is interesting that different types of immune cells, immune cells from different parts of human body (peripheral blood, lymphoid, and mucosa), or immune cells from different donors (healthy and infected patients) may have different performance in cryopreservation. Scheiwe et al. found that granulocytes were fragile and exquisitely sensitive to freezing/thawing and osmotic stress. Granulocytes could not survive cryopreservation like lymphocytes, and the damaged granulocytes might be the reason of aggregation [84]. Thus far, there is still no clinically available method to preserve granulocytes. Lymphoid dendritic cells were shown less robust than macrophages to stresses [85]. For comparison between different lymphocyte donors, more losses of T cell responses in HIV-infected individuals were detected than healthy individuals. T cells from HIV-infected donors were more fragile and more susceptible to freezing and thawing [78]. Differences were also found in the cryobiological characteristics of the immune cells. Hallak et al. demonstrated that cell membrane permeabilities to water depended on the donors' health condition and age [86]. This difference in cell membrane property may explain the different performance of those immune cells in surviving cryopreservation.

Further optimization of cryopreservation protocols for immune cells is still needed to maximize the retention of their functionality after freezing/thawing. More intensive studies of the injury mechanisms to the immune cells are desired, including in cryobiology and physiology.

Some investigators suggested a few changes to attempt to improve lymphocyte function after cryopreservation. For example, Jennes et al. found incubation of the thawed T cells with a cytokine cocktail was helpful [64]. Stroh et al. demonstrated the function of caspase inhibitors in preventing cryoinjury and improving cell recovery [80].

## 5. Summary

In summary, cryopreservation of stem cells and immune cells is essential for both clinical treatments and fundamental researches. Numerous studies have been done to optimize the cryopreservation protocols. The currently optimal procedure of cryopreservation of stem cells and immune cells is composed of several critical procedures: slow addition of CPA (5–10% DMSO in basal medium), slow cooling at 1–2 °C/min to a low temperature with cooling rate controlled freezer, storage in -80°C freezer or liquid nitrogen tank, fast thawing in 37°C water bath with agitation, and gradual removal of CPA prior to infusion. In the future, desired work includes further optimization of the cryopreservation protocols especially for lymphocytes; standardization of the optimized protocols with temperature monitoring and quality control; exploration of DMSO-free, serum-free or even xeno-free media for cryopreservation; development of simple, reliable, and cost-effective devices that can be used at field sites for CPA addition/removal, controlled cooling and transportation (cold chain); and more fundamental cryobiological studies to avoid injuries to the cells.

## Acknowledgements

The authors are grateful to Dr. Florian Hladik and Mr. Sean M. Hughes for the enlightening discussions and their help in manuscript editing. The work was supported by NIH grants (P30 DK56465, P01 CA18029, and P30 CA15704).

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