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Review Article

Cryopreservation of Stem Cells in Tissue Engineering and Regenerative Medicine

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Abstract
Cryopreservation is a critical enabling technology in stem cell-based therapies, tissue engineering and regenerative medicine that provides stable and long-term storage of organelles, cells, tissues, or any other biological constructs. However, this technology faces challenges, including decreasing cell survival rates and using dimethyl sulfoxide (DMSO), a cytotoxic agent. Moreover, cryopreserving methods are time-consuming and expensive. Various cells and tissues, due to some reasons, such as different metabolic and functional characteristics, respond differentially to the cryopreservation protocols which cause diversities in viability after thawing. This review discusses methods currently used for optimized cryopreservation of hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), adipose-derived stem cells (ASCs), and their advantages and disadvantages. Also, here we discuss about changing the, DMSO, freezing rate, pre-freeze storage, and storage temperature that can improve the cryopreservation outcomes. Further studies are still needed to find better cryopreservation methods for stem cells.

Keywords: Cryopreservation, Regenerative Medicine, Tissue Engineering, Stem Cells, Hematopoietic Stem Cells, Mesenchymal Stem Cells
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Introduction

Cryopreservation is a critical enabling technology in stem cell-based therapies, tissue engineering, and regenerative medicine that provides stable and long-term storage of organelles, cells, tissues, or any other biological constructs. However, some problems are associated with cryopreservation, which are mentioned below: 1) Cells must be taken from a metabolic state to a frozen state for storage and then from a frozen state back to a metabolic state for use. During this transition, intracellular ice crystals could be formed and damage the cell, so cryopreservation reduces cell viability; 2) Using dimethyl sulfoxide (DMSO) in the cryopreservation procedure as a common cryoprotectant can prevent the formation of intracellular crystals. However, DMSO is cytotoxic for cells in their metabolic state. Although various cryoprotectants with less toxic affects have been suggested for stem cell freezing, DMSO is still used as a conventional cryoprotectant. Some of the compounds used are mentioned in Table 1; 3) Cryopreservation is a time-consuming and expensive method.[1,2] In addition, the decrease in temperature before the freezing stage or hypothermia can affect stem cell proliferation and differentiation. Hypothermia by changes in the expression of some genes affects cell metabolism and mechanisms.[3] Therefore, developing optimal stem cell freezing methods is essential, given that we have to use cryopreserved cells for research, diagnosis, and especially therapies.

Hematopoietic stem cell transplantation (HSCT) has been successfully implemented in the treatment of blood cancers such as leukemia and lymphoma. Despite the conventional use of HSCT for the treatment of hematological malignancies, their clinical uses have recently been developed for diseases such as severe scleroderma, diabetes, treatment of metabolic disorders, and gene therapy. Bon marrow obtained through aspiration from the cavity of the ilium (hip bone), peripheral blood harvested by leukapheresis, and umbilical cord blood (UCB) gathered from the placenta

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are the three main sources of hematopoietic stem cells (HSCs). HSCT can be performed with autologous HSCs or allogeneic HSCs. HSCs Cryopreservation is an affective method to maintain and preserve HSCs during the treatment process. But as mentioned, the main challenge of this method is the reduction of cell viability.[4,5]

Table 1. Two Classes of Cryoprotectants Used for Different Cells[4]

Penetrating Cryoprotectants	Non penetrating Cryoprotectants
Glycerol for iPSCs[6] AdMSCs[7]	Polyvinyl pyrrolidone for AdMSCs[5]
1,2-propanediol for BMSCs[8]	Trehalose for Peripheral blood stem cells,[4] pluripotent stem cells[4]
Dimethyl sulfoxide for MSCs and other types of stem cells[10]	Fructose for BMSCs[9] Sucrose for Hematopoietic stem cells[11] and Spermatogonial stem cells[12]
	Glucose for Spermatogonial stem cells[12]

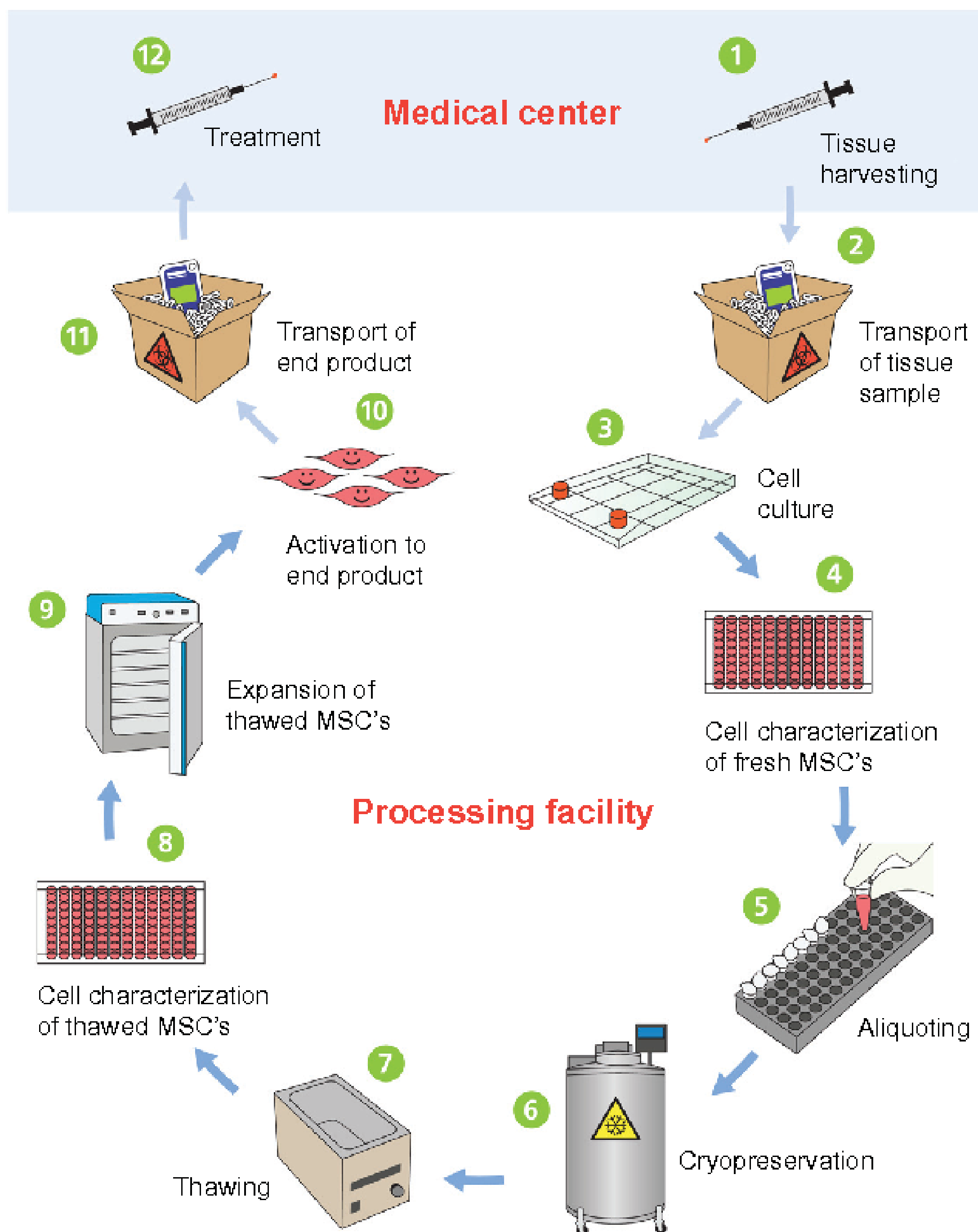

Figure1. Stages of Cryopreservation of MSCs[78]

Mesenchymal stem cells (MSCs) have the ability to differentiate into several various tissue lineages and also do not have undesirable immunogenic potential. These characteristics make them an efficient option in regenerative medicine. Despite being popular, there is no identical method for MSCs preparation. In primary MSC-based clinical trials, cryopreserved cells were assumed to be the failure source. Moreover, the variability in the result of MSC-based clinical trials was suggested to mostly be because of the functional changes that the freeze-thaw process makes in MSCs rather than the freezing method itself (Figure 1).[6-9]

Embryonic stem cells (ESCs) have an infinite capacity for proliferation and differentiation *in vitro*. Hence, these cells are ideal in cell-based therapies. There is a diversity of challenges in the bioprocess design of ESCs. According to a study, ESCs cryopreserved in feeder cells' absence could be thawed and expanded in a stirred bioreactor.[10]

Adipose-derived stem cells (ADSCs) are a group of mesenchymal stem cells (MSCs) with self-renewal and potential plasticity abilities. Because of their characteristics like immunomodulation, anti-inflammatory, and angiogenesis affects, they are paid attention to in regenerative medicine. To isolate and obtain these cells, most of the time, surgery is needed, which is an invasive and expensive method, on the other hand, the number and quality of ADSCs decrease with age. Freezing these cells can help overcome these limitations, but as mentioned, this process comes with consequences.[11-13]

Cryopreservation of cord blood stem cells (CBSCs) is also faced with a challenge because the source and number of these stem cells are often limiting. Also, osmotic shock, dehydration, and solution affects may lead to the loss of function in cryopreserved cells.[14]

Regaeding the studies, all cells and tissues don't answer equally to a identical cryopreservation process. variety in physical and biological composition, like membrane permeability and surface to volume ratio, make different answers to the cryopreservation protocol and cause diversities in viability after thawing. Moreover, the metabolic and functional “health” of the cells used in cryopreservation method has affect on the results.So, different cells and tissues respond to the same cryopreservation process differently.[15]

Hence, in addition to the optimization of cell isolation and culture, the cryopreservation process optimization for various cell types rather than adopting an off-the-shelf protocol is essential.[15]

In this review, we investigated and summarized methods that are currently suggested or used for HSCs, MSCs, ESCs, ADSCs, and CBSCs. Almost all of these methods are based on changing freezing rates, DMSO, pre-freeze storage, and storage temperature to improve cryopreservation. Moreover, the advantages and disadvantages of different methods are discussed.

Cryopreservation of HSCs

HSCs are the first tissue-specific stem cells that prospectively isolated and are the only stem cells which use in routine clinical to date. Today, HSC-containing grafts are widely used to treat various blood cell diseases such as leukemias and autoimmune disorders.[16] The optimal cryopreservation of HSCs requires considering several factors, including the composition of the cryoprotectant solution, cell concentration, freezing rate, and storage temperatures. But, most of the research has focused on cryoprotectant solutions. Despite evidence of detrimental side affects, DMSO remains the gold standard for cryopreservation of HSCs.[17]

DMSO

Multiple studies have noted adverse reactions following infusion of DMSO-preserved HSCs, ranging from mild events like nausea and vomiting to life-threatening conditions such as cardiac arrhythmia.[18-20] Due to the toxicity of DMSO in patients who received DMSO-preserved HSCs, most research has focused on reducing or removing DMSO from cryoprotectant solutions.

Clinical studies have successfully reduced the amount of DMSO used in cryoprotectant solutions from 10% to as low as 3.5%. Moreover, removing DMSO from cryopreserved HSCs before infusion could reduce its side affects. The classical method of removing DMSO is based on immediately centrifuging thawed cells, removing the supernatant, and replacing it with a fresh solution several times.[21] Turney et al., compared three different cryoprotectant media to optimize DMSO concentration and improve cryopreservation of HSCs. They used PRIME-XV FreezIS (Irvine Scientific) and two standard cryoprotectants: Std10 and Std5. Based on their results, average CD34+ recovery with Std10, Std5, and PRIME-XV FreezIS were 39%, 74.7%, and 73.6%, respectively.[22] Additional clinical studies have shown that washing cells before infusion into patients can decrease DMSO-related side affects. Quality control studies have found that HSCs can be stable in liquid nitrogen for up to 10 years. Clinical results have highlighted that post-thaw cell assessment is vital in predicting engraftment success.[17]

Freezing Rate

Different cells have different optimal freezing rates. The cryoprotectant type and its concentration affect the optimal freezing rate. The higher the cryoprotectant concentration, the slower the optimal freezing rate.[23] Cryopreservation of HSCs is generally carried out using slow freezing rates (approx. 1 °C/min). The viability of these cells may be achieved by increasing the freezing rate.

Pre-freezing Storage

Delay in cryopreservation after the collection of cells may adversely affect cell viability. It is suggested that hematopoietic

progenitor cells be maintained at a refrigerator temperature (4-8 °C) before freezing to avoid significant loss of cell potency.[24,25]

Storage Temperature

Today, controlled rate of freezing and storage in liquid nitrogen vapor are standard techniques for cryopreservation of HSCs. To explore and validate easier and more cost-affective methods for cryopreservation of HSCs, freezing and storage of cells in –80 °C mechanical freezers have been examined. However, the recovery and viability rates of CD34+ cells were decreased for peripheral blood stem cell (PBSCs) cryopreservation. Calvet et al.,[26] found that regeneration of hematopoietic and immune systems with PBSCs cryopreserved at –80 °C for less than 6 months is satisfactory, although a profound CD34+ T lymphocyte deficiency persists for 1 year. Sputtek et al.[27] compared the recovery of PBSCs frozen at –80 °C versus –170 °C for 3 and 6 months. Results showed that storage at –80 °C results in a significant loss of cellular membrane integrity and clonogenic capacity. Yuan et al.[28] successfully stabilized pluripotent stem cells for up to 1 year at –80 °C by adding the minimally toxic polysaccharide Ficoll 70 to the cryoprotectant solution.

Long-term Storage

Seo et al.[29] compared cord blood samples that had been preserved for 1 and 2 years. Total nucleated cell count (TNC), cell viability, and CD34+ count showed no significant difference between cells frozen for 2years and those frozen for 1 year. However as expected, both of them showed a decrease in viability and CD34+ cells count compared to fresh samples. Of course, cryopreserved hematopoietic progenitor cell in 5% and 10% DMSO for 9 years showed no difference in cell viability.[30]

Cryopreservation of MSCs

MSCs are easily extracted from various tissues of the human body like bone marrow, fat, and synovial fluid. These cells can differentiate into cells of various lineages. Since MSCs are not detected by the immune system, they can prevent graft rejection. Because of this property, MSCs are counted as competent biomedical candidates, especially in tissue engineering.[31] In order to achieve a sufficient number of cells for clinical applications, cryopreservation is an affective method for the preservation and pooling of MSCs.[32]

The collection, processing, and cryobiological properties of MSC isolated from human dental pulp were investigated in a study. Based on the results, minimal processing may be required for the sample banking with no instant programs for development and use of them.[33] The affect of using chitin scaffolds in the cryopreservation process on cell survival was investigated by V.Mutsenko et al. Although the viability of cells cryopreserved on chitin scaffolds was decreased about 30%, the surviving cells had ability to maintain their differentiation potential.[34] The proliferation and osteogenic differentiation of MSCs on scaffolds after cryopereservtion in vitro and in vivo were investigated. Regarding the results, these potentials on partially demineralized bone matrix in vitro were not affected by cryopreservation and it can be deduced that cryopreservation allows hMSCs to be available for potential treatment application in bone tissue engineering.[35]

It has been reported that human bone marrow-derived MSCs are sensitive to cryopreservation methods. During the freeze-thaw process, a significant decrease in cell viability occured, which is a technical obstacle to achieve sufficient cell count.[31,32] In a study, the affect of a Rho-associated kinase (ROCK) inhibitor, Y-27632, was investigated on the post-thaw survival of cryopreserved MSCs. The frozen-thawed cell suspensions were re-plated on new cell culture dishes for 24 hr. It was observed that the proportion of viable adherent cells increased with increasing inhibitor concentration.[36]

DMSO

Carvalho et al. used culture media supplemented with 10% fetal calf serum (FCS) and 5% DMSO to freeze rat bone marrow-derived MSCs.[37] In continued efforts to develop a more appropriate solution for clinical use, Pal et al. used different parenteral solutions (e.g., saline solution, Plasmalyte A) supplemented with 5% human serum albumin (HSA) and 10% DMSO.[38] Moreover, Moon et al. used a vitrification solution of 40% ethylene glycol (EG) + 18% Ficoll 70 + 0.3 M sucrose to preserve amniotic fluid-derived MSCs.[39]

According to the relationship between DMSO and the occurrence of a variety of cardiovascular-related adverse reactions (hypotension, arrhythmia, etc.,), it is necessary to removing DMSO before infusion.[20,40-43]

While there was no significant difference between using 2% and 10% DMSO in terms of cell viability after 1 month freezing period, a significant decrease in cell viability observed after long-term cryopreservation with 2% DMSO.[44] Some researchs, in order to reduce the toxicity related to DMSO, reduced the percentage of DMSO, or used secondary CPAs (such as trehalose, sucrose, and boron).[45-47] A study used 5% DMSO / 5% HES for MSCs cryopreservation. Based on the results, HES keeps the character of the cell surface markers. However, HES alone did not provide adequate cryoprotection for rat-MSCs, but combined with DMSO provides suitable cryoprotection while reducing DMSO levels to 5%.[48]

Freezing Rate

Some studies have used “controlled-rate freezing” for MSCs preservation.[37,38] Carvalho et al. evaluated some cooling rates (3, 5, 10 °C/ min) but did not observe any variation in

the survival of MSCs.[37] Some studies used mechanical freezers. Heng et al. placed samples directly into a -80 °C freezer,[36] and Kotobuki et al.[49] placed the samples into a -30 °C mechanical freezer followed by freezing at -80 °C.[39]

In another study, four various methods for MSCs cryopreservation were examined: conventional slow freezing (following a standard protocol), program freezing (using Consarctic BV65 (Consarctic GmbH) equipment), and two vitrification protocols. The results indicated that the program freezing method was more affective than vitrification in low-temperature storage.[50] Additionally, the affect of different freezing rates during MSCs cryopreservation was studied by Naaldijk et al. results showed that using the straight freeze method was more efficient than the "controlled-rate freezing" method in post-thaw viability of MSC.[48] To date, the role of storage conditions (temperature and duration) or the influence of warming conditions on post-thaw recovery has not been examined. Comparing results of post-thaw recovery studies illustrated several challenges. First of all, two studies noted that the measured post-thaw viability varied with the assay timing/time post-thaw.[37,49] Specifically, the viability of the MSCs declined with post-thaw time. The frozen-thawed MSCs showing a decrease in viability from >80% at 2 hours to<40 % at 8 hours when maintained at 4 °C.[51] Secondly, measuring viability with different viability testing methods may give different results. Carvalho et al. noted that measuring viability with trypan blue gives significantly different results than measuring by 7-AAD (90.6% versus 66.3%).[52]

Long-term Storage

In order to modulate the freeze-thaw stress responses after long term storage, cryopreservation or post-thaw wash solutions are sometimes supplemented with additives (e.g., apoptosis inhibitors to reduce apoptotic cell death related to post-thaw).[53,54] Heng et al. used a Rho-associated kinase (ROCK) inhibitor to improve the long-term post-thaw recovery.[63]

Cryopreservation of hESCs

HESCs have great potential to use as a source for cell therapy and regenerative medicine. Low-temperature stabilization (cryopreservation) is one of the key elements for the eventual clinical applications of hESCs.[55] HESCs have ability to use as a new source in heart failure treatment and drug screening. In a study, an affective generation of cardiomyocytes from hESCs was made. In addition, the ability of cryopereservation of the differentiated cells was investigated. The differentiated cells showed suitable cardiomyocyte markers and hold contractility in culture. Further, the cardiomyocytes survived after cryopreservation, and viable cardiac grafts were found after transplantation of cryopreserved cells into rat hearts after myocardial infarctions.[56]

It should be noted that effective cryopreservation protocols for hESCs did not established yet. Generally, recovery of viable hESC after freezing is very low, and due to the low growth rate of hESC, the time from thawing to having suitable cultures for experiments may take weeks to months.[55] The low survival rates of hESCs in conventional slow-cooling cryopreservation protocols are mostly due to apoptosis rather than cellular necrosis.

Xu et al. investigated the affect of cryopreservation on cell apoptosis, which leads to the low cell recovery rate after cryopreservation. Based on the findings, reactive oxygen species (ROS) level was notably raised, F-actin content and distribution was changed, and caspase-8 and caspase-9 were activated after cryopreservation. Also, p53 was activated and translocated into nucleus.[57] In another study, the results showed that hESCs adherent to microcarriers in cryovials makes a higher undifferentiated cells recovery than cryopreservation of cells in suspension.[58] Heng et al. investigated the affect of a synthetic inhibitor on the post-thaw survival rate of hESCs. For this purpose, the synthetic inhibitor was supplemented into either the freezing solution, the post-thaw culture media, or both. The results showed that adding an inhibitor to the freezing solution leads to a greater increase in the survival rate than the post-thaw culture media.[59]

Xu et al. examined the affects of a new combination of freezing solution (DMSO: polyethylene glycol (PEG) 3:1) on hESC survival rate and recovery after cryopreservation. Also, they investigated the role of the combination of two types of inhibitors on cell recovery during the subsequent culture. The results showed that the new freezing solution leads to 30% more hESCs recovery than the conventional slow-freezing method. Additionally, hESC maintained undifferentiated status by this novel protocol.[60]

Since collagen IV and laminin key components of ECM can improve the proliferation and differentiation rates of hESCs, Kim et al. evaluated their affects on survival and differentiation rates of hESCs after slow freezing and rapid thawing. The results showed that the survival rate of hESCs after thawing was significantly increased. Frozen-thawed hESCs were cultured in more than 70 passages but had key properties of hESCs like morphological characteristics, normal karyotype, similar pattern of marker expression, basement membrane-related gene expression, and the ability to differentiate into derivatives of all three germ layers, which showed that ECM treatment is a reliable and affective cryopreservation method for these cells.[61]

Since the efficiency of the traditional cryopreservation method is low, Zhou et al. used the vitrification method to evaluate its efficiency. For this purpose, an identical hESCs line was randomly used to be cryopreserved by vitrification or slow freezing. The results showed high percentages of hESC were recovered after vitrification, while the recovery

was low after slow freezing. So, vitrification was affective in cryopreserving hESCs, and during prolonged culture, hESCs maintained pluripotency capacity after cryopreservation.[62]

The affect of using the open pulled straw (OPS) vitrification method for the cryopreservation of hESCs were studied by Reubinoff et al. They demonstrated that the vitrified hESCs were cultivated for prolonged periods while maintaining the pluripotency properties.[63] Since cryopreservation of a large quantity of hESCs at one time is not reachable by using the traditional vitrification method, a newly invented vitrification carrier for cryopreserving - bulk vitrification (BV) method - was introduced compared with the popular OPS vitrification method. According to the results, cryopreservation of cell clumps at one time with the BV method was 30 times as high as those for the OPS method.[64]

DMSO

An affective, consistent, and xeno-free cryopreservation technique was tested in two different laboratories for 3 years. The method was based on an increasing concentration of DMSO with a slow or a rapid cooling system. Almost 60 colonies per cryovial were cryopreserved.[65] Thay developed a new slow freezing-rapid thawing method in serum-free conditions for the cryopreservation of a large number of colonies without the use of a programmable freezer.

Freezing Rate

Two freezing protocols were currently applied to cryopreservation of hESCs, including slow freezing and vitrification. Slow-freezing using 10% DMSO as a cryoprotectant is commonly used successfully to cryopreservation of primary cells,[66] human MSCs,[36] and mouse ESCs.[67] However, this protocol has not been successfully transferred to hESs. It leads to low cell survival rates after freezing.[59,65] On the other hand, the vitrification of hESs by the OPS method is more affective than the slow freezing. A higher cell survival rate, 70–90%, has been reported after vitrification.[62-64]

In a study, three cryopreservation methods (slow-rate conventional, programmable, and vitrification methods) were used and the attachment and recovery rates of cryopreserved hESCs compared. According to the results, in the conventional cryopreservation method, the cells had a lower attachment and recovery rates after thawing than programmed and vitrification methods. In the vitrification method the cells had the highest attachment and recovery rates. So, the conventional slow-rate freezing method was unsuitable for cryopreservation of hESCs.[68]

Cell Concentration

In contrast to HSCs and MSCs, hESCs cryopreserved in small aggregates of a few hundred cells to prevent cell loss from apoptosis during cell detachment and dissociation.[69]

Long-term Storage

Most of the hESC lines for research are maintained in the presence of xenoproteins also during long-term storage of ESCs, affective cryopreservation methods that prevent or limit the accidental contact of hESCs with nonsterile liquid nitrogen should be used. So a safe, xeno-free cryopreservation protocol involving vitrification in closed sealed straws and long-term storage in the vapor phase of liquid nitrogen was introduced by Richards et al. Results showed high survival and low differentiation rates after thawing.[70]

Several investigators have attempted to improve the post-thaw recovery of hESCs by modifying the cell configuration. Martin-Ibanez et al. used ROCK to improve the recovery of hESC after freezing. Results showed that post-thaw recovery was improved (60%), but colonies overally decreased.[71]

In a study immediately after cryopreservation, cell viability was detected at different conditions using propidium iodide (PI) (BD Bioscience, UK) staining method, which is an indicator of cell death based on membrane integrity. For this issue, cells were stained with PI for 1 min in the dark after removing CPA.[60]

A standard cryopreservation protocol (slow cooling with 10% DMSO) for the hESC line was used by Katkov et al. After cooling to -80 °C, cells were transferred to a -80 °C freezer. Cells were kept at -80 °C for 3 days ("short-term storage") and for 3 months ("long-term storage"). Then, cell vials were thawed in 36 °C in a water bath, and cells were cultured for 3, 7, or 14 days. Control cells were passaged on the same day. Results showed the majority of cultured control cells were alive during the study. While only 50% of the cultured frozen cells were live after 3 days. So, in long-term storage, the harmful affect of freezing was further observed.[55]

Freezing Rate

To better study of freezing rate, the rate of cooling within the Styrofoam box when placed in the -80 °C refrigerator was measured with a temperature probe. The cooling rate of the box was approximately –3 to –4 C in 1 min, reproducible and consistent.[72] To achieve a gentler temperature drop, the 12-well plates were placed within an insulated styrofoam box instead of an isopropanol container.[72] In one study the hESCs were cryopreserved in clumps with some modifications by conventional slow-rate freezing and rapid thawing methods. Briefly, about 20-25 clumps (over 100 cells per clump) of hESCs were collected and transferred into a cryo-vial (Nalge Nunc; Naperville, IL, USA) containing 0.5 ml of pre-cooled (0 °C) freezing medium as a new protochole for hESCs cryopereservtion.[59]

Cryoprotectant Concentration

It has been reported that high cryoprotectant concentrations

and flash-freezing in liquid nitrogen yield higher survival rates. Of course, such protocols are tedious to perform manually and unsuited for handling bulk quantities of hESC that would almost certainly be required for various clinical and non-clinical applications.[59]

Cryopreservation of ADSCs

Adipose tissue is an abundant accessible source of tissue-specific adult stem cells. Adipose-drived stem cells (ADSCs) are able to differentiate into some functional cells such as osteoblasts and then use in bone tissue engineering. Hence, a trustworthy method is essential for ADSCs storage in research and clinics. In a study, the proliferation potential and osteogenic differentiation of ASCs studied in two groups; the cryopreserved and non-cryopreserved populations.[73] The results showed that cryopreservation has no affect on the proliferation and osteogenic differentiation of hADSCs.

Zhang et al. evaluated the cryopreservation impact on the endothelial markers of differentiated ADSCs. After cryo-preservation, no remarkable change in morphology and proliferation of ADSCs was observed. Based on their results, ASCs were resistant to cryopreservation in terms of keeping endothelial cell characteristics and retention on a vascular graft.[74] Viability and osteogenic differentiation of ASCs in vitreous cryopreservation process for 1 week and 12 weeks, short term and long term, was studied. Liu et al. findings showed that Cryopreservation time has no considerable affect on the cell viability and osteogenic function.[75]

Since freshly collected adipose tissue is not always available, having improved and reproducible cryopreservation methods to maintain ADSC viability and in long-term storage is always needed.[76] In a study, the efficiency of conventional DMSO cryopreservation methods was examined by measuring the maintenance of differentiation potential of ADSCs after one freeze cycle. Based on the results, the post-thaw viability of ADSCs was affected by concentration of DMSO.[77] Moreover, in order to understand the affects of cryopreservation on ADSCs, the differentiation potential of adipocytes was compared before and after one freeze cycle by colony-forming unit (CFU) and alkaline phosphatase assays. The results showed that differentiation potential of adipocytes donot change significantly.[77]

Cord Blood-Derived CD34+ Hematopoietic Progenitor Cells

Cord blood (CB) is an unlimited source of hematopoietic stem and progenitor cells (HSPC). Using cryopreserved CB-derived CD34+ HSPCs is successful in children and usually leads to rapid hematopoietic recovery upon transplantation.[78]

Sodian et al. used cryopreserved umbilical cord blood cells (UCBCs) for tissue engineering of heart valves. The cells grown in the pores and finally, a confluent tissue layer was observed by electron microscopy. Moreover, formation was seen with biochemical examination.[79] *In vitro* and in vivo osteogenic capacity of human UCB-MSCs were performed by Liu et al. In fact they explained the cryopreservation impact on the osteogenic differentiation of green fluorescent protein (GFP)-marked UCB-MSCs. According to the results, cryopreservation had no impact on the cell phenotype, GFP expression or UCB-MSCs osteogenic differentiation, and also, showing that cryopreserved GFP-labeled UCB-MSCs might be used for bone tissue engineering.[80]

DMSO

In order to decrease DMSO concentration, Bueno used and examined the affect of Y-27632 on the post-thaw survival and recovery of CB cells. According to the results, Y-27632 inhibited expansion of (fresh and cryopreserved) CB cells and impaired the survival and recovery of cells upon thawing.[78] Another method for reducing DMSO concentration in the cryopreservation mixture is decreasing the amount of DMSO and adding another component instead. A study by Hayakawa et al. used 5% DMSO and 5% pentastarch in the cryopreservation of UCBCs. Preserved cells in 5% DMSO with pentastarch had higher post-thaw viability than frozen cells in 10% DMSO.[81]

Freezing Rate

The most widely used cryopreservation method is adding 10% v/v DMSO and a constant cooling rate of 1 °C per minute. Current methods for ex vivo expansion of HSPCs still result in losing multilineage differentiation potential. Current freeze-thawing protocols result in significant cell death and losing required CD34+ HSPC for engraftment. The apoptosis induced directly by cryoinjury is the major cause of loss of viability after slow freezing.[78] A comparison between slow cooling (2 °C/min) and rapid cooling (Vitrification) of human UCBCs have performed by Djuwantono et al.[82] They found that cell viability and CD34+ enumeration after rapid cooling was significantly higher than slow cooling. These results suggest that rapid cooling is a promising cryopreservation method for CB.

Long-term Storage

Guttridge et al. found that pre-cryopreservation storage time significantly affected the viability of UCB-derived CD34+ cells after cryopreservation, suggesting that extended pre-cryopreservation should be avoided.[4] Lisenko et al. found that the storage duration of PBSCs, ranging from several months to several years, has no impact on hematopoietic system recovery following transplantation. It means the quality of long-term cryopreserved CB units for hematopoietic stem cell transplantation (HSCT) showed no difference between cryopreserved cells for 10 years and 1 month.[83] Broxmeyer et al. found that frozen CB-derived

CD34+ cells can be engrafted in mice for up to 21 years.[84]

Cryopreservation of PBSCs

Peripheral blood stem cells (PBSCs) are one sources of HSPCs. Regarding the continuously rising number of autologous and allogeneic stem cell transplantations, cryopreserving PBSCs collected by apheresis has become a necessity for many institutions worldwide. In Figure 2 the process of using and cryopreservating BMSCs for stem cell therapy is presented.[85,86]

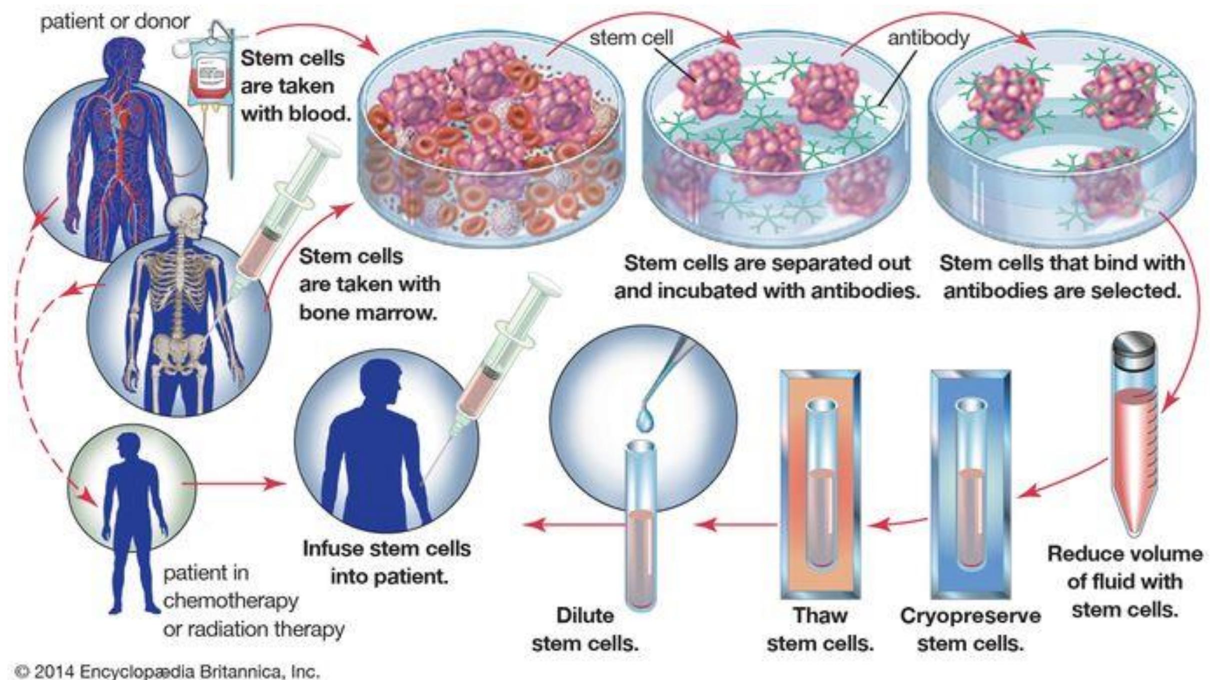

Figure 2. Cryopreservation of BMSCs for Stem Cell Therapy

Mobilized peripheral blood (MPB) bone marrow cells with having the differentiation potential into a diversity of cell types are useable in tissue engineering. For the first time, isolation, characterization, and post-in vitro culture thawing of CD90+ MSCs from MPB in sheep was investigated by Landa-Solís et al. Thay showed that obtained cells can be used as a MSCs source for experimental models in tissue engineering studies.[87] Also, Martinetti et al. used trehalose as a cryoprotectant to cryopreserving pure PBSCs. Based on the results, using trehalose enhances cell survival after thawing compared with the standard freezing procedure.[88]

Cell Concentration

The influence of cell concentration on cryopreservation outcome is not well studied.[89] Two studies have demonstrated that cryopreservation of PBSCs at a concentration of 2 × 108 cells/mL still yields high recovery of viable cells[90] and successful engraftment after autologous PBSC transplantation. It is noteworthy that a very high cell concentration may lead to cell loss and cell clumping after thawing or seizures during the cell infusion. As a result, special care should be taken with high cell concentration products.[91,92]

Long-term Storage

The CD34+ cells were cryopreserved for short (20 days) and long (3 months) durations. Closed bag systems consist of tightly connected sterile bags for collection and cryopreservation of PBSC without reopening the system during the cryopreservation procedures. So that all steps from stem cell collection until the freezing procedure were done in a "closed system". These systems had quality and safety for transplantation.[86] Humpe et al. used a closed system using a sterile filter similar to cleanroom-based methods in terms of the quality and sterility of cellular products. This closed system consisted of three cryopreservation bags, different tubing, a DMSO-resistant sterile filter, a tubing line for sterile connecting, and a syringe for air removing.[93]

Conclusion

Up to now, there is no universal method for preserving various stem cells. The impossibility of using the method of cryopreserving HSCs for MSCs, as well as the difficulty of cryopreserving hESCs, is a proof of this claim. so that cryopreservation protocols for each cell type should be appropriate to its unique biology. Since DMSO has many adverse and epigenetic affects on cells, it is not approved for

application in human in order to cryopreserving cells used in therapise. Therefore, affective methods of preservation without using DMSO must be developed. As the clinical use of stem cells continues to grow, the need to develop preservation methods will continue to grow. Current preservation methods are very operator-dependent and labor-intensive and may require equipment that is not available. Generally, new technology development is still needed to facilitate the high-efficiency preservation of cells. Moreover, protocols for affectively preserving cells in different contexts must be developed.

Authors' Contributions

MAT, PA, MHT, and FGh wrote the manuscript; AA conceived the original idea and supervised the project; AA and ZS reviewed and edited the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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